Dietary Supplements of Plant Origin

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A nutrition and health approach

Edited by Massimo Maffei

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Foreword

The field of dietary supplement research has not kept pace with the burgeoning interest by consumers in the use of these products, particularly those of plant origin. Consumers and patients increasingly are demanding better evidence to support the claims that are made for effectiveness, safety and quality of these ingredients. Such scientific evidence exists, of course, but it is of widely variable quality. The abundance of information to support reasonable claims for some ingredients is easily matched by the enormous gaps in knowledge about others.

Part of the reason for that exists in the way that herbal use has been translated to modern society. Many herbal ingredients have been used in traditional healing systems (e.g. traditional Chinese medicine, Ayurveda, and many others) for centuries, sometimes millennia. This should offer comfort and security for their incorporation into conventional Western models of healthcare, health promotion, disease prevention, and even disease treatment. However, given the patterns of use of these ingredients – especially when available as dietary supplements – there are now circumstances in which they are used chronically when their traditional use called for acute symptom management, or when their application is completely different from anything that commended their use in traditional healing.

Coupled with this are the other challenges that exist in this field: the lack of information about mechanism of action for many herbal products; the array of regulatory authorities that govern the marketing of herbal products around the world; the variability in manufacture of products; and the paucity of validated analytical methods, reference materials and standards.

A book that attempts to address these issues should be celebrated, not only because of the challenges that it confronts, but also because of the promise that some herbal products can offer, e.g. in health promotion, disease prevention and disease management. This book represents collaboration between many specialists from very different disciplines. In addition, it offers the reader a comprehensive look at many aspects of the roles that these ingredients can play in human health.

From legislative mandates to biochemistry, from functional foods to industrial applications, this book covers many topics crucial to understanding both the promise and the challenge associated with dietary supplements of plant origin.

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1 An introduction to dietary supplements of plant origin

Definitions, background and an overview of this volume

Bernadette M. Marriott

Introduction

Dietary supplements are in widespread use in the United States (Eisenberg *et al.*, 1993, 1998; Kessler *et al.*, 2001). This use continues to grow as more people, particularly the baby-boomers, become concerned about their access to health care, and the quality of that care, and seek complementary and alternative medical practices (Eisenberg *et al.*, 1998; Foster *et al.*, 2000; Kessler *et al.*, 2001; Rainey and Cason, 2001). With the increasing advent of food that is heavily synthetic and influenced by technology, a significant segment of the US population is turning towards foods and medicines, including dietary supplements of plant origin, that they see as more natural (Kaptchuk and Eisenberg, 1998; Greger, 2001). Information on supplement use comes from many sources but there are limited data that incorporate the reasons for supplement selection and employ sampling techniques that are representative of the breadth and diversity of the United States' population.

At present, the best data on use of supplements of plant origin in the US is based on emerging data from the ongoing National Health and Nutrition Examination Survey (Radimer *et al.*, 2000) and specific populations such as cancer survivors (cf. Richardson *et al.*, 2000; Boon *et al.*, 2000). The number of meta-analyses, clinical studies and trials of the health outcomes of botanical supplements has grown exponentially in the last six years. These data have not only underlined the potential health value of supplements of plant origin, as, for example, the dramatic expansion of research on phytotherapeutic agents in the treatment of benign prostatic hyperplasia (cf. Lowe and Fagelman, 1999), but identified that the choice and dose of supplements can directly interact with prescription drugs (Miller, 1998; Fugh-Berman, 2000), and impact nutrient bioavailability from food as well as other treatment regimes (Drew and Myers, 1997; Marriott, 1997; Ernst, 1998; Miller, 1998; Barone *et al.*, 2001; and see Cott in this volume). In addition, reports continue to emerge that demonstrate inconsistency in supplement content, labelling and quality (cf. Shibata and Asetai, 1996; Hahm *et al.*, 1999; Harkey *et al.*, 2001), as well as incidents of toxicity (Brent, 1999; Ritter and Dembicki, 2000). While data on why individuals chose to use botanical supplements, the demographics of their use, and related health behaviours are still developing, it is critically important to build a strong basic scientific understanding of the chemistry, biology and biotechnology of these supplement ingredients and disseminate these results to inform scientific research methodology, policy and regulatory processes.

This volume combines an overview of the latest research on plant bioactive

compounds, their biochemistry, and the bioengineering and biotechnology of supplement ingredients of plant origin with current information on the economic and regulatory situations that govern consumer health protection in Europe and the United States. The authors have been cognizant of the diverse audience that are interested in botanical supplements, and have been careful to write in an accessible fashion and include references that are both general and specific.

This chapter will give a brief review of the history and current situation of dietary supplements in the United States including the role of the Office of Dietary Supplements (ODS) at the National Institutes of Health (NIH), followed by an overview of the other chapters in this volume.

A brief overview of dietary supplement ingredient regulation in the United States

In 1994 the United States Congress passed the Dietary Supplement Health and Education Act (DSHEA, Public law 103-417, October 25, 1994, 103rd Congress). This law modified the Food, Drug, and Cosmetic Act and expanded the definition of dietary supplements to include botanical ingredients, hormones, and a diverse array of related products in addition to vitamins and minerals. The DSHEA also specified the role of the United States Food and Drug Administration (FDA) in regulating dietary supplements, mandated the creation of a Presidential Commission on Dietary Supplement Labeling, and authorized the establishment of the Office of Dietary Supplements at the National Institutes of Health (NIH). The main elements of the definition of dietary supplements from the DSHEA are listed in Box 1.1.

For products that are regulated by the FDA, the 'intended use' of a product or ingredient is the turnkey that determines whether an item is broadly classified as a food or a drug. Dietary supplements, no matter how they are presented, if intended to be used to supplement the diet, are reviewed by the Office of Special Nutritionals and related offices in the Center for Food Safety and Applied Nutrition (CFSAN) at FDA. The DSHEA included a number of provisions that apply to dietary supplements alone. As a result of the DSHEA the pre-market safety evaluations that are required for new food ingredients or new uses of approved food ingredients do not apply to dietary supplements. The DSHEA authorized the FDA to establish good manufacturing practice (GMP) guidelines for dietary supplements and dietary supplement ingredients and the DSHEA provided guidelines for the display of literature used to market dietary supplement products (Box 1.1).

When a manufacturer wishes to market a dietary supplement or dietary supplement ingredient, they must submit information attesting to the safety of the new ingredient to the FDA 75 days prior to the product's availability. This petition must indicate that the ingredient does not present a significant risk of illness or injury based on the conditions of use specified on the product label. This DSHEA-based process for dietary supplements differs from the requirements for food additives and new food products, ingredients, or uses for food ingredients. These products must be reviewed through FDA's pre-market approval process, which includes the submission to FDA of the results from a specified array of safety studies. Once a dietary supplement is on the shelf, for the FDA to take action it must demonstrate that the product or its ingredients are unsafe. Since the passage of the DSHEA, there have been a number of instances in which the FDA has taken direct public action regarding ingredients that

A *dietary supplement* can be:

- *a product* (other than tobacco) intended to supplement the diet that bears or contains one or more of the following dietary ingredients:
  - a vitamin, mineral, amino acid, herb or other botanical; OR
  - a dietary substance for use to supplement the diet by increasing the total dietary intake; OR
  - a concentrate, metabolite, constituent, extract or combination of any ingredient described above;

Use:

- intended for ingestion in the form of a capsule, powder, softgel or gelcap; and not represented as a conventional food or as a sole item of a meal or the diet.

Box 1.1 Key elements of the definition of a dietary supplement from the DSHEA

are marketed as dietary supplements. One early example occurred in 1997 when the FDA requested manufacturers to limit the amount of ephedrine alkaloids in dietary supplements as a result of reports of adverse events including cases of reported heart attacks, strokes, seizures, and death. Also in 1997, the presence of the harmful herb *Digitalis lanata* as a contaminant in selected batches of a herbal product containing plantain, resulted in the industry and FDA joining forces to identify and remove the contaminated products from stores nationwide (Slifman *et al.*, 1998).

Both manufacturers and the FDA have been moving forward to assure the safety of dietary supplements. While the FDA has made progress to fulfil the mandates of the DSHEA regarding guidance, labelling, and GMPs, most companies have begun to self-regulate their products and adopt the same good manufacturing practises used for foods. To aid this effort, the American Herbal Products Association (AHPA), a trade association composed of growers, processors, manufacturers and marketers of herbal products, developed a reference book that pulls together in one place the published safety information on over 600 herbs commercially available in the United States (AHPA, 1997).

Of particular concern to the consumer is that botanical supplements and supplement ingredients are derived from plant materials that may be grown throughout the world and shipped to the United States for capsulation and packaging. Collection and processing of natural plant materials introduces additional concerns that need to be addressed for good consumer information on the quality of the products. There are plant variables such as identification of the plant species, the plant part (young leaf versus mature leaf, stem, etc.), the presence of potentially toxic pesticide residues, and purity concerns such as the potential for contamination with bacteria, fungal growths, etc. There are manufacturing variables, including the processing of the plant material, extraction procedures, product formulation, and product packaging (exposure to light). Also to be considered are post-manufacturing variables, including appropriate shipping and product stability during storage or on the shelf. A shelf-life designation is not required for dietary supplements.

The Presidential Commission on Dietary Supplement Labeling issued its report in 1997 and the resulting labelling requirements for dietary supplements went into effect on 23 March 1999. The label is modelled after the label on food products and

includes a 'Supplement Facts' box of specified size that must include information about the serving size, amount of primary ingredient, other ingredients in descending order and the name and contact information of the supplier. The identity of the product, net quantity and any structure–function claims for the product are elsewhere on the label. If the manufacturer chooses to include a structure–function claim, there must also then be the disclaimer: 'This statement has not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure, or prevent any disease.'

A structure–function claim on a dietary supplement is a statement about how the product may affect the structure or function of the body. A structure–function claim that is used as an example by the FDA is 'Calcium builds strong bones'. Another that might be used related to the functioning of the body is 'Amino acids are essential for normal brain functioning'. Structure–function claims do not have to be approved by the FDA.

In contrast, health claims on products must be authorized by the FDA. Health claims link the product or ingredients in the product, whether food or supplements, to a disease or health condition. The FDA has authorized a number of health claims for foods, several of which may be used for dietary supplements if the product meets the minimum content requirements. At present, health claims can be found on supplements if they include a sufficient quantity of calcium, folic acid, oat bran and *Psyllium* seed husk. These claims may associate calcium with the reduction in risk of osteoporosis, folic acid with reduction in risk of neural tube defects, oat bran with reduction in cholesterol levels, and *Psyllium* husk fibre with reduction in risk of heart disease.

While the steps for handling the regulatory process for supplements are evolving, there have been product classes introduced into the market that further confuse the picture. Standard snack foods such as potato chips have been marketed with added botanical ingredients such as ginseng, kava kava, etc. Beverages, performance-enhancement food bars, and teas also boast the addition of botanical ingredients with structure–function claims on the products. According to the DSHEA definition of dietary supplement, most of these products are foods with non-regulated food additives incorporated into them. However, most are being marketed as dietary supplements.

As an example, on 26 October 1999 the FDA authorized the use of health claims for soy protein in reducing the risk of coronary heart disease (CHD) through cholesterol lowering. To qualify for the health claim the food item must include 6.25 grams of soy protein per serving. This amount is based on the assumption of four servings per day and because 25 grams per day have been shown to significantly lower cholesterol. This health claim is an example of one in which the amount of soy protein in the product may result in health claims for dietary supplement food products such as snack bars, beverages, etc. How the FDA will continue to regulate these types of products as well as the growing market of functional foods remains to be seen. It appears, however, that the approach of 'intended use' may no longer be a clear turnkey for distinguishing among the ever-evolving array of products that are blurring the distinctions between food, drug and dietary supplements.

Definitions of dietary supplements

The DSHEA defines dietary supplements for regulatory purpose within the United States. The Office of Dietary Supplements at the NIH needed to implement research priorities using this definition and yet in some manner group the wide array of supplement ingredients available in the marketplace into more manageable categories. A definition was needed that also could be interpreted more readily in terms of research projects. An operating definition was requested in meetings with the NIH Institute Directors and other agencies who expressed difficulty in understating whether or not specific ingredients would be supported in the research and education programmes developed by the ODS. The resulting *operating definition* and *categorization* of dietary supplement ingredients was developed in collaboration with the staff of the Office of Special Nutritionals at the Food and Drug Administration (FDA), who had oversight for dietary supplement regulatory affairs, and a large group of ad hoc advisers who assisted the ODS in developing its strategic plan (see description below). Since dietary supplements found in the marketplace are not only available as single nutrients or plant-derived compounds but often in combinations, the ODS refers to dietary supplement *ingredients* in its writings and only uses the term 'dietary supplement' to refer to a specific product. This operating definition was developed with the understanding that the ODS would be addressing its activities to only those supplement ingredients that met the 'intended use' as dietary supplements and delivery methods described in the DSHEA. The final operating definition is given in Box 1.2.

In operationally defining dietary supplements, the ODS indicated that some ingredients may in fact be whole plants, plant parts, plant-derived, or modified foods that are termed 'functional foods' or compounds that are referred to as 'nutraceuticals' if in fact these compounds meet the requirements of the DSHEA. The ODS staff made this decision with care because the terminology in the late 1990s in the US was very confused, and the same compounds, particularly plant-derived materials, were termed differently by manufacturers, yet all were being marketed as dietary supplements in order to fit under the DSHEA regulatory umbrella. By designating the intended use of a product as a dietary supplement the manufacturer could avoid the more lengthy and costly review of the product as a drug or food. In addition, new terms were proliferating in an attempt to carve out market share among competing companies. The most common of these terms which have emerged with distinctive definitions that currently have become well understood in the marketplace are 'functional foods' and 'nutraceuticals' (see Box 1.3). The development of both of these product domains has led to extensive, high-quality research areas that are producing exciting new information related to the links between plant-derived materials, synthetic compounds and human

> Following DSHEA, a *dietary supplement* is viewed by the ODS as any substance that is consumed in addition to the regular diet – that is, in addition to meals, snacks and beverages – and follows the methods of delivery clauses outlined in the Act. Food items, in any physical form (such as a liquid or a powder intended to be added to a liquid, etc.) that are intended to be a sole source of nutrition, meal replacements or conventional foods are not dietary supplements as defined in DSHEA, and thus are outside of the scope of ODS (ODS, 1998, p. 7).

Box 1.2 ODS operating definition of dietary supplement

health. Other terms such as 'holistic medicines', 'herbal medicines', 'healthful herbs', continue to emerge and are confusing to the consumer in the United States. None of these terms have been accompanied by the development of clear definitions or research approaches that have been long-lived. None were incorporated into the operating definition or categories for research of the ODS.

The ODS reviewed the situation with functional foods and nutraceuticals as part of their strategic planning process. Based on the DSHEA, the operating definition of dietary supplements, and the then current definitions of functional foods, nutraceuticals and other market terminology, the ODS included in its strategic plan a statement of its assumptions and general approach to supporting research and related activities (see Box 1.4). This approach acknowledges that key dietary supplement ingredients that warrant research on efficacy and safety may be termed or contained in nutraceuticals or derivatives from functional foods. Such a clarification may not be warranted if the ODS strategic plan were written now, since these terms, in particular, have become more specifically defined and are much more widely understood through the growth in research and research publications (cf. Corridan *et al.*, 2001; McCusker *et al.*, 2001; Murray *et al.*, 2001; Velmurugan *et al.*, 2001; Zeisel, 2001). A recent discussion of the definition and regulations regarding nutraceuticals can be found in Zeisel (2001).

In the mid-1990s identification and verification of plant parts was problematic. Many companies were importing raw ingredients for dietary supplements as powders from outside the United States and the speciation was unclear. Rarely were pressed

Functional food – any modified food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains (Thomas and Earl, 1994).
Nutraceutical – any substance that may be considered a food or part of a food and provides medical or health benefits, including the prevention and treamtent of disease (DeFelice, 1993).
Nutraceutical – those diet supplements that deliver a concentrated form of a presumed bioactive agent from a food, presented in a nonfood matrix, and used to enhance health in dosages that exceed those that could be obtained from normal foods (Zeisel, 2001)

Box 1.3 Definitions of functional foods and nutraceuticals in common use in the United States

Therefore, the ODS will identify and foster research on the health benefits and risks of substances based on the merit of the underlying scientific evidence regardless of how they might be currently incorporated into the different categories of commercial products or their regulatory status in the commercial marketplace. This approach allows the ODS flexibility to address the scientific questions relevant to the role of specific substances in promoting health without being unnecessarily constrained by the nuances that bear on whether a substance may be lawfully used in a dietary supplement, or whether specific information on the label/labeling causes a product to be marketed under regulatory frameworks other than as a dietary supplement. (ODS, 1998 p. 8)

Box 1.4 ODS approach to the variability of terminology of dietary supplements and dietary supplement ingredients

voucher plant specimens sent with the powders to verify the taxonomy. Often, plant material was identified only by a common name from the country of origin, not by the standard Latin binomial. Common names for the same plant species vary from country to country and often across regions within the same country.

In 1992 the American Herbal Products Association (AHPA) published a compendium that linked all common plant names they could identify with appropriate internationally recognized scientific names (AHPA, 1992). The goal of AHPA was to reduce the confusion in the marketplace of the diversity of common names and outdated scientific nomenclature by advocating for the establishment of a 'uniform common name with a uniform Latin name' (p. I). The publication, *Herbs of Commerce*, included 550 primary species with 1800 cross-referenced synonyms. The book was prepared primarily to support the plant-derived dietary supplement, spice and herb industry and, as a result, it provided a rough definition of the diversity of the plant-derived products available in the US market in the early 1990s.

In order to deal with this diversity of dietary supplements of plant origins coupled with other substances that were defined by the DSHEA, the ODS used the expertise of the individuals who participated in their strategic planning process to group supplements into categories that could be discussed and addressed by research in a more meaningful manner. Since dietary supplements typically contain one or more ingredients, the ODS grouped these ingredients into three categories: botanicals, nutrients and other dietary substances as defined in Box 1.5. These categories therefore provided further elaboration on the operating definition and stated approaches. Clearly, the dietary supplement ingredients of plant origin far overshadowed the number of ingredients in the other two categories. Botanical supplement ingredients also included the

Botanical ingredients include all plant-derived materials whether fresh, preserved, or dried full plants, plant parts, plant species mixtures, plant extracts, and compounds found in such materials. Thus, items that are commonly termed 'herbs' or 'herbal products', regardless of whether they meet the dictionary definition of herb[1] or that are comprised of parts, extracts, or preparations of woody plants will be included as botanical ingredients.

Nutrient ingredients include all essential and non-essential nutrients and other food constituents, that are typically described in standard nutrition reference texts or that fall within the review parameters of the Food and Nutrition Board, National Academy of Sciences in consideration of Dietary Reference Intakes (DRIs). Thus, this category would include substances recognized as essential nutrients (i.e., iron, vitamin C, essential amino acids, etc.) and substances not generally recognized as being essential but that have or may have a dietary or nutrient role in humans.

Other dietary substances comprises a broad and diverse group of substances that are neither of plant origin nor alone could be viewed as 'nutrients' within the common-sense meaning of the term. For example, such substances could include animal or plant metabolites or constituents, microorganisms and certain of their constituents, etc. The substances subject to inclusion in this category are limited by the statutory definition of 'dietary supplement' in the DSHEA (i.e., not an approved or investigational drug, not a conventional food or meal replacement, and intended to be used to supplement the diet, etc.).

1 Herb – a flowering plant whose stem above ground does not become woody.

Box 1.5 Categories of dietary supplement ingredients

most diverse preparations, sources of origin and questions regarding the consistency of all aspects of production and marketing. As a further indication of the growth in the diversity of the dietary supplement ingredients of plant origin, AHPA recently published the second edition of *Herbs of Commerce*, which includes over 2,000 plant species (AHPA, 2000).

Because the focus of this present book is dietary supplements of plant origin, the authors use the term 'dietary supplement' to roughly equate to botanical supplements or supplement ingredients. While some authors discuss dietary supplement products that contain multiple ingredients, most are focused on the science behind the bioactive compounds in one plant species or specific plant parts of one or related plant species.

Sales and use of dietary supplements in the United States

The sale and use of dietary supplements in the United States initially grew dramatically after the passage of the DSHEA. Figures of individual and mixtures of botanical supplements drove the market. The growth and demand led to a consolidation of manufacturing and retail corporations as well as new growth in botanical supplements produced by the major pharmaceutical corporations in the US.

Supplement sales in the United States

It is estimated that from 30–53 per cent of Americans or 100 million people use dietary supplements on a regular basis (several times each week) (Aarts, 1998). In 1996 sales of dietary supplements totalled $9.8 billion and represented 51 per cent of the total sales in the nutrition industry in the United States. Sales of dietary supplements in natural food stores comprised 44 per cent of the total, and mass-market retail accounted for 26 per cent of the total with the remainder in direct marketing through the mail or Internet. Overall sales for dietary supplements grew 9 per cent in 1996 (Aarts, 1998). Sales continued to grow in 1997 and 1998 to a new record level for total sales of dietary supplements of $13.9 billion in 1998, which was a 10 per cent increase over sales in 1997. Of this market, for specialty supplements, glucosamine grew 23 per cent, botanical supplements sales increased 13 per cent, and sports-oriented supplements, as exemplified by energy bars, grew 12 per cent. In contrast, according to the *Nutrition Business Journal* (*NBJ*), vitamin and mineral sales grew less dramatically at the rates of 6 per cent and 8 per cent respectively (*NBJ*, 1999a). Online Internet sales for dietary supplements represented the 'fastest growing distribution channel' which totalled $40 million in 1998 and was a significant increase over the $12 million online sales total for 1997 (*NBJ*, 1999b).

In 1997 botanical supplements sales from all distribution channels totalled $3.6 billion with multi-herbal products comprising 27 per cent of the total. The top five single botanical products sold in 1997 were *Echinacea* (9 per cent, $324 million), ginseng (8 per cent, $288 million), *Ginkgo* (7 per cent, $252 million), garlic (6 per cent, $216 million), and St John's Wort (6 per cent, $216 million) (*NBJ*, 1998).

In 2000 the sales slowed significantly for this industry, but recent projections indicate continued growth in the future. At present, the nutrition industry, overall, represents $48 billion in annual sales with an expected growth rate of 6.2 per cent from

2001 to 2003. The nutrition industry is divided as follows: functional foods accounting for 36 per cent, dietary supplements accounting for 35 per cent, natural and organic foods accounting for 21 per cent and the remaining 8 per cent representing personal care products. In 2000, dietary supplement sales totalled $16.8 billion with vitamins and minerals accounting for 42 per cent, herbs and botanicals accounting for 25 per cent, and other supplements including meal supplements accounting for 31 per cent. From 2001 to 2003 mineral supplement sales are expected to grow 7.8 per cent, vitamins, 2.0 per cent, herbs and botanicals 2.4 per cent, while other supplements range from 4.7 per cent (meal supplements) to 10 per cent (specialty supplements/other). (Source: the *Nutrition Business Journal* as cited on the National Nutritional Foods Association website on 14 February 2002.)

Use of dietary supplements based on representative population samples in the United States

The first well-stratified statistical samples of the use of botanical supplements by the United States population are currently being conducted as part of the National Health and Nutrition Examination Survey (NHANES), which began in 1999, and includes questions on dietary supplement use. The other information on supplement use in the United States other than what is emerging from the NHANES, is garnered from market information and studies that have focused on specific populations.

The National Health and Nutrition Examination Survey (NHANES) and the Continuing Survey of Food Intakes by Individuals (CSFII) have traditionally been important sources for information on the relationship between health outcomes and dietary practices in the United States. These series of diet, nutrition and health surveys, which are managed by the federal government, utilize statistically driven samples of the diverse population of the United States. The data collected by these surveys prior to 1999 included questions on dietary supplements, but the focus was on vitamin and mineral supplements.

The NHANES is run from mobile health units that travel to specific locations throughout the country to reach the identified samples of the population. The data collected in each of the surveys prior to 1999 combined questionnaires with a series of measurements and samples on each individual that varied with the survey questions and government groups supporting the particular survey. In spring, 1999 the NHANES became a continuous survey that has the goal of visiting 15 locations and collecting data on 5,000 Americans each year (http://www.cdc.gov/nchs/nhanes.htm). As of January 2002, the NHANES merged with the CSFII and became the National Food and Nutrition Survey (NFNS). The goal of this new tool is to 'provide comprehensive information on health and nutrition characteristics of the US'. (http://www.cdc.gov/nchs/about/major/nhanes/current.htm). Since 1999 the NHANES has included questions about the use of supplements of plant origin and will provide a rich source of data to address health questions and their relationship to supplement intake.

Data from earlier national surveys provides information on Americans' use of vitamin and mineral supplements. For example, Slesinski and colleagues reviewed the data from the National Health Interview Surveys conducted in 1987 and 1992 to look at nutrient supplement use over the previous 12-month period. These authors found that the prevalence of supplement use remained unchanged between the two surveys.

Forty-six per cent of adults stated that they used vitamin and mineral supplements in the last year and 24 per cent reported regular use (Slesinski *et al.*, 1995). Those who used nutrient supplements, as recorded by the 1992 survey, had overall diets that were lower in fat, higher in fibre and higher in specific vitamins and minerals than non-users of supplements (Slesinski *et al.*, 1996).

Data from the early NHANES (prior to 1999) similarly indicated that nutrient supplement users had a higher mean intake of nutrients from their diets, higher incomes and higher education levels than non-users (Koplan *et al.*, 1986; Looker *et al.*, 1998). In a survey that assessed the health habits of a representative sample of older residents in the state of Georgia, which included persons in their sixties and eighties, and 76 individuals over the age of 100, those who used nutrient supplements were more physically active and more consistently followed the United States Dietary Guidelines for Americans (Houston *et al.*, 1997).

In contrast to nutrient supplement users, those who use botanical supplements present a different profile. In a random sample of 1,035 Americans, those who used botanical supplements were more highly educated but in generally poorer health; specifically they reported a higher prevalence of back pain, chronic pain and anxiety than those who did not use supplements (Astin, 1998).

Overall, representatives of health professional groups tend to use nutrient supplements in similar patterns to the general population. Of 1,732 nurses surveyed in the Nurses Health Study, 38 per cent reported regularly taking multivitamins. Use of single-nutrient supplements was less prevalent: vitamin C (23 per cent), vitamin E (15 per cent) and vitamin A (4 per cent) (Willett *et al.*, 1981). Forty-seven per cent of 692 pharmacy students queried in a study by Ranelli *et al.* (1993) reported using nutrient supplements within the previous two weeks. Among 181 cardiologists, 44 per cent reported routinely taking antioxidants, 42 per cent were routinely taking aspirin and 28 per cent reported regular use of both products (Mehta, 1997). Dietitians appear to use supplements to a greater degree than other health professionals do. Of 665 dietitians polled in Washington State, 60 per cent reported using some form of dietary supplement (Worthington-Roberts and Breskin, 1984).

Two nationally representative random household telephone surveys in the 1990s that measured the use of alternative medicinal practices by Americans included questions on dietary supplements. Between 1990 and 1997 there was a dramatic increase by adults in the use of high-dose vitamins (130 per cent increase) and botanical supplements (380 per cent increase) (Eisenberg *et al.*, 1998).

Not unexpectedly, a large percentage of cancer patients incorporate alternative medical therapies as adjuvants to conventional treatments. In a cohort of 480 patients who were newly diagnosed with early-stage breast cancer, 10.6 per cent of the women had used alternative medicine prior to their diagnosis and 28.1 per cent initiated the use of alternative medicine after surgery. In this study by Burstein and colleagues, alternative medicine included botanical supplements. There was no difference between alternative medicine users and non-users on quality-of-life measures at the time of surgery; however, at three months post-surgery, use of alternative practices was associated with depression, fear measures, lowered mental health ratings, and lowered sexual satisfaction (Burstein *et al.*, 1999).

Rock and colleagues incorporated information on botanical supplement use as part of a secondary breast cancer prevention trial of 435 women. These women had completed their medical treatment and replied to four 24-hour dietary recalls over a two-

week period to identify their dietary patterns. In this study, 80.9 per cent of the women reported dietary supplement use on a regular basis, which is approximately double that of the general population. Women who used supplements, whether nutrient, botanical or biological, consumed diets with more dietary fibre and less dietary fat than non-users (Rock *et al.*, 1997). In a localized breast cancer follow-up trial in postmenopausal women, 71 per cent of the first 724 patients randomized on the study were regularly taking dietary supplements. In a subset of 116 consecutive women on the same study, 82 per cent were taking at least one dietary supplement but the variety of the supplements taken each day varied from 1 to 10 and the number of pills consumed per day ranged from 1 to 26. Of those women who reported regular intake of dietary supplements, 22 per cent were taking botanical supplements and biologicals (Winters *et al.*, 1997). Using data from comprehensive cancer centres, Richardson *et al.* (2000) reported that 38 per cent of survivors used botanical supplements and 60.3 per cent reported using vitamins. Ongoing work by these and similar groups are the main source of growing understanding of the parameters of use of dietary supplements by cancer patients. A good review of specific botanical supplements in use by cancer patients can be found in Spaulding-Albright (1997).

Much of the other data on botanical supplement use in the United States are from industry-sponsored, consumer-based surveys. In a 500-household survey conducted by the Celestial Seasons Company in partnership with the Harris Organization, 33 per cent of households regularly used botanical supplements (cited by Brevoort, 1998). A larger survey of 43,000 households reported the use of the botanical supplements: garlic (19 per cent), ginseng (10 per cent), *Ginkgo* (9 per cent) and *Echinacea* (7 per cent) (as cited in Brevoort, 1998). In a Gallup interview of 704 individuals the three most frequently cited reasons for using botanical supplements were to combat fatigue, stress and menopause/PMS (Gallup, 1997, as cited in Brevoort, 1998). A survey of their readership by *Prevention Magazine* indicated that 32 per cent of those who responded used botanical supplements and spent an average of $54 per year on botanical supplement purchases. Sixty-five per cent of the respondents indicated that they used botanical supplements because they believed these products were safer than drugs (*Prevention Magazine*, 1997, as cited in Brevoort, 1998).

Belief structure rather than scientific evidence appears to continue to drive dietary supplement use. In a recent survey, dietary supplement users were more likely than non-users to believe that supplements were beneficial for persons with cancer, and most surveyed reported positive attitudes towards the health benefits of supplements (Blendon *et al.*, 2001). Yet data from well-controlled studies points to variability of product quality (cf. Shibata and Asetai, 1996; Hahm *et al.*, 1999; Harkey *et al.*, 2001), lack of beneficial results and the need for caution with regard to prolonged supplement use (King *et al.*, 1999).

In general, comprehensive information on the use of dietary supplements, as defined in the DSHEA, is limited to select population-based studies. The current NHANES includes questions on the type and amount of supplement used and will therefore provide a stratified sample of these factors within the sampling model of the overall project. However, the necessary breadth of the NHANES and the complex nature of its design (as described previously) result in a limited set of questions related to dietary supplements. Targeted research is needed to develop more detailed information on determinants of supplement selection, rationale for supplement use, relation of price to choice patterns, specifics of use, etc.

The Office of Dietary Supplements at the National Institutes of Health

The Office of Dietary Supplements (ODS) was authorized at the NIH as part of the DSHEA legislation. This office was formally started in late 1995 with the mandate to serve as a source of research support, inter-government advice, and science-based information on dietary supplements. The ODS has worked extensively with the other NIH institutes, centres and offices to partner in identifying the most fruitful areas for research in dietary supplements and to serve as a source of information for scientists, industry and the public.

While the Congressional mandate for the ODS was specified broadly, the office staff needed to take this mandate and transform it into specific achievable goals and objectives. In order to do this, the office assembled over 125 scientists and professionals from academia, government, industry and public-interest groups and held a series of seven strategic planning meetings in autumn and winter 1996–1997. Different individuals were involved in each of the seven meetings. The initial three meetings worked with the definition of dietary supplements included in the DSHEA and further identified the scope and terminology of different working subsets of this diverse set of ingredients. The participants in the three meetings recommended an operating definition, developed the three categories of supplement ingredients, and recommended that research priorities be focused on the broad categories: vitamin and mineral ingredients, botanical ingredients and all other ingredients. The next three meetings each involved a mixture of experts from within these three supplement ingredient categories. These individuals independently used the priorities identified by the first groups to develop mission statements, goals and objectives for the ODS. Subsequent to these meetings, the ODS staff combined the recommendations and developed a draft plan that was reviewed at the seventh and final meeting by representatives of the institutes, centres and divisions of NIH and from various federal agencies. The revised draft plan was sent to all participants for their comments. The final ODS strategic plan reflects over 700 comments, concerns and suggestions that were sent to the ODS from the meeting participants and is thus representative of the breadth of expertise represented by the strategic planning participants.

The resulting ODS strategic plan, *Merging Quality Science with Supplement Research: A Strategic Plan for the Office of Dietary Supplements,* was released in September 1998 and includes the ODS mission[1] and five goals with six to nine objectives that address each goal. In addition, the plan identifies operating principles that underlie all of the activities of the office and defines the types of activities that will be undertaken by the office as well as how the progress towards the goals will be evaluated (ODS, 1998).

In addressing the goals and objectives of the strategic plan the ODS has sponsored or co-sponsored research studies, workshops and related scientific support activities on the efficacy, safety and basic science of supplement ingredients of plant origin. These studies are listed in periodic reports, such as the *Status Report: The First Years of the Office of Dietary Supplements 1995–1998* (ODS, 1999) and on the ODS website at http://ods.od.nih.gov/accomplish/accomplish.html. Publications or links to publications that have resulted from many of these activities can also be found on the website.

'To greatly advance the scientific base of knowledge about botanicals, including issues of their safety, effectiveness, and biological action' (ODS press release 6 October 1999; see also http://ods.od.nih.gov/news/releases/funding_rel99.html) the ODS in

collaboration with the National Center for Complementary and Alternative Medicine (NCCAM), has provided five-year grants to establish four Centers for Dietary Supplement Research. These centres are targeted towards research on different supplement ingredients of plant origin to support interdisciplinary research on the health benefits of the supplement ingredients.

To address the Congressional mandate of providing science-based information on dietary supplements, the ODS has developed a number of products that are widely accessible to scientists and the public. The general ODS website (http://ods.od.nih.gov/index.asp) is the gateway to two databases: CARDS (Computer Access to Research on Dietary Supplements) and IBIDS (International Bibliographic Information on Dietary Supplements).

CARDS contains an easily searchable database of research projects on dietary supplements that have been funded by the National Institutes of Health, beginning in 1999. As the data become available, information from projects on dietary supplements that are funded by other US federal agencies will be incorporated into CARDS.

IBIDS is a database that pulls together in one place the scientific literature that has been published in US-based and international scientific journals on dietary supplements, including botanicals. IBIDS is easy to search and the user can access three databases from one web portal: the full database, a subset of the database that is limited to peer-reviewed scientific journals, or a consumer-oriented database. IBIDS is updated quarterly through incorporation of citations and abstracts from over 2,000 journals. IBIDS represents a partnership between two federal agencies the NIH and the US Department of Agriculture (USDA) with direct staff partnerships between the ODS and the Food and Nutrition Information Center, National Agricultural Library, USDA.

The ODS continues to involve scientific experts in dietary supplements as ad hoc advisers and peer reviewers in all aspects of its activities. Two projects that utilize this expertise are the *Annual Bibliographies of Significant Advances in Dietary Supplement Research* and a series of consumer-oriented fact sheets on individual dietary supplements. Both of these activities also represent partnerships of the ODS with the Consumer Products Health Care Association and Warren Grant Magnuson Clinical Center, NIH, respectively.

In addition to the activities mentioned, the ODS staff interact widely within the NIH, across the federal agencies, and with national professional societies and industry groups to foster science-based activities that support understanding of dietary supplement ingredients. In the complexity of dietary supplements, the ODS plays an important role in the United States in promoting and supporting hypothesis-driven research and then disseminating and interpreting the results of that research to the public.

The chapters that follow

The chapters in this book have been compiled to provide an in-depth focus on supplements and supplement ingredients of plant origin. In the first half of the book, authors from the United States and Europe provide contrasting perspectives on the history, use, regulation and sources of information about these products. In particular, this volume includes chapters written by European scientists who have focused their research on plant bioactive compounds long before the DSHEA legislation in the US

in 1994 or the establishment of the European Scientific Cooperative on Phytotherapy (ESCOP) in 1989. These scientists present a comprehensive view of the earlier and current approaches to studying plant-derived compounds that are used as dietary supplements. They also have placed their research into the worldwide perspective of plant biodiversity, ecology and the increasingly politically fraught arena of genetically modified foods.

The European marketplace is characterized by Valerio Bombardelli in Chapter 2. Dr Bombardelli clearly describes the perspective of European countries where compounds derived from plants have a long tradition of use as medicines and have been regulated from distinctly different perspectives in different countries. Dr Bombardelli discusses the complexity of harmonization of regulations of supplements within Europe that has resulted from the historic variability of the approaches of the countries involved.

Drs Franco Vincieri and Antonella Riva describe the establishment and goals of the European Scientific Cooperative on Phytotherapy (ESCOP) in Chapter 3. Their chapter incorporates a list and description of the ESCOP monographs. These monographs include the therapeutic indication, dose, and pharmacological properties for individual medicinal plants. The ESCOP monographs and related activities represent the joint harmonization activities of 13 European nations.

Dr Can Baser approaches dietary supplements of plant origin from the perspective of the plant industry in Chapter 4. He discusses the active compounds with potential health benefits in a number of food products such as soybeans, tomatoes, etc.

In Chapter 5 Giovanni Appendino and Orazio Taglialatela-Scafati take what they describe as a 'pharmaceutical point of view' towards diet. In this carefully organized chapter Drs Appendino and Taglialetela-Scafati walk the reader through major developments in the scientific understanding of active pharmaceutical ingredients that occur naturally in food plants and spices. They conclude their chapter with a description of a number of dietary secondary metabolites that show exciting promise for health.

Marco Mucciarelli begins his chapter (Chapter 6) with a clearly written description of the tools and techniques currently used in plant biotechnology. Dr Mucciarelli then details the research history and current knowledge base of the biotechnological advances with plants used for herbal medicines and dietary supplements. Throughout his chapter and particularly in its concluding pages, Dr Mucciarelli incorporates a thoughtful discussion of the benefits and concerns surrounding genetic modification of plants.

In Chapter 7 Massimo Maffei presents a picture of the biochemistry of the key groups of bioactive compounds found in common plants marketed as dietary supplements. Dr Maffei further summarizes the current knowledge of the beneficial physiology of these compounds, their botanical sources, as well as their possible toxicity. His chapter incorporates figures and tables which illustrate the pathways of action and chemical transformations of these compounds.

The public concern that innocent ingestion of botanical supplements might cause unexpected adverse interactions with prescription drugs is addressed in Chapter 8 by Jerry Cott. Specifically, Dr Cott provides an accessible overview of some potential pharmacokinetic interactions of medications with plant-derived dietary supplements. He uses a review of the known interactions of the popular herbal supplement *Hypericum perforatum* as an example of a herbal medicine that can interact significantly with specific prescription drugs.

In the final chapter, Chapter 9, Gail Mahady provides a comprehensive description of information resources for scientists interested in dietary supplements of plant origin. Dr Mahady has been actively involved for many years with the World Health Organization's Traditional Medicine Programme (WHO-TRM) and she has been instrumental in the development of the WHO monographs on medicinal plants. Dr Mahady divides her chapter between official and scientific information sources and includes current information on Internet links to key databases and websites. This chapter provides an excellent resource of scientific information for all investigators. Finally, the book ends with a useful appendix describing the use of plants in cancer prevention.

Conclusion

Dietary supplements in the United States include a wide array of ingredients and products that are broadly available in the marketplace. While these products are presented in formats that give the appearance of standard over-the-counter (OTC) medications such as aspirin, their manufacturers are not required to provide the same level of premarket safety data as manufacturers of OTC products. Botanical supplements, in particular, are directly derived from wild, or in some cases, commercially grown plants and therefore are more subject to product variability due to plant contamination, and manufacturing and post-manufacturing variables that are currently not regulated.

The United States and countries within Europe have widely differing approaches to regulation and marketing of dietary supplements of plant origin. While these aspects may differ, scientists worldwide share concern with the need for careful hypothesis-driven research on the efficacy and safety of dietary supplements, and, as is illustrated in this book, a commitment to high-quality research in plant bioactive compounds.

Note

1 The Office of Dietary Supplements (ODS) strengthens knowledge and understanding of dietary supplements by evaluating scientific information, stimulating and supporting research, disseminating research results and educating the public to foster an enhanced quality of life and health for the US population.

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2 Herbal dietary supplements in the European market

Valerio Bombardelli

Introduction

Plants have been a source of medicines in past centuries and even today scientists and the general public recognize their value as a source of new or complementary medicinal products. A significant percentage of the active compounds of currently prescribed medicines in Western countries continues to be derived from higher plants. Over the past 20 years interest in medicinal plants has grown enormously, from the use of herbal products as natural cosmetics and for self-medication by the general public to the scientific investigation of plants for their biological effects in human beings.

Even traditional herbal medicinal products offer unique possibilities to develop a self-medication approach and at the same time to boost research projects. As a matter of fact the traditional use of a herbal medicine may serve as an initial screening of the biological activity of the product and of its compounds.

Beyond this pharmaceutical approach to plants, there is a wide tendency to utilize herbal products to supplement the diet, mainly with the intention of improving the quality of life and preventing the diseases of elderly people.

This tendency has its main development in the USA, where, since 1994, with the Dietary Supplement Health and Education Act (DSHEA), a new category of dietary supplements (not only herbs but also vitamins, minerals, amino acids, etc.), is marketed with the declared aim of supporting well-being and helping to prevent certain diseases. In this way, on the US market there are three types of products containing herbs: pharmaceuticals, dietary supplements and food products.

The European market

On the European market there cohabit pharmaceutical and food products containing plant derivatives. As a general rule if a herbal product has a therapeutic value in itself or in its use, it should be marketed as a medicinal product. Herbal products sold as food supplements should have nutritional properties to enrich the normal diet. Moreover, herbal medicinal products are considered on a risk/benefit basis when evaluating their safety and efficacy. A food-based product must be absolutely safe.

Of course, this is a too simple vision of the market. Nowadays, you can find the same herbal product sold at the same dosage in the same member state as a medicine, as a food supplement and, simply, as a food. Also, licensing requirements, permissible claims and routes of sale vary from country to country. The reason can be found in the lack of a common legal framework at a European level applicable to food products and

in the lack of coordination between the medicine and food offices of the member states' health authorities. This means that, for instance, if a herbal medicinal product is sold as food, the medicines authority simply ignores the problem, because it cares about registered medicines only, and the foods authority considers the medicines authority competent to act, because the product is truly a medicine. This lack of coordination allows the presence on the market of borderline products, sometimes dangerous or with a very low quality profile.

Due to the uncertain interpretation of rules and the lack of a harmonized legislation, anyone who has ever tried to market food supplements containing herbs, having in mind to sell the same product in the majority of EU countries, will experience the range of complications and practical difficulties which may arise. For instance, the first problem may be the legal status of the herbal product: in certain countries it will be deemed as a medicine, in others as a food or a food ingredient. Talking about dietary supplements in Europe means considering both the above-mentioned categories because of the different legal basis that a single product can have in different states. As a matter of fact, we must not limit our discussion to food products. From the description of a herbal product as a medicine or as a food can derive the practical feasibility of marketing it in a number of countries. A good example of this can be marketing a *Ginkgo biloba* product, claiming its capability to improve brain blood flow, in the United Kingdom. The medicine control agency could consider it a medicine and ask the producer to submit a full pharmaceutical dossier as for a new chemical entity.

After a brief overview of the EU legal models, we will examine the regulatory schemes for herbal products in the main member states.

European Union legal models

First of all it is necessary to make a clear statement regarding products containing plant derivatives. The pharmaceutical approval scheme has been designed for pure chemical compounds. For this reason it is actually very difficult to register a herbal medicine, collecting all the data needed for the marketing authorization. This is true if the herbal medicine is a multi-compound product, like an extract. But it is false if a single compound, which is the sole cause of the pharmacological effect, has been isolated from the herbal derivative. In this case, the normal pharmaceutical registration way does not present any insurmountable obstacle and there are no reasons not to follow it. The product is always a plant derivative, but as a single compound it is very similar to a new chemical entity. A good example is the oncological products obtained from *Taxus baccata*.

Basically, herbal products are regulated under food law or under pharmaceutical law sometimes with a simplified pharmaceutical registration dossier. The major trend in the member states is to regulate herbs as medicines with the possibility of a simplified pharmaceutical registration procedure. To obtain marketing authorization it is enough to get a bibliographical dossier of the pharmaco-toxicological and clinical aspects and good chemical and pharmaceutical documentation demonstrating the safety and quality of the product. Following this trend the European Commission is preparing a directive on traditional herbal medicines. Herbal products marketed in Europe for more than 30 years, can be registered with an abridged dossier. The safety of the product is based on the 30 years' safe usage and efficacy and does not have to be proven.

The other very strong trend, but not at the European level, is the inclusion of herbs in positive or negative lists. This means that herbs included in (or excluded from) the lists can be sold under food law and the others are regarded as medicines. Some countries, even if the herb is considered a food, require a notification with the health ministry prior to marketing.

The European Parliament is preparing a food supplements directive. The impact of this directive on herbal products is, as a matter of fact, not very clear. At the beginning the directive regulated only vitamins, minerals and amino acids, but now it is applicable even for substances capable of having a physiological action on the body, not dependent on its nutritional properties. The physiological function can imply that even certain herbal products shall be deemed as food supplements. The directive proposal has an annex with a positive list of vitamins, minerals and amino acids that can be contained in a food supplement. The Scientific Committee on Food will be competent to evaluate which substances to add to the list in a five-year time frame. In this time frame substances not present in the list can be marketed on a national basis.

Starting now our little tour of the member states' regulations for herbal products, we will examine the legal framework, distribution channel and the real marketing practice of the most significant (in population size) countries.

Regulations for herbal products in the member states

France

France has developed a list of 34 plants regarded as foods. These plants can be sold without registration. Combination of the herbs present in the list is allowed up to a maximum of eight herbs, but they must have the same claimed pharmacological indications or usage.

There is also the category of traditional herbal medicines: a list of 196 herbal ingredients that can be registered through a simplified pharmaceutical registration procedure. Mixtures containing herbs on this list are allowed if their association is justifiable: the product must consist of a maximum of four herbs with similar or synergic usage.

All other products containing herbs are deemed as medicines and must follow the full pharmaceutical registration process.

Food supplements, whether or not they contain herbs, are sold freely in all distribution channels. Herbal medicinal products, including traditional ones, are sold only through pharmacies. Actual market practice sees many herbal food supplements for which the legal status is unclear sold freely thanks to the tolerance of the local health authority. These products contain as 'excipients' herbals that are not on the positive list.

Germany

In Germany herbals are usually marketed as medicines, because the local health authority considers even the most traditionally used herbs as having therapeutical properties. Beyond medicinal products, there is a category of traditional herbal remedies. These products were on the German market before 1978 and contain prophylactic or mildly effective herbs of German or other European origin. A limited number of

herbal medicines are covered by pharmacopoeia monographs. Products which fully comply with these monographs can be authorized with a very simplified dossier.

Herbal products sold as food are tolerated if their use is intended to supply a nutritional deficiency.

The absolute lack of clarity in the product qualification compels most manufacturers to contact health authorities prior to marketing the product in order to get authoritative advice on the regulations.

Food supplements containing herbs and traditional herbal remedies are sold freely. The selling of the other categories is restricted to pharmacies.

Greece

Herbal food supplements are not regulated with a dedicated regulation. The criteria for their classification under the food or medicine law is their intended use – in the case of food the purpose should be to supplement the daily diet. In any case most herbal products are regarded as medicines and fall under the 1994 regulation on herbal medicines. For the traditional use of herbs the legislation sets lower documentation requirements. The pecularity of the official Greek market is that even food supplements are sold through pharmacies. Market practice is slightly different in that there are food supplements sold outside pharmacies. The Greek Ministry of Health monitors the situation and from time to time bans some products deemed to be medicines.

Italy

In Italy medicinal plants should be registered as medicines. Plants with a nutritive value only can be sold as food. The market practice is, nevertheless, very different. To get the pharmaceutical registration is very difficult, and, as a matter of fact, a very small number of herbal medicinal products are approved. For this reason the wide tendency is to add herbal preparations to food supplements based on vitamins or minerals. This tendency is tolerated by the local department for food and nutrition. Those food supplements require a notification prior to being marketed with the labelling of information on safety and intended use (not therapeutical). Beyond food supplements, we can find herbals sold as food without any such labelling.

At the moment there is a law pending in the Italian Parliament which could give a basic regulation to all the herbal sector. This proposal, which Parliament has not yet passed, in its original version contained two lists of herbs, one for toxic or dangerous plants, marketed and registered as medicines, and another for safe plants. The plants of the second list should have been sold freely in pharmacies and in health stores and could claim to have a coadjuvant effect on the physiological functions of the human body. Now the above-mentioned proposal states that a committee will be in charge of preparing the lists. Finally, the Italian Health Ministry issued, in 2002, a decree that states that all products containing herbs have to be labelled as such and their production sites authorized and inspected under the food supplement decree (D. Lgs. N. 111/1992).

The Netherlands

In the Netherlands at the moment there is no law regulating herbal products. This means that herbs can be used in food products or alone as long as they are deemed safe for general consumption. The distribution channel is free except for products with a pharmaceutical licence. In this case their sale is restricted to pharmacies or authorized drug stores. In the near future it is very likely that some basic rules will be issued for herbal products. The main change is that there will be a negative list of plants that will not be allowed to be sold under food law. In 1996 the Health Ministry issued a draft list divided into three sections: plants containing pyrrolizidine alkaloid in a toxic amount, plants of the *Aristolochia* family and a list of 56 plants deemed to have medicinal properties for which a medicinal product licence is needed.

Spain

The legislation on food supplements is limited to the Royal Decree n. 3176/1983. This decree regards mainly 24 herbs used for infusions. It is possible to sell other edible herbs in supplements if they are combined with vitamins or minerals. Herbs are generally deemed to be medicines.

At the moment there are two draft regulations, one for food supplements, the other on traditional herbal medicines, but it is very likely that the Spanish government will wait for the EU directives on food supplements and traditional herbal medicines before issuing any new law on this matter.

The United Kingdom

In the United Kingdom plants are considered as medicines. In fact the Medicine Control Agency monitors the market and has the power to judge if a product is or contains a medicine by function or by presentation. Nevertheless there are a lot of borderline products still on the market. Basically a herbal product can be a medicine or a food. If it is a real food, it can be freely sold in any outlet. If it is a medicine, it can be a licensed medicine or an unlicensed one. As a licensed one, it can be a general sale list product, which means a low-profile medicine (old products that are not very effective but have a good safety profile) that can be sold practically everywhere. The other possibility is the full pharmaceutical route, but of course very few herbal medicines can have this status. As an unlicensed medicine, it can only consist of powdered or comminuted herbs not in a pharmaceutical form (e.g. capsules or tablets) and can be sold freely without any claim written on the package. Market practice is very different. Certainly, there are products presented as medicines without having a registration, but the real uncontrolled area belongs to ethnic medicines. On the market there are a lot of Ayurvedic, Chinese and Indian products, mainly mixtures, containing in the same dosage form plants, minerals and sometimes synthetic medicines. The wide use of this kind of product by the numerous Indian and Chinese population is becoming a problem very difficult to solve through only enforcement action.

The future

What about the near future? As you can see, the regulatory framework in the various member states is quite different. It is likely that the Traditional Herbal Medicines

Directive and the Food Supplements Directive, once finally approved, will not remove the differences, because member states will be able to choose slightly different ways to implement the directives. In any case, having a common regulatory framework will help the market to grow and, hopefully will raise the quality of the products. Certainly, the directives will not be able to create competition in the free movement of these products.

From the point of view of the legal status of herbal products, the Food Supplements Directive would create a category of food supplements, with few herbals, in addition to vitamins or minerals alone as inserted in the attached positive list. The Traditional Herbal Medicine Directive will apply to a restricted number of herbal medicines, having the characteristics of being considered traditionally used. For the major part of herbal products, not covered by the directives, the scenario in the near future seems to be still undefined. A radical ban on all the products not qualified as food supplements or traditional herbal medicines is probably not feasible because of their large number.

Concluding remarks

Probably we must come to a pessimistic conclusion. Just to try to find a way to ameliorate the scenario, but without expecting to know the whole truth, it could be a reasonable proposal to extend as much as possible the Traditional Herbal Medicine Directive to give rules for most products. Furthermore, the application of this directive to herbal products will, probably, ameliorate the quality and safety level of these products. Herbals and herbal extracts, as a matter of fact, should be considered in a different way from food products because of their peculiarities. The main problem regarding herbal products is their safety. This fundamental aspect cannot be guaranteed by current food production rules (basic hygiene requirements plus the Hazard Analysis and Critical Control Points, HACCP, system). Generally speaking, a fundamental initial step to get a valid proof of safety (non-toxicity) regarding botanicals starts from a chemically defined raw material. To obtain a safe botanical product (herbs and herbal extracts category), means to obtain, first of all, a quality product and, consequently, being able to monitor the whole production chain, starting from the control of the growing plants, a botanical and chemical check of the biomass to be used, a quality control process, certain stability limits and a release of the finished botanical derivatives under strict quality controls. As you can easily see, the HACCP system and food production requirements do not set rules on all of the above-mentioned items. For these reasons, the plain application of the food supplements directive is probably to be considered inadequate. As regards establishing new regulations, it may be considered advisable that the new rules should be very similar (or equivalent) to pharmaceutical good manufacturing practice (GMP) and be applied even to the production of the active ingredients. Performing all the steps necessary to have a safe product, of course, is quite expensive and requires a big investment also in terms of organization. It is very likely that small companies (and larger ones) will not accept such a proposal, because, at the moment, it is too difficult or unappealing to them.

Nevertheless, the current market requires a major change in terms of levels of quality and safety offered to the consumer. The consumer is starting to be very confused about herbs, and, probably, is becoming very worried about their real efficacy. Being able to offer to the customer quality and safe products is the first step in having the opportunity to introduce an initial discussion about innovative herbal products.

Further reading

Consultation letter MLX 283: UK Medicine Control Agency seeks comment on the Proposal for a Directive of the European Parliament and of the Council amending the Directive 2001/83/EC as regards traditional herbal medicinal products, 17.01.2002.

Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to medicinal products for human use (O.J. n. L 311, 28.11.2002).

Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to food supplements (O.J. n. L 183, 12.07.2002).

Marketing food supplements, fortified and functional foods in Europe, European Advisory Services, 2000.

Proposal for a Directive of the European Parliament and of the Council amending the Directive 2001/83/EC as regards traditional herbal medicinal products, 17.01.2002 (O.J. n. C 126 E, 28.5.2002).

3 ESCOP, the European Commission, consumer policy and health protection

Franco Vincieri and Antonella Riva

Introduction

Medicinal plants and their preparations have been used since the earliest history of humanity and have formed one of the foundations for healthcare in cultures throughout the world.

Although herbal medicinal products have a long tradition, recognition of their clinical, pharmaceutical and economic value and the public's interest in them are still growing. The European Union represents the largest single commercial market for medicinal plants and herbal medicines in the world. The EU market for licensed herbals is estimated around $1.1 billion while estimates of the sales of herbal remedies, dietary supplements and functional foods combined exceed $7.5 billion (Commonwealth Secretariat, 2001).

In 1987, the European Parliament acknowledged in a resolution

> that there do exist a large number of well-known medicinal plants traditionally used by the population and fully described in scientific literature whose very slight pharmacological effect allows their authorisation on the basis of a list to ensure correct identification of the species, variety, conservation, labelling and specified dosage and application; this group would not require the same kind of monitoring as proprietary medicinal products.

Furthermore, the European Parliament called on the European Commission

> to help to ensure that, profiting by the results of research work to date, a scientific approach to phytopharmaceuticals is developed, which will not only incorporate centuries of practical experience but will also respect the indispensable requirement of scientifc precision.
>
> (*Official Journal of the European Communities* (*OJEC*), 1987)

As a matter of fact, in these last ten years, numerous scientific articles on medicinal plants have been published and many European universities have been involved in research into herbal drugs. In parallel, the legislative framework for these kinds of products has been changing continuously. In this chapter we are going to present a brief update on the regulatory status of herbal medicinal products in the European Union and the ESCOP contribution to the harmonization process.

Consumer policy and health protection are the main targets of health authorities.

The growth in the consumption of plant-based medicines is a worldwide phenomenon. The development of safe and efficacious herbal medicines is hence an important objective to which the European Community must pay increasing attention.

ESCOP

The foundation of ESCOP

In order to provide scientifically based assistance for a harmonized assessment of herbal medicinal products, ESCOP, the European Scientific Cooperative on Phytotherapy, was founded in 1989 by six national scientific associations, and over the years a large number of other associations have joined. The main objectives of this European scientific umbrella organization are to establish harmonized criteria for the assessment of herbal medicinal products, to give support to scientific research and to contribute to the acceptance of phytotherapy on a European level (Steinhoff, 1999). The following national associations are members of ESCOP (2000):

Österreichische Gesellschaft für Phytotherapie	Austria
Société Belge de Phytothérapie	Belgium
Dansk Selskab for Fytoterapi	Denmark
Association Française pour le Médicament de Phytothérapie	France
Gesellschaft für Phytotherapie	Germany
Irish Phytotherapy Association	Ireland
Società Italiana di Fitochimica	Italy
Nederlandse Vereniging voor Fytotherapie	The Netherlands
Norwegian Society for Phytotherapy	Norway
Associação Nacional de Fitoterapia	Portugal
Svenska Sällskapet för Fytoterapie	Sweden
Schweizerische Medizinische Gesellschaft für Phytotherapie	Switzerland
British Herbal Medicine Association	United Kingdom

The aims of ESCOP are to advance the scientific status of phytomedicines and to assist with the harmonization of their regulatory status at the European level. The objectives are:

- to develop a coordinated scientific framework to assess phytomedicines;
- to promote the acceptance of phytomedicines, especially within general medical practice;
- to support and initiate clinical and experimental research in phytotherapy;
- to improve and extend the international accumulation of scientific and practical knowledge in the field of phytotherapy;
- to support all appropriate measures that will secure optimum protection for those who use phytomedicines;
- to produce reference monographs on the therapeutic use of plant drugs;
- to further cooperation between national associations of phytotherapy to advance these aims and objectives.

Definition of phytomedicines

For ESCOP, phytomedicines, or herbal medicinal products, are 'medicinal products, containing as active ingredients only plants, parts of plants or plant materials, or combinations thereof, whether in the crude or processed state'.

The following notes on the definition of phytomedicines were given (ESCOP, 2000):

- Medicinal products are defined in European Directive 65/65/EEC.
- The production of phytomedicines is formally determined in 'Quality of Herbal Remedies' in 'The rules governing medicinal products in the European Community, Volume III, Guidelines on the quality, safety and efficacy of medicinal products for human use' (ISBN 92–825–9619).
- Plant materials include juices, gums, fixed oils, essential oils, and any other directly derived crude plant product. They do not include chemically defined isolated constituents, either alone or in combination with plant materials.
- Phytomedicines may contain excipients of plant or non-plant derivation.

ESCOP constitution and structure

Members of ESCOP meet formally at Annual General Meetings (AGMs) or Extraordinary General Meetings (EGMs).The Board is constituted by the Honorary Directors appointed at General Meetings, including a Chairman and Treasurer, plus an Honorary Secretary appointed by the Directors.

The Scientific Committee of ESCOP, comprising delegates from each member country, embarked at the outset on a programme of compiling proposals for European Monographs summarizing the medicinal uses of plant drugs (including their safety), an area which ESCOP considers to be of prime importance for scientific harmonization.

The Research Committee comprises at least one Board member and other invited individuals.

The Secretariat is constituted by the Honorary Secretary appointed by the Board, supported by an employed Administrator and other staff as necessary.

The new European marketing authorization system

On 1 January 1995, new Community procedures concerning the authorization and surveillance of medicinal products came into force, superseding various procedures based on voluntary cooperation between the relevant national authorities. The European Agency for the Evaluation of Medicinal Products (EMEA) plays a central role in this system. Its aims include pooling the scientific expertise of member states in order to ensure a high degree of protection for public health, ensuring free movement of pharmaceuticals, and making certain that Europeans have access to new generations of medicinal products.

To achieve free movement of medicines within the common market of the European Union, in addition to a centralized system of marketing authorization, e.g. for new chemical entities on the one hand, and the possibility of an application on a national level only on the other hand, the so-called decentralized procedure has been established. This represents a system of mutual recognition of marketing authorization decisions and provides as a general rule that the assessment by one national authority should be sufficient for subsequent registration in other member states. Within this decentralized procedure, the 'Summary of Product Characteristics (SPC)' approached by the first authority is taken into account. It represents the main characteristics of the

product, e.g. active ingredient, indication, dosage, contraindications, side-effects, shelf-life, etc. The SPC is one of the basic elements of the application procedure.

As herbal medicinal products have, in most cases, well-known active ingredients of which much experience exists in several countries, performance of new clinical and pharmacological/toxicological studies does not seem necessary. In these cases an option for a so-called bibliographic application is provided by Article 4.8 (a) (ii) of Directive 65165/EEC: 'The applicant is not required to provide the results of pharmacological and toxicological tests or clinical trials if he can demonstrate by detailed references to published scientific literature that the constituent(s) of the medicinal product have a well-established medicinal use, with recognized efficacy and an acceptable level of safety.' That means that principle data from literature can be used to answer the questions on safety and efficacy when the expert report and dossier are prepared.

ESCOP monographs

In October 1990, the first monographs were presented at a symposium in Brussels and were officially handed over to representatives of the European Community. From the very beginning the ESCOP Scientific Committee has been working on proposals for monographs on individual plant drugs, primarily those for which European or national pharmacopoeial monographs exist.

In preparing drafts, the ESCOP Scientific Committee has the advantage that it can incorporate the views, knowledge and experience, not only of different nationalities, but also of individuals from different scientific and professional backgrounds, such as medical doctors, practising phytotherapists, pharmacognosists, pharmacologists and regulatory affairs specialists. The Committee, operating as two subcommittees, assesses information from published scientific literature on each plant drug with the assistance of leading researchers on specific plants, who are invited to meetings of the Committee for discussion and critical evaluation of the literature.

To be consistent with the requirements laid down in European guidelines, and on the advice of the Committee for Proprietary Medicinal Products (CPMP), the format of SPC was adopted for subsequent documentation as an integral part of an application for marketing authorization. Therefore, the ESCOP Scientific Committee has been working on monographs, structured like SPCs, for individual plant drugs and their preparations (primarily those for which European or national pharmacopoeial monographs exist). An ESCOP monograph describing a medicinal plant and its preparation refers to a pharmacopoeia monograph with respect to quality, and it lists the most important constituents which are possibly able to contribute to the claimed effect. The most important parts of the monographs (as for an SPC) are the therapeutic indication, the dosage and the pharmacological properties. The latter paragraph gives as many details as possible on pharmacodynamic properties, pharmacokinetic properties and preclinical safety data; each statement is supported by references. In the forthcoming edition the clinical session will report the so-called level of evidence. The text is followed by a detailed reference list including all the papers that have been used for the evaluation of safety and efficacy of each medicinal plant and its preparations.

Drafts prepared by the subcommittees are then circulated to an independent Board of Supervising Editors for appraisal. These eminent academic experts in phytotherapy and medicinal plant research are invited to comment on and criticize each draft and, where appropriate, their comments are incorporated into the final version.

Thus, each SPC proposal and ESCOP monograph takes into account the available scientific literature, national viewpoints and the advice of many authorities on the subject.

The first six fascicules, each containing ten ESCOP monographs, have already been published (Table 3.1). The work of the Scientific Committee was also supported by the European Union through its BIOMED programme.

ESCOP monographs represent an overview of the current scientific data on a medicinal plant, but they can replace neither an expert report nor documentation. They can be used as a 'harmonized' scientific background for an application, whereas the questionnaire (i.e. the requirements of the expert report) has to be answered item by item by the applicant. In 1997 the HMPWG (Ad Hoc Working Group on Herbal Medicinal Products) of EMEA (European Agency for the Evaluation of Medicinal Products) was established and in the November 1997 meeting considered drafting of so-called core-SPCs, the most appropriate way to develop general guidance on criteria to assess efficacy data based on bibliographic documentation. General agreement had already been reached during the discussion on a core-SPC for Valerianae radix based upon a proposal submitted by ESCOP. Furthermore, the HMPWP (Herbal Medicinal Products Working Party) which replaced the HMPWG in 1999, has proposed an amendment to Notice to Applicants, Vol. 2A of May 1996, stating that

> scientific monographs on certain substances, e.g. those drafted by ESCOP and WHO, offer a valuable and updated overview on published scientific literature, which together may be used in support of the demonstration of the safety and efficacy of a medicinal product in a bibliographical application in accordance with Article 4.8 (a) ii of Directive 65/65/EEC.

The ESCOP monographs were regarded as helpful in avoiding duplication of work and in bringing about gradual harmonization in the evaluation of herbal medicinal products. Therefore it was suggested that the Commission and member states recommend that both applicants and competent authorities should make use of these monographs.

Conclusion

ESCOP represents a scientific umbrella organization which gives support to scientific research and contributes to the acceptance of phytotherapy on a European level. Moreover, since 1992 ESCOP has been working on monographs, structured like SPCs, for individual plant drugs and their preparations. For a harmonized assessment, each monograph takes into account the available scientific literature, national viewpoints and the advice of many authorities on the subject.

According to the HMPWP of the EMEA, these scientific monographs may offer a valuable and updated overview of published scientific literature, which together may be used in support of the demonstration of the safety and efficacy of a medicinal product in a bibliographic application.

References

Commonwealth Secretariat (2001) *A Guide to the European Market for Medicinal Plants and Extracts*. London: Commonwealth Secretariat.

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Steinhoff, B. (1999) Monographs for herbal medicinal products: A way to reach the harmonization. *Pharmaceutical Policy and Law*, 2, 37–46.

Table 3.1 The first six ESCOP fascicules

Fascicule 1	Althaeae radix (Marshmallow root)
	Betulae folium (Birch leaf)
	Boldo folium (Boldo)
	Calendulae flos (Calendula flower)
	Foeniculi fructus (Fennel)
	Hyperici herba (St John's wort)
	Lini semen (Linseed)
	Orthosiphonis folium (Java Tea)
	Thymi herba (Thyme)
	Zingiberis rhizoma (Ginger)
Fascicule 2	Harpagophyti radix (Devil's Claw)
	Melissae folium (Melissa leaf)
	Plantaginis ovatae semen (Ispaghula)
	Plantaginis ovatae testa (Ispaghula husk)
	Salviae folium (Sage leaf)
	Solidaginis virgaureae herba (Golden Rod)
	Tanaceti parthenii herba/folium (Feverfew)
	Taraxaci folium (Dandelion leaf)
	Taraxaci radix (Dandelion root)
	Urticae radix (Nettle root)
Fascicule 3	Allii sativi bulbus (Garlic bulb)
	Anisi fructus (Aniseed)
	Carvi fructus (Caraway)
	Juniperi fructus (Juniper berries)
	Lichen islandicus (Iceland moss)
	Menthae piperitae aetheroleum (Peppermint oil)
	Menthae piperitae folium (Peppermint leaf)
	Polygalae radix (Senega root)
	Primulae radix (Primula root)
	Rosmarini folium cum flore (Rosemary)
Fascicule 4	Absinthii herba (Wormwood)
	Arnicae flos (Arnica flower)
	Gentianae radix (Gentian root)
	Lupuli flos (Hop strobiles)
	Meliloti herba (Melilotus)
	Passiflorae herba (Passiflora)
	Ribis nigri folium (Blackcurrant leaf)
	Salicis cortex (Willow bark)
	Urticae folium/herba (Nettle leaf and herb)
	Valerianae radix (Valerian root)
Fascicule 5	Aloe capensis (Cape aloes)
	Frangulae cortex (Frangula bark)
	Hamamelidis folium (Hamamelis leaf)
	Ononidis radix (Restharrow root)
	Psyllii semen (Psyllium seed)
	Rhamni purshiani cortex (Cascara)
	Sennae folium (Senna leaf)
	Sennae fructus acutifoliae (Alexandrian Senna pods)
	Sennae fructus angustifoliae (Tinnevelly Senna pods)
	Uvae ursi folium (Bearberry leaf)
Fascicule 6	Centaurii herba (Centaury)
	Crataegi folium cum flore (Hawthorn leaf and flower)
	Echinaceae pallidae radix (Pale Coneflower root)
	Echinaceae purpureae radix (Purple Coneflower root)
	Eucalypti aetheroleum (Eucalyptus oil)
	Hippocastani semen (Horse-chestnut seed)
	Matricariae flos (Matricaria flower)
	Myrrha (Myrrh)
	Rhei radix (Rhubarb root)

4 Industrial plants as sources of dietary supplements

K. Hüsnü Can Baser

The situation in Europe

In Europe, dietary supplements account for 15–20 per cent of the herbal market. Although regulations vary from country to country, in Europe, herbal products are generally considered as medicines. Therefore, full registration with a full dossier on quality, safety and efficacy has to be submitted for pre-market approval. Bibliographic documentation can be based on clinical trials, and the monographs of the European Pharmacopoeia (EP), World Health Organization (WHO), German Commission E and the European Scientific Cooperative on Phytotherapy (ESCOP). There is a simplified way of registering a herbal product as medicine with proof of efficacy based on long-term traditional use in countries like Germany, Austria, Belgium and France. In such a case, the medicine has to carry a disclaimer on the label stating that it is 'traditionally used' (Lawson and Bauer, 1998; Stott, 1998; Grünwald, 1999; Steinhoff, 2001).

In short, the EU has, at present, no legislation like in the USA to regulate dietary supplements (Grünwald, 1999).

New terminology

The expanding world of dietary supplements especially in the USA, has learned new terminology such as 'functional foods', 'novel foods', 'nutraceuticals', 'designer foods', 'pharmafood', 'phytoceuticals'. These terms are used to describe

> one or more phytochemical(s) acting individually, additively or synergistically, usually as component(s) of whole food, that have the characteristic of providing protective, preventive and possibly curing roles in the pathogenesis of cancer and other chronic disease progression. The perceived benefits may be based on the result of epidemiological *in vivo*/*in vitro* cellular organ system on whole organism assay, and may possibly include human clinical trials.
>
> (Shahidi, 1999)

In other words, these terms describe specific foods fortified with ingredients that have or are perceived as having health-promoting effects.

The terms 'nutraceuticals' and 'functional foods' apply to foods or food components that provide a health benefit over and above basic nutrition. Nutraceutical has a broader meaning because it has been applied to foods and food components in both

conventional and non-conventional form (e.g. pills). Functional food, however, refers to products in the form of conventional food (Betz, 1999).

In other words, a nutraceutical is any non-toxic food extract supplement that has scientifically proven health benefits for both the treatment and prevention of disease. Functional food is food that should have a relevant effect on well-being and health or result in a reduction in disease risk (Dillard and German, 2000).

At present, in the USA, there is no legislation governing the use of nutraceuticals or functional foods. The FDA states that existing regulations for foods are sufficient and that companies must decide on intended use (conventional food or dietary supplement) early in product development (Anon., 2000).

'New ingredients' may be regarded as adulterants in foods unless they are naturally occurring or GRAS (generally recognized as safe). An expert panel in the FDA grants GRAS status to food ingredients. Such components are classified as food additives. Food additives that are not GRAS are regarded as unapproved food additives, and their presence in food renders it adulterated (Anon., 2001).

The mechanism of action of the various nutraceuticals varies. Free-radical scavengers and antioxidants can interfere with a number of biochemical mechanisms and may also enhance immune function.

Antioxidants

The main action of antioxidants in human health is realized through their free-radical scavenging and chain breaking features. Oxygen is a potentially toxic element for living systems since it can be transformed by metabolic processes into more reactive forms such as superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radicals, collectively known as active oxygen. In living cells, an enzyme called superoxide dismutase converts superoxide into H_2O_2. Hydrogen peroxide is able to cross all biological membranes. Excessive production of oxygen radicals, particularly hydroxyl radicals can cause lipid cell membranes to reduce lipid peroxides. The hydroxyl radical is very reactive as it combines with almost all molecules found in living cells. Formation of the hydroxyl radical from active oxygen requires catalysis by metal ions, e.g. iron and the copper. The ability of the copper – H_2O_2 system to do severe damage to proteins and DNA is well established. Lipid peroxidation finally leads to loss of membrane function and integrity leading to cell necrosis and death. Hydroxyl radicals can also interact with bases in DNA to cause mutations. Reactive oxygen species (ROS) appear to be an important cause of chronic inflammatory joint diseases such as rheumatoid arthritis of cataracts and of cancer. When antioxidants are present in the body, they dramatically reduce the damage caused by oxidative stress (Cadenas and Packer, 1996).

Antioxidants act as hydrogen atom donors and transform chain-forming radicals to less reactive species. The antioxidant radical so formed is stabilized by delocalization of the unpaired electron over the aromatic ring and over the oxygen atom. Therefore, antioxidant molecules generally possess a phenolic function (Kahkönen *et al.*, 1999).

Antioxidants intervene on lipid peroxidation, protein cross-linking and DNA mutation, thereby reversing the effects of tissue damage. As free radicals also cause cancer, most antioxidants prevent the initiation of cancer and inhibit tumour promotion. Through their action on Ca^{2+} homeostasis, phenolic antioxidants also play a pre-

ventive role in the development of coronary heart disease (CHD) (Rauha *et al.*, 1999; Summanen *et al.*, 2001).

The antioxidant activity of plant phenolics is mainly due to their redox properties through which they act as reducing agents, hydrogen donors and singlet oxygen quenchers and also because of their metal-chelating potential. Plant phenolics include phenolic acids, phenyl propanoids, monoterpenic phenols, flavonoids and tannins. (Shahidi and Naczk, 1995; Cadenas and Packer, 1996; Packer *et al.*, 1999).

Fruits and some vegetables contain a wide range of flavonoids, phenolic acids, carotenoids and terpenoids. The US National Cancer Institute has produced a pyramid showing possible cancer-preventing food and ingredients (Figure 4.1).

Other useful chemicals for human health found in foods are as follows: glucosinolates (Mithen *et al.*, 2000), phenolics (Parr and Bolwell, 2000; Clifford, 2000c), flavones, flavonols, flavanols (Hollman and Arts, 2000), anthocyanins (Clifford, 2000a), proanthocyanidins (Santos-Buelga and Scalbert, 2000), isoflavones, lignans, stilbenes (Cassidy *et al.*, 2000), ellagitannins (Clifford and Scalbert, 2000), hydroxybenzoic acid derivatives (Tomas-Barberan and Clifford, 2000), folic acid and folates

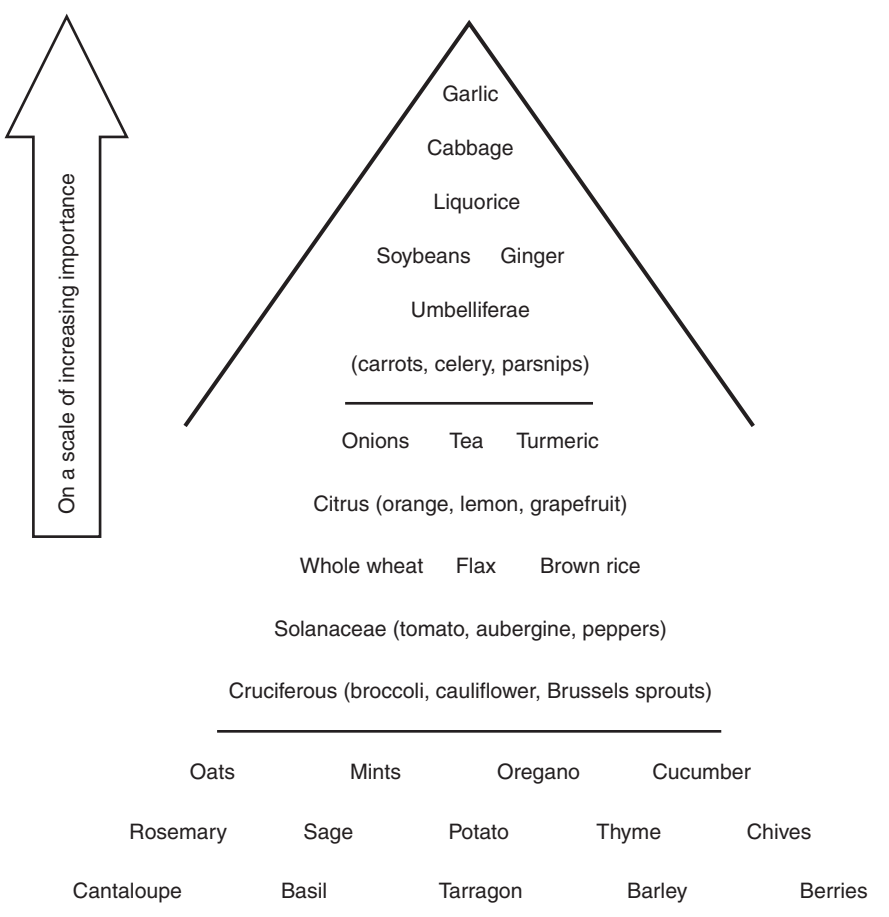

Figure 4.1 Possible cancer-preventive foods and ingredients.

(Scott *et al.*, 2000), chlorogenic acid and cinnamates (Clifford, 2000b), carotenoids (Stahl and Sies, 1999; Khachik *et al.*, 1999; Nishino, 1999), thiosulfinates (Koch and Lawson, 1996), monoterpenoids (Loza-Tavera, 1999; Elson *et al.*, 2000).

As research into antioxidants and other health-beneficial phytochemicals has intensified in recent years, the scientific literature is abounding with numerous reports (Dillard and German, 2000). Therefore, in this review, only those industrial crops used as dietary supplements or nutraceuticals will be covered.

Tea

Tea is the most widely consumed beverage in the world. Of *c.*2.5 million tons of tea 78 per cent consists of black tea, 20 per cent comprises green tea and there is a small percentage of oolong tea (2 per cent). Black tea is produced by controlled fermentation of fresh tea leaves (*Camellia sinensis*) (Hasler, 2000).

Polyphenolic compounds constitute up to 35 per cent of dry weight of tea. Major polyphenolics are flavanols (catechins), flavonols, flavones and phenolic acids; both black and green tea contain catechins as the most significant phytochemicals. Four main catechins and their content in fresh tea flush (percentage dry weight) are (−)-epigallocatechingallate (EGCG) (9–13), (−)-epicatechingallate (ECG) (3–6), (−)-epigallocatechin (EGC) (3–6) and (−)-epicatechin (EC) (1–3) (Shahidi and Naczk, 1995; Mitscher and Dolby, 1998).

These compounds have been shown to possess marked antioxidant activity. The antioxidant action of tea polyphenolics is reported to be related to inhibition of lipid peroxidation and due to free-radical scavenging (Mukai *et al.*, 2000). Epidemiological studies have found that tea consumption reduces the risk of strokes, coronary heart disease, certain cancers and liver disorders (Wiseman *et al.*, 1999). Inhibition of carcinogen in mice by tea constituents has been demonstrated (Yang *et al.*, 2000; Lin *et al.*, 2000). Tea catechins isolated from green tea and theaflavins, especially theaflavin-digallate have been shown to be antiviral (e.g. influenza), antibacterial (e.g. cariogenic bacteria, *Streptococcus mutans, Helicobacter pylori*), and an oral deodorant for suppressing bad breath (Packer *et al.*, 1999).

Soybean

Soybean (*Glycine max* L.) is widely used in the food industry as a source of vegetal protein and dietary supplement. It has been intensely investigated for its ability to treat and prevent various chronic diseases, including cancer (Shahidi and Naczk, 1995).

Its flour has been shown to have antioxidant properties. α-tocopherol and δ-tocopherol are the main antioxidant components of soybean oil. The other antioxidant components of soybean include isoflavones, phospholipids, amino acids, phytic acid and peptides. Phenolic acids such as syringic, vanillic, caffeic, ferulic, p-coumaric and p-hydroxy benzoic acids are also present in soybean products. These compounds also contribute to the antioxidant activity of soybean (Packer *et al.*, 1999).

Soybean is the most significant dietary source of isoflavones. The most important biologically active isoflavones in soybean are genistein and daidzein. Being structurally similar to oestrogenic sterols such as 17 β-oestradiol, these compounds are also known as phyto-oestrogen. These non-steroidal compounds possess both oestrogenic and enzyme-inhibitory actions as well as antioxidant activity. Therefore, they can be used

in treating menopausal symptoms such as hot flush, insomnia, migraine and joint pain. They also reduce cholesterol and protect against osteoporosis. There is also epidemiological evidence for the prophylaxis or therapy of neoplastic diseases (Shahidi and Ho, 2000; Moll and Montalban, 2001).

Rosemary

Leaves of *Rosmarinus officinalis* contain rosmarinic acid and carnosic acid as potent antioxidants. Carnosic acid has been shown to be the most potent antioxidant for animal fat. Carnosic acid and carnosol were found to be responsible for over 90 per cent of the antioxidant activity of rosemary. The other compounds with the same activity include rosmanol, epirosmanol, isorosmanol, rosmaridiphenol, rosmadial and miltirone. Crude and refined extracts of rosemary leaves are commercially available (Shahidi and Naczk, 1995).

Rosemary extracts are largely used for the preservation of food products and have also been shown to inhibit skin, lung, fore stomach, breast, ovary, uterus and colon cancers and leukaemia in mice (Ho *et al.*, 2000).

Tomato

Lycopene, a carotenoid, has recently been shown in epidemiological studies to possess cancer prevention properties. It has been shown that men who consumed processed tomato products such as tomato paste and ketchup, about 10 times a week had statistically less than one half the risk of developing prostate cancer. The action is thought to be related to its antioxidant activity since it has been shown to be the most efficient quencher of singlet oxygen in biological systems. Similar epidemiological evidence has shown that tomato intake may also be related to lesser incidence of other types of cancer such as those that may occur in the digestive tract, pancreas, bladder, cervix, skin and lung (Hasler, 2000).

Lycopene is also present in pink grapefruit, guava and water melon. Since it is one of the rare carotenoids absorbed through the intestines, it is also the most abundant carotenoid found in plasma. As humans are unable to synthesize carotenoids, they have to get them exclusively from diet. At least 85 per cent of our dietary lycopene comes from tomato and its products. Lycopene contents in various fruits and tomato products are shown in Table 4.1 (Bramley, 2000).

Table 4.1 Lycopene content of fruit and tomato products (Bramley, 2000)

Fruit or tomato product	*Lycopene content (μg/g wet weight)*
Tomato paste	54.0–1,500.0
Tomato ketchup	99.0–134.0
Pizza sauce	127.1
Tomato juice	50.0–116.0
Water melon	23.0–72.0
Tomato sauce	62.0
Pink guava	54.0
Papaya	20.0–53.0
Fresh tomato	8.8–42.0
Pink grapefruit	33.6

Lycopene induces gap junctional communication between cells, which is considered to be a basis for protection against cancer development (Zhang *et al.*, 1991). This property is independent of its antioxidant activity. Lycopene has also been reported to alleviate coronary heart disease. This action is thought to be related to its antioxidant activity through preventing the oxidation of serum lipoproteins (Bramley, 2000; Packer *et al.*, 1999).

Garlic

Garlic (*Allium sativum* L.) bulbs have been used as a food with medicinal properties since ancient times. Many consider garlic as a cure-all. Animal and clinical studies have shown that garlic is effective in treating atherosclerosis, bringing down high blood pressure and high cholesterol levels, especially low-density lipoprotein (LDL) cholesterol, the so-called bad cholesterol. It also provides relief from gastrointestinal disorders (Hasler, 2000; Robbers and Tyler, 2000).

Garlic contains organosulphur compounds or thiosulphinates as active ingredients. Allicin has been shown to be responsible for most of its antimicrobial, antimycotic, antiviral, hypocholesterolaemic, hypotensive and antioxidant activities. Allicin is also responsible for the typically strong odour of garlic. It evolves from odourless sulphur containing the amino acid derivative alliin by the action of the enzyme alliinase. Alliin, which is present in garlic at *c.*1 per cent, is immediately converted to allicin by bruising or crushing garlic cloves. One mg of alliin produces *c.*0.5 mg of allicin (Lawson, 1998; Blumenthal *et al.*, 2000).

Distillation of garlic bulbs yields an essential oil (0.1–0.4 per cent) rich in allylsulphides (e.g. diallyldisulphide), ajoenes and vinyldithiins as decomposition products of allicin and other related thiosulphinates. Ajoene, a self-condensation product of allicin, is an antithrombotic agent of garlic, at least as potent as aspirin (Koch and Lawson, 1996; Lawson, 1998).

Epidemiological and clinical studies to prove the anticancer activity of garlic have produced inconclusive and conflicting results. This is largely due to the use of a wide variety of garlic products in investigations. Garlic consumption substantially increases the anticoagulant effects of warfarin and is not recommended for people on warfarin treatment. It is also not recommended during lactation (Blumenthal *et al.*, 2000; Mahady *et al.*, 2001).

Flax seed

Flax (*Linum usitatissimum* L.) is one of the earliest cultivated crops. Apart from textile use of its fibres, flax seed has been used for food for the last *c.*10,000 years. Worldwide, flax seed is used as a mild laxative as well as a poultice for topical application to treat inflammations and similar skin conditions. In the USA, it is consumed as a component of health food or nutraceutical products such as baked foods, breads, bars, breakfast cereals, granolas, etc. (Hasler, 2000).

Flax seed contains fixed oil (30–45 per cent) with 52–76 per cent linolenic acid, proteins (20–25 per cent), mucilage (3–10 per cent), steroids, cyanogenic glycosides (0.1–1.5 per cent) composed mostly of linustatin, neolinustatin, linamarin, lotaustralin, high content of soluble and insoluble fibre and secoisolariciresinol diglucoside (SDG) and matairesinol, precursors of lignans, enterodiol and enterolactone (Westcott

and Muir, 2000). Lignans are diphenolic compounds containing a 2,3-dibenzylbutane structure. Enterodiol and enterolactone possess weak oestrogenic and anti-oestrogenic activities suggesting a possible preventive role in oestrogen-dependent cancers. Preventive effects on colon cancer have been shown in animal studies. Flax seed supplementation has been shown to lower serum cholesterol (Oomah and Mazza, 2000; Blumenthal *et al.*, 2000).

Clinical studies suggested that flax seed supplementation has been successfully used in treating various disorders such as types of cancer and lupus nephritis. It reduces atherogenic risk in hyperlipidemic patients, improves arterial function and positively affects platelet composition and function (Oomah and Mazza, 2000).

Anticarcinogenic, hypolipidemic activities of flax seed as well as its beneficial effects in treating systemic lupus erythematosus (SLE) and rheumatoid arthritis have been put down to its high content of α-linolenic acid and secoisolariciresinol (Blumenthal *et al.*, 2000).

Ginkgo

A standardized dry extract of the dried leaves of *Ginkgo biloba* L. (GBE) contains 24 per cent flavonoids (mostly flavone glycosides and quercetin) and 6 per cent terpenes (mainly ginkgolides composed of 2.9 per cent bilobalide and 3.1 per cent ginkgolides A, B, C and J) (Braquet, 1988; Ahlemeyer and Krieglstein, 1998).

GBE is widely used and is sold as a prescription and over-the-counter (OTC) drug both in solid and liquid dosage forms for its beneficial effects on the circulatory system as a safe and efficacious drug. It is used in the treatment of cerebral dysfunction symptoms, dementia, vertigo, tinnitus and peripheral arterial occlusive disease. There are also indications that it is an effective antioxidant with free-radical scavenging activity (Hori *et al.*, 1997; Foster and Tyler, 2000).

Due to the necessity to use high doses, GBE instead of the herb itself is used. Therefore, unless standardized extracts are used no beneficial effects of *Ginkgo* can be achieved. Recommended doses are 120–240 mg standardized dry extract (2 times or 3 times a day) in solid or liquid forms for oral use (Halpern, 1998; Blumenthal *et al.*, 2000; Mahady *et al.*, 2001).

St John's wort

Leaves and flowering tops of *Hypericum perforatum* L. (Hypericaceae) have in recent years become quite popular for their antidepressant properties. The plant has been known to mankind since time immemorial for the wound-healing properties of its olive oil macerate which acquires a reddish colour when exposed to sun for several weeks. This red oil is highly valued since it can be applied externally and internally to relieve inflammations and promote healing. The colour and the activity are related to the presence of hypericin, a reddish dianthrone pigment. Excessive use of this herb induces photosensitivity on exposure to direct sunlight. The damage is characterized by dermatitis and inflammation of the skin and mucous membranes. In Germany, St John's wort is an approved medicine and licensed as a medicinal tea. It is marketed in various dosage forms to treat mild depression Leung and Foster, 1996; Foster and Tyler, 2000).

In the USA, it is widely used as a dietary supplement in various forms such as

alcoholic tincture, aqueous infusion for oral use, oil infusion for topical use and standardized dry extract in capsules and tablets. United States Pharmacopoeia (USP) notes the safety of St John's wort and has published a monograph for assessing its purity. USP requires not less than 0.2 per cent of hypericin and pseudohypericin combined and not less than 3.0 per cent of hyperforin (Anon., 1999).

Several clinical studies have confirmed the antidepressant action of St John's wort extracts in humans. St John's wort contains 6.5–15 per cent catechic tannins and proanthocyanidins (e.g., catechin, epicatechin, leucocyanidin); flavonoids (e.g. hyperoside – up to 2 per cent; rutin – up to 1.6 per cent; quercetin – up to 0.3 per cent); biflavonoids (*c.*0.26 per cent biapigenins); phloroglucinol derivatives (e.g. hyperforin – up to 4 per cent); 0.05–1.0 per cent essential oil containing mainly higher *n*-alkanes; 0.05–0.15 per cent naphthodianthrones (e.g. hypericin and pseudohypericin); xanthones (up to 10 ppm); sterols; and vitamins (A and C) (Reuter, 1998; Blumenthal *et al.*, 2000; Mahady *et al.*, 2001).

For the constituents of St John's wort the following activities have been reported: hypericin (antiviral), pseudohypericin (antiviral), hyperforin (antibacterial, wound-healing, neurotransmitter inhibitor, potential anticarcinogenic), biapigenins (anti-inflammatory, antiulcerogenic, probably sedative), proanthocyanidins (antioxidant, antimicrobial, antiviral, vasorelaxant), xanthones (antidepressant, antimicrobial, antiviral, diuretic, cardiotonic, monoamine oxidase inhibitor), quercitrin (*in vitro* MAO inhibitor) (AHP, 1997).

Recommended doses for St John's wort products are as follows: powdered or chopped herb: 2–4 g daily; fluid extract 1:1 (g/ml): 2 ml, 2 times daily; dry extract 5–7:1:300 mg, 3 times daily; oily macerate: directly apply on affected areas (Blumenthal *et al.*, 2000).

Echinacea

Echinacea has been a popular remedy since the early 20th century in the USA and Europe for preventing and treating the common cold, influenza and upper respiratory tract infections. It is considered as a potent immunostimulant and also used to treat vaginal candidiasis (Bauer and Wagner, 1990; Foster and Tyler, 2000).

Roots and herbs of *Echinacea purpurea, E. pallida* and *E. angustifolia* are used as sources of echinacea products. Fresh pressed juice of the aerial parts with 22 per cent ethanol by volume as a preservative and alcoholic extract of the roots are approved for use. Echinacea polysaccharides are responsible for its immune-stimulant effect. Stimulation of phagocytosis is apparently enhanced by the alkamides (isobutylamides), glycoproteins and cichoric acid. Roots and herbs of official *Echinacea* species contain caffeic acid derivatives, alkamides and polysaccharides as active ingredients (Bauer, 1998).

Ginseng

Korean ginseng (*Panax ginseng* C.A. Meyer) (Araliaceae) and American ginseng (*P. quinquefolius* L.) are two species used as the principal sources of ginseng. The former grows in Korea, northern China and parts of Siberia where it is harvested from the wild. In this form it is known as 'white ginseng'. It may sometimes be bleached and dried, or sometimes processed and sold as 'red ginseng'. American ginseng is harvested from wild sources and cultivated, especially in China (Blumenthal *et al.*, 2000).

In Chinese medicine, it is used as a general tonic to provide energy and considered as a panacea (cure-all). However, recent randomized, controlled clinical trials did not provide substantive evidence to support its purported ergogenic effects in healthy subjects. Some evidence is available for treating erectile dysfunction and age-associated memory-impairment with ginseng. It is also an antioxidant and immune stimulant. Ginseng is generally considered as an aphrodisiac (Robbers and Tyler, 2000).

Active principles responsible for most of ginseng's activities are believed to be ginsenosides (or panaxosides). These are a series of triterpenoid saponins. Ginseng root contains 2–3 per cent ginsenosides with Rg1, Rc, Rd, Rb1, Rb2 and Rb0 being the most important (Sticher, 1998).

The German Commission E suggested a daily dose of ginseng as 1–2 g of root per day for up to three months, or 200–600 mg of standardized extracts (4–7 per cent ginsenosides) (Blumenthal *et al.*, 2000; Mahady *et al.*, 2001).

Dietary fibre

Dietary fibre is composed mainly of non-starch polysaccharides and lignin. Water-soluble forms of dietary fibre includes pectins, beta-glucans, gums and mucilages while the insoluble portion consists of cellulose, lignin and hemicelluloses. Barley and oats have greater contents of soluble fibre than do wheat and maize. The use of dietary fibre in human diet has been shown to have association with heart disease, cancer, diabetes and obesity. Wheat bran may have an effect on oestrogen metabolism as well (Kritchevsky and Bonefield, 1994).

Phytates and phytate derivatives (e.g. phytic acid) are found in flax, wheat germ and bran (Thompson, 1994). These compounds possess antioxidant properties with potential use in food preservation. They also have hypocholesterolemic, anticarcinogenic, hypolipidemic and hypoglycemic effects (Oomah and Mazza, 2000).

Concluding remarks

In this short review, I have attempted to cover most industrial plants that are commonly used as dietary supplements. There are, certainly, many more such materials. However, it is almost impossible to limit the growing number of plant materials currently used to enrich food to provide health benefits. Therefore, I conclude with the prophecy that the future of plant-based dietary supplements looks very bright and that many more new materials in the form of plant extracts and essential oils will enter the global marketplace within the next decade.

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5 Drug-like compounds from food plants and spices

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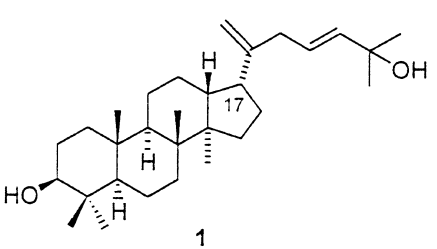
Introduction

Since nutritional research has traditionally focused on macronutrients (proteins, lipids, sugars) and essential micronutrients (vitamins, minerals), food plants have long been assumed to contain few if any secondary metabolites apart from those revealed by our senses and responsible for their taste and flavour. Over the past few decades, evidence has, however, been mounting that food plants also contain a host of secondary metabolites which, though generally undetected by our senses, can nevertheless contribute to human well-being and play a role in the maintenance of health (Pisha and Pezzuto, 1994). Evidence for the dietary intake of biologically active small molecules can be traced to two distinct lines of research, namely the study of the detrimental effects of the inordinately large consumption of single food plants, and the recognition that food and medicines can interact, sometimes with dramatic consequences. Research in these areas has always been intense, and recent highlights are the identification of the configurationally unusual (17-α side-chain) dammarane **1** as the immunosuppressive principle of Palmyra palm flour (Révész *et al.*, 1999), and that of the furanocoumarin **2** (bergamottin) as the main cause of the 'grapefruit effect' which plagues the pharmacokinetics of several important drugs (He *et al.*, 1998).

The other side of the coin, namely the health-promoting effects of dietary secondary metabolites, has so far received relatively little systematic attention. This is most surprising, not only because of the vast anecdotal literature on the subject ('An apple a day keeps the doctor away'), but also in consideration of the fact that the first two 'controlled' clinical trials were carried out with food plants, namely the investigation of the benefits of crushed onions as a wound dressing by the French army surgeon Ambroise Paré in the 16th century, and that of the anti-scurvy properties of *Citrus* fruits by the British physician James Lind in 1747 (Watts, 2001). Food and medicine have undoubtedly shaded into each other for a long time. The full continuum from



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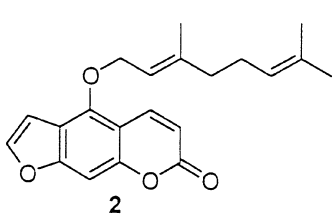

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food to medicine, an integral part of Chinese and Indian medicine, was well recognized in the Greco-Roman tradition, and certain food plants (garlic, onion, parsley, pepper) have always enjoyed a 'more than a food, less than a drug' status. Nevertheless, mainstream pharmaceutical research has never considered food as a likely source of pharmacologically active compounds.

Several explanations can be put forward as to why food has been positioned outside the domain of therapeutics. First, the majority of the health-promoting effects of dietary secondary metabolites are in the realm of chronic and degenerative diseases (cancer, diabetes, Alzheimer's and cardiovascular diseases). These are the result of the combined effect of several genes and environmental factors, and variation in their incidence can only be evidenced by large and costly epidemiological studies. Furthermore, the identification of an individual nutrient responsible for a diet-related disease which can be reproduced in laboratory animals is methodologically much easier than tracing a nutrient or a class of nutrients which reduce the incidence of a disease in humans. Next, data on the occurrence of non-nutritional phytochemicals in food are rare, information regarding their absorption and metabolism in humans is minimal, and the elucidation of the molecular bases for the observed activity can be potentially plagued by entourage effects, which make it difficult to identify a single 'active principle'. The current renaissance for the therapeutic application of mixtures of compounds is cogently exemplified by the drug cocktails used in the treatment of cancer, AIDS and hypertension, but combinations of active principles are more easily planned following mechanistic clues rather than 'decoded' from an active matrix. Last, but not least, most health-promoting activities of food plants are preventive rather than curative. Prevention has always produced less fanfare than the latest medical breakthrough, though transition from therapeutic to preventive medicine is making great strides with the current huge investments for the discovery of 'high-tech' cures, with an increased dependence on the wealth of information available from the human genome project.

Despite these difficulties and limitations, diet can still be a productive hunting ground for pharmaceutical leads with a definite biological activity, and we have tried to overview the area from a pharmaceutical point of view. Currently, most research in this field is fuelled by the growing dietary supplements industry, but the diet–medicine interface is also rich in opportunities for the pharmaceutical industry. The discussion of the 'natural' occurrence of mainstream drugs in food will show how blurred this interface is, and we will next move on to review the basic chemistry and biochemistry of small molecules of dietary origin currently under clinical development, concluding with an overview of a selection of further agents which might soon enter clinical studies.

Mainstream drugs from edible plants

The background 'pharmaceutical noise' of dietary origin

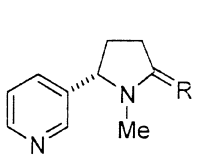
Certain active pharmaceutical ingredients occur naturally in food plants and spices. With a few notable exceptions (statins, xanthines, capsaicinoids), the concentrations are very low and unlikely to exert any direct significant pharmacological activity. This background dietary 'pharmaceutical noise' can, however, become significant under particular conditions, especially in the realm of exposure to recreational drugs like tobacco and opioids.

The interference of dietary nicotine (**3a**) in studies of exposure to environmental tobacco smoke has long been debated. Nicotine is widely distributed, and has been found in twelve plant families, with detectable amounts in many food plants. Thus, levels up to 100 μg/kg have been measured in aubergines, and also non-solanaceous vegetables like cauliflower can accumulate nicotine (Domino *et al*., 1993). The dietary intake of nicotine is unlikely to produce any direct pharmacological or toxicological effect. For comparison, a cigarette contains on average 8–9 mg nicotine, 1 mg of which is delivered to the circulation of the smoker (Benowitz and Henningfield, 1994). This amount corresponds to the consumption of 10 kg of unprocessed aubergine or 20 kg of puréed tomatoes, and a dietary addition to nicotine is clearly inconceivable. Nevertheless, the amount of nicotine to which passive smokers are exposed can be comparable to that of dietary origin. The possibility therefore exists that dietary uptake underlies the puzzling detection of nicotine and its metabolite cotinine (**3b**) in biological fluids of non-smokers, mimicking the inhalation of nicotine from passive smoke and complicating the interpretation of studies of exposure to environmental cigarette smoke. The vegetable equivalent of a toxicologically meaningful exposure to tobacco smoke is difficult to determine. Thus, while studies on the availability of inhaled nicotine are straightforward because of the efficacy of this way of administration, the bioavailability of nicotine after dietary ingestion is difficult to assess. Removal of vegetable skin, where most nicotine is stored, significantly reduces the exposure. On the other hand, nicotine survives a variety of processing operations, from preparation of tomato ketchup and sauce to frying and boiling of potatoes, and higher concentrations are found in these processed products. The absorption of ingested nicotine is poor and erratic, and most of the compound entering the circulation from the gastrointestinal tract is subjected to a significant (up to 70 per cent) first-pass metabolism in the liver (Henningfield, 1993). To complicate the situation even further, furanocoumarins from parsley and related vegetables can inhibit the metabolic oxidation of nicotine to cotinine, resulting in a much higher half-life of this alkaloid and a reduced urinary level of cotinine, the hallmark of nicotine exposure (Johnston, 2000). For all these reasons, it is not clear if, and to what extent, the dietary uptake of nicotine can interfere with studies of exposure to passive smoke. Contamination from the use as a pesticide was suggested to be responsible for the detection of nicotine, sometimes at levels higher than in solanaceous fruits, in tea leaves (Sigmund *et al.*, 1999). Intake of nicotine via tea consumption is therefore controversial, also because nicotine, presumably because of the interaction with polyphenolics, is not efficiently extracted during tea brewing.

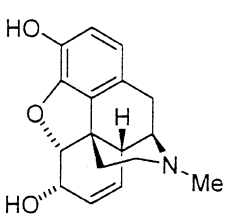
Aubergines, tomatoes, potatoes and tea are the four major dietary sources of nicotine. Based on data on the averaged daily consumption of these plants and products thereof, the highest dietary intake of nicotine (*c.*2.25 μg/day) should take place in Italy and Portugal (Sigmund *et al.*, 1999).

A situation somewhat similar to that of nicotine exists for morphine (4). This and related alkaloids are synthesized *ex novo* by many organisms, including several mammals, invertebrates and insects, but they are accumulated in sizeable amounts only by a few representatives of the poppy family, and notably by the opium poppy (*Papaver somniferum* L.) (Kosterliz, 1987). The small black seeds of some varieties of this plant are a common garnish on rolls and muffins, and are also used in making jams. The seeds contain low amounts of morphine (4–250 mg/kg) and codeine (0.4–60 mg/kg), mostly from contamination by the latex, and are a legal source of opiates. It has been shown that consumption of a single (!) poppy seed muffin can lead

to a positive GC-MS urinary assay for opiates, mimicking the recreational use of heroin (Perrine, 1996), while morphine addiction was reported in a baker who was regularly drinking a tea made from poppy seeds (King *et al.*, 1997). The so-called poppy seeds defence has often been claimed by people accused of abusing opiates, and has been the subject of considerable forensic investigation. These considerations do not apply to the common corn poppy (*Papaver rhoeas* L.), whose leaves are eaten just like spinach, since this plant contains tetrahydrobenzazepine alkaloids and lacks morphine and related alkaloids (Brunetton, 1999).



R
3a H,H
3b O



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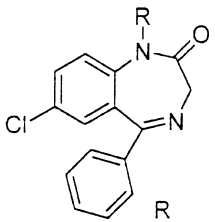


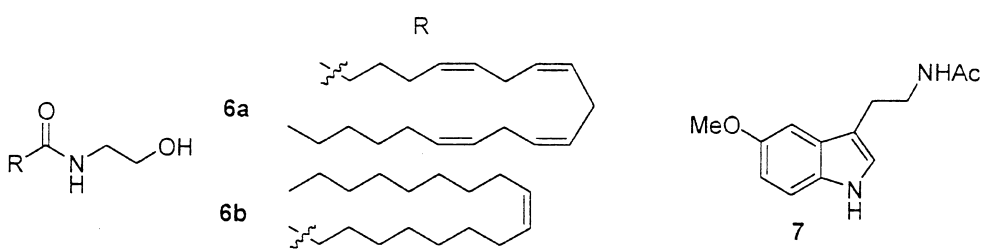
R
5a Me
5b H

Nicotine and morphine are not the only psychoactive drugs for which a dietary source exists. Over the past few years, evidence has been growing that benzodiazepines (BDZs), and especially diazepam (**5a**) and desmethyldiazepam (**5b**), occur naturally in plants, with levels in the range 0.05–1 μg/kg measured in several fruits and vegetables, including potatoes, cherries, corn, soy and various cereals (wheat, oat, rice) (Klotz, 1991). Since benzodiazepines could be found also in food products and brain samples pre-dating the introduction of these compounds into the clinic in the early 1960s, it is very unlikely that a sort of worldwide industrial contamination similar to that of DDT or of the synthetic musks can be blamed. The observation that the content of BDZs increases during the germination of potatoes and wheat grains suggests a specific and hitherto unknown role for these compounds (Klotz, 1991). Indeed, a forebear of the benzodiazepine receptor has been detected in the pond-dwelling bacterium *Rhodobacter spaeroides*, where it acts as a sort of 'oxygen sniffer', regulating the balance between photosynthesis and oxygen respiration (Yeliseev *et al.*, 2001). Remarkably, this is achieved by modulating the production of cytochromes, a well-known activity of BDZs in mammal cells. BDZs can be formed endogenously by higher plant cells in the absence of microorganisms (Kavvadias *et al.*, 2000), and food intake seemingly accounts for their detection in brain tissues, where these compounds might be incorporated via the food chain. The amounts of natural BDZs in food plants are very small, and unlikely to exert any pharmacological effect. Thus, 25,000 kg of wheat grains or 10,000 kg of potatoes would have to be ingested to attain the exposure level corresponding to the average pharmacological dose of diazepam (Klotz, 1991). Nevertheless, the possibility exists that selective trapping might lead to accumulation into specific compartments of the CNS or in specific subcellular structures.

A further interesting case is the occurrence of cannabinomimetics in cocoa and chocolate. The lipid fraction of cocoa pods has been reported to contain anandamide (*N*-arachidonoylethanolamine, **6a**), the major endogenous cannabinoid, and related amides (di Tomaso *et al.*, 1996). These compounds also occur in chocolate, where the ethanolamides of unsaturated acids, mainly oleic (**6b**) and linoleic acids, were detected

in concentrations ranging from 0.5 to 90 mg/kg. Oleyl and linoleyl ethanolamides do not activate brain cannabinoid receptors, but can inhibit the metabolic inactivation of anandamide, and a contribution to the hedonic properties of chocolate was postulated for these compounds (di Tomaso *et al.*, 1996).

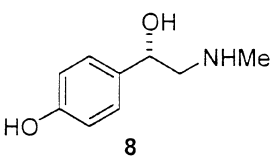
These data are puzzling, since higher plants cannot produce arachidonic acid. Furthermore, the amounts of *N*-acylethanolamides later detected in cocoa powder and chocolate were much lower (0.01–5.8 mg/kg), and comparable to those present in other dietary lipid matrices, like hazelnuts and milk (Di Marzo *et al.*, 1998). The latter, incidentally, contains a relatively high concentration of another endocannabinoid, 2-arachidonoylglycerol, as well as of the sleep-inducing lipid oleamide. *N*-Acylethanolamides have a very low oral bioavailability (<5 per cent of the ingested dose), presumably because of hydrolysis by the enzyme fatty acid amide hydrolase (FAAH), expressed in high concentration in the gastrointestinal tract. It is therefore unlikely that cannabinomimetics are involved in the satisfying and craving-inducing effects of chocolate (Di Marzo *et al.*, 1998). Cannabinoids stimulate appetite, and are involved in weight homeostasis. Thus, knock-out mice for leptin, a hormone which curbs appetite, have abnormally high levels of endocannabinoids in their brains, and injection of leptin brings them to normality (Di Marzo *et al.*, 2001). The cannabinoid antagonist SR141716 is under clinical evaluation as an antiobesity treatment, and it has been suggested that factors other than the sheer caloric intake are responsible for the development of obesity (Pool, 1997). For the reasons discussed above, it is, however, unlikely that endocannabinoids and cannabimimetics in food plants play a role in abnormal weight gain.



Melatonin (7) and related indoleamines are involved in the regulation of the circadian rhythm of vertebrates. These compounds also occur in a large number of fruits and vegetables, and high level (>1 μg/kg) have been found in cereals (oat, rice) and corn, where a possible role in the protection of germ tissues has been postulated (Dubbels *et al.*, 1995). The effect of dietary melatonin on the plasma level of this hormone was investigated, showing that dietary melatonin is efficiently absorbed from the gut and can cross the haematoencephalic barrier (Hattori *et al.*, 1995).

A final example of a 'dietary drug' is synephrine (**8**), a vasoconstrictor α-adrenergic agent which is used, as the tartrate salt of the racemate, to relieve nasal congestion and as a mild cardiac stimulant. Certain plants have the capacity to hydroxylate the benzyl position of phenylethylamines. Adrenaline has not been found outside the animal kingdom, but (*S*)-noradrenaline is accumulated, along with dopamine, in banana skins, contributing to their browning, while (*S*)-synephrine (**8**) and *N*-methyltyrosine (**9**) are found in *Citrus* leaves and fruits. Concentrations of **8** up to 1 g/L have been detected in the juice of certain varieties of tangerine (*Citrus reticulata* Blanco), while

the peel and the immature fruits of certain *Citrus* species contain 0.2–0.4 per cent synephrine (Kusu *et al.*, 1995). *Aurantii nobilis pericarpum* and *Aurantii fructus immaturus* are used as drugs in China and Japan as expectorants and antitussives as well as 'regulators of vital energy'. *Citrus* fruit extracts have also become popular in Western countries as antiobesity agents (Calapai *et al.*, 1999). The alleged lipolytic properties are seemingly mediated by the activation of the β_3-receptors of fat deposits, but this claim appears largely unsubstantiated, and concerns about the safety of the *Citrus* preparations have been raised because of the cardio-circulatory effects of synephrine (Calapai *et al.*, 1999). Natural synephrine is the *S*-enantiomer, but partial thermal racemization can occur during the extraction from orange peel (Kusu *et al.*, 1995). Since the two enantiomers have different pharmacological activities, this adds a further layer of complexity in the evaluation of the pharmacological and toxicological profile of *Citrus* dietary supplements. The natural occurrence of trace amounts of *S*-naproxene (**10**) in banana has also been reported. This compound is structurally unrelated to natural naphthalene derivatives, and its biosynthesis is unknown (Luis *et al.*, 2000).



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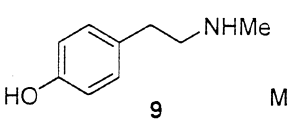

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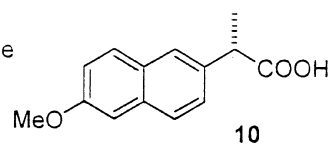

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Dietary pharmaceutical leads

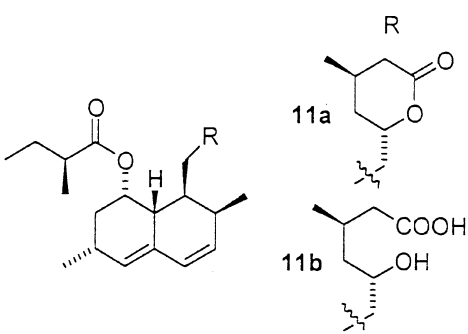
Though interesting, the 'natural' occurrence of trace amounts of pharmaceutical active ingredients in food plants has no practical relevance for the drug discovery process, since it is realized *a posteriori* after the introduction of an agent into the clinics. On the other hand, the discovery of important drugs can be traced back to their occurrence in our diet. The most important examples are statins, xanthines and capsaicinoids, which are the archetypal compounds of important classes of pharmacological agents.

Statins

Statins are polyketides which lower blood cholesterol with a mechanism related to the blocking of the enzyme HMG-CoA reductase, the principal step in the biosynthesis of isoprenoids. Because of their *in vitro* inhibitory activity on cholesterol biosynthesis, statins were introduced into the clinic as a first-line therapy for patients at risk of myocardial infarction because of hypercholesterolemia. Successive studies have shown that these compounds substantially fail to reduce cholesterol synthesis *in vivo*, and rather exert their hypocholesterolemic effects via an increased expression of the low-density lipoprotein (LDL) receptors (Moghadasian, 1999). Statins show only low cholesterol-lowering activity in common experimental animals such as mice and rats. This, coupled with their substantial lack of *in vivo* inhibition of cholesterol biosynthesis, makes us ponder how roundabout and logic-defying the process of drug development can be. Statins have revolutionized the treatment of lipid abnormalities, and show outstanding effectiveness and safety. Their market has now expanded to a substantial 10 billion dollars per year, and millions of patients all over the world are taking them daily to fend off the risk of myocardial infarction. These compounds are

useful in decreasing cardiovascular morbidity and mortality in patients at risk as well as in people with a normal cholesterol level and no sign of cardiovascular disease. Furthermore, meta-analyses have shown that statins can also reduce death from all causes, suggesting a pleiotropic pattern of activity and beneficial effects which transcend their cholesterol-lowering activity (Vogel, 1999). Thus, statins can promote bone formation, and are of enormous potential for the treatment of osteoporosis (Mundy *et al.*, 1999), while preventive activity against Alzheimer's disease has also been demonstrated (Gorman, 2000). The blocking of other lipids involved in cell function seemingly underlies the expanding range of indications of statins.

The archetypal statin is lovastatin (monacolin K, **11a**), a polyketide originally isolated in Japan from various *Monascus* species, including the red yeast of rice (*Monascus ruber* van Tieghen), an ingredient of oriental cuisine used to give the typical colour and taste to Peking duck and other dishes (Endo, 1979). The medicinal use of red yeast rice has been documented since AD 800, and the cholesterol-lowering efficacy of its extracts has been confirmed in double-blind clinical trials (Chang, 2000). The concentration of lovastatin (**11a**) and its lactone-opened active form (**11b**) in red yeast rice extracts is *c.*0.3 per cent, with a total monacolin content higher than 0.4 per cent (Ma *et al.*, 2000).



The monacolin fraction is seemingly responsible for the cholesterol-lowering activity of red yeast rice. Apart from lovastatin, six additional compounds have been isolated, with structural variation involving semireduction of the diene system and the dehydration of the γ-lactone ring (Ma *et al.*, 2000). Red yeast rice also contains a series of polyketide pigments, including trace amounts of the mycotoxin citrinine. Further constituents of this traditional Chinese food are a series of polyunsaturated fatty acids with up to five double bonds, which might contribute to the cholesterol-lowering effect of the extracts.

Red yeast rice has recently been at the centre of a legal battle between the US Food and Drugs Administration (FDA) and Pharmanex Inc., a company producing and commercializing a red yeast rice extract for the healthfood market. This product, known as Cholestin, is prepared from *Monascus purpureus* Went, a variety of *M. ruber*. According to the FDA, any product containing a constituent approved or under investigation as a drug is legally a drug and not a dietary supplement. Claiming that the strain of *Monascus* used to prepare Cholestin had been selected for its high production of lovastatin, the FDA tried to ban the sale of Cholestin and other supplements based on red yeast rice in the USA. The import of red yeast rice from China was blocked in 1998, but Pharmanex was then granted a partial relief, allowing it to import the

amount of red yeast rice sufficient to meet its sales projections. In February 1999, the FDA's ban on the import of red yeast rice was lifted, but it appealed the decision, and the saga is still going on (Israelsen, 1999). At the same time, Merck, which manufactures lovastatin, and Bristol-Myers Squibb, which produces pravastatin, a successful analogue, requested permission to sell to the general public their pharmaceutical-grade products at doses comparable to those found in the red yeast rice extracts (Gorman, 2000). These requests were, however, turned down.

The Cholestin issue will certainly become a test case for the dietary supplement industry, shaping its future legal state. Hopefully, it will also foster interesting research. Thus, at the doses indicated by the producer (2.4 g per day, corresponding to *c.*10 mg lovastatin), Cholestin contains lovastatin in insufficient quantity for a significant reduction of the cholesterol blood level, which requires dosages of 20–80 mg per day. Nevertheless, a random double-blind study carried out in China on hyperlipidaemic patients to compare the effects of a *Monascus* extract and lovastatin, showed a comparable activity. Furthermore, a decrease of the triglyceride level was only observed for the *Monascus* extract, whose activity apparently increased in the course of the treatment (Chang, 2000). These observations were substantially confirmed in further studies, but larger trials, financially challenging for dietary companies, are needed. The bioavailability of lovastatin as a pure active principle is poor. Only 30 per cent of an oral dose is absorbed, and hepatic activation by hydrolysis to the δ-hydroxy acid form is required for activity (Moghadasian, 1999). It is therefore possible that certain constituents of *Monascus* extracts boost the bioavailability of lovastatin, which is well known, for instance, to be markedly increased by meals. Another attractive hypothesis is that *Monascus* constituents like fatty acids and sterols interact in a synergistic way with the lipid-lowering properties of statins (Heber, 1999).

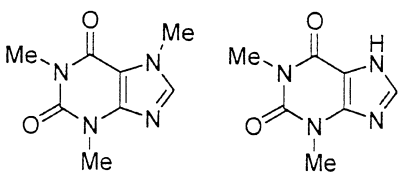
Myopathy, the major side-effect of statins, was apparently not observed with *Monascus* extracts, but the number of patients enrolled in the studies and the low frequency (<0.2 per cent) of this lovastatin side-effect make the observation of limited, if any, value. Lovastatin has teratogenic effects in animals, and is contra-indicated in pregnant women and in women of child-bearing age who might become pregnant. Furthermore, statins were introduced in therapy only in the late 1980s, and their long-term side-effects are not known. The cost of a treatment with Cholestin is only a quarter to a tenth of one with statins, and the 'Cholestin affair' is obviously more than an entangled regulatory matter, opening questions having far-reaching ethical implications.

Xanthines

Purine alkaloids have a rather limited distribution in nature, but are accumulated by the coffee and tea plants, as well as in the seeds of the cacao and cola trees and the leaves of the maté bush (Dewick, 1997). The enormous and global relevance of these plants reflects in the thousands of articles available on all aspects of their history, botany, chemistry, pharmacology and social relevance (Hobhouse, 1987). We will therefore only highlight the biomedical relevance of the two most important dietary purine alkaloids, namely caffeine and theophylline.

Coffee is the most important source of caffeine (**12a**) in the Western diet, and the 'more than a food, less than a drug' status of this beverage has long been recognized, with the stimulating effects rather than the flavour having largely contributed to its

popularity (Wills, 1994). Caffeine is used today mainly in association with antipyretic and antalgics, to treat fever, aches and the symptoms of flu. Caffeine is also employed to increase the intestinal absorption of ergotamine in anti-migraine preparation and, topically, to activate lipolysis and reduce subcutaneous fat deposits (Rall, 1990). Caffeine is efficiently absorbed after oral ingestion, and beverage consumption provides the major route of dietary exposure. A cup of coffee contains from 30 to 150 mg caffeine, depending on the method of preparation and the variety employed. Also tea and cola drinks provide significant amounts of this alkaloid (from 10 to 100 mg per serving) (Wills, 1994). It should be pointed out, however, that the bioavailability of caffeine from a food matrix can be strongly reduced by polyphenols, which are abundant particularly in tea.



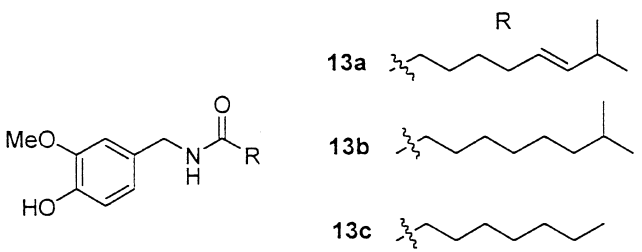
12a **12b**

Caffeine is readily available as a by-product of the preparation of decaffeinated coffee. Theophylline (**12b**) is only a minor component of tea leaves and cola nuts, and is more conveniently synthesized than isolated. The major pharmaceutical use of theophylline is for the treatment of acute attacks of asthma and of bronchospasm associated with chronic obstructive pulmonary disease. These applications can be traced back to the seminal observation by Salter in 1860 that strong black coffee can relieve the breathing problems of asthmatic patients. Interestingly, theophylline is much more potent as a bronchodilatator *in vivo* than *in vitro*, and one therefore ponders if this important drug might ever have been discovered without the clinical observations on coffee drinkers by the physicians of the nineteenth century (Sneader, 1996). The two best characterized molecular actions of caffeine and theophylline are the antagonism of adenosine and the inhibition of phosphodiesterases. Natural xanthines lack discrimination between the various subclasses of adenosine receptors, but suitable substitution can produce selective agents. They also show phosphodiesterase inhibiting activity, and have served as a lead for the synthesis of analogues, one of which (pentoxyfylline) is currently employed as a peripheral vasodilator (Rall, 1990). The increased knowledge of the molecular mechanism of the activity of xanthines undoubtedly underlies the resurgence of interest in the natural purine alkaloids.

Capsaicine

With more than 20 per cent of the human population consuming it daily and almost 8,000 km^2 dedicated to its cultivation, chilli pepper (*Capsicum annuum* L.) is the most popular spice on the planet, and its pungent principle capsaicin (**13a**) is possibly the most important pharmacological agent we get from our diet (Naj, 1992). No compound better than capsaicin exemplifies the blurred boundaries between food and medicine. Hot pepper contains a mixture of *N*-acylvanillamides (NAVA) which are responsible for its pungent taste and most of the pharmacological activity traditionally

ascribed to this spice. The contents of NAVA depends on the variety of pepper, and can reach 1 per cent in the variety Habanero. The major (*c.*50 per cent) NAVA of hot pepper is capsaicin, a compound first isolated from chilli in 1849. Among the analogues, the dihydro derivative (**13b**) and the nordihydro derivative are the most abundant (*c.*35 per cent and 7 per cent, respectively) (Suzuki and Iwai, 1984), but the so-called synthetic capsaicin (*N*-pelargonylvanillamide or nonivamide, **13c**) has also been isolated from the chilli oleoresin (Constant *et al.*, 1996). Interestingly, these compounds are replaced by the corresponding non-pungent esters (capsiates) in sweet pepper, where, apparently, transamination of vanillin is blocked, while the polyketide route to extend a valine starter is still operative.



Apart from pungency, the first pharmacological property discovered for capsaicin was the capacity to induce hypothermia (Szallasi and Blumberg, 1999). Capsaicin can induce a profuse perspiration, so-called gustatory sweating, which leads to a decrease of body temperature and is probably responsible for the popularity of hot pepper in warm climates, where heat loss is beneficial. Capsaicin is a pleiotropic pharmacological agent that shows activity in an impressive variety of pharmacological assays. However, some of these activities, like neurotoxicity, can be evidenced only at high doses, and are probably aspecific and of no dietary or practical pharmacological relevance. Nowadays, research on capsaicin focuses mainly on its analgesic and appetite-suppressant activities. Capsaicin is the archetypal vanilloid, a class of compound which targets a heat-sensitive ion channel (the so-called vanilloid receptor-1, VR1) involved in pain sensation and neurogenic inflammation (Szallasi and Blumberg, 1999). Under physiological conditions, the channel is activated by an increase of temperature and a fall in pH. Binding of capsaicin lowers the temperature threshold of the channel, which is activated at room temperature also by what would otherwise be subliminal stimuli (Caterina *et al.*, 1997). Expression of VR1 is not limited to the nerve endings of the lingual epithelium, but is typical of a set of neurons involved in pain sensation and neurogenic inflammation.

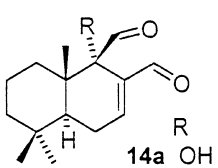
Binding to VR1 underlies the biological activities of capsaicin. This compound acts as a sort of 'heat surrogate', and ultimately causes desensitization of the receptor, with block of transmission of algic information (Caterina *et al.*, 1997). The endocannabinoid anandamide shows vanilloid activity, and has been suggested as the endogenous modulator of VR1 (Szolcsanyi, 2000). Both the murine and the human versions of the receptor have been cloned, and knock-out mice for VR1 have also been generated (Caterina *et al.*, 2000). These animals have an impaired pain sensation, but are otherwise normal, an observation which validates VR1 as a pharmacological target. The sheer frequency at which hot pepper has been featured on the front cover of the major scientific journals testifies the current excitement on vanilloid research.

Data on the oral bioavailability of capsaicin are contradictory, and this compound is

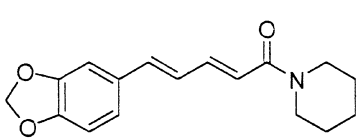
partly eliminated with the faeces, causing intense anal burning. For these reasons, capsaicin is employed only in topic forms, as a constituent of pain-relieving preparations. The initial strong burning sensation causes problems with patients' compliance, greatly limiting the use of these products, whose efficacy is obviously difficult to assess in double-bind studies. Furthermore, capsaicin is poorly absorbed from the human skin, where it is extensively metabolized by amidases (Szallasi and Blumberg, 1999).

Despite these limitations, the manipulation of the vanilloid receptor has great relevance to the discovery of new analgesics, and capsaicin has indeed been at the centre of three major industrial medicinal chemistry efforts (Procter & Gamble, Sandoz, Glaxo) (Wrigglesworth and Walpole, 1998). These investigations failed to substantially improve the natural product, but detailed the structure–activity relationships, and led to the discovery of the non-pungent analogue olvanil and the vanilloid antagonist capsazepine. Orally active analogues have been synthesized, and one of them, capsavanil, is currently undergoing pharmaceutical development in the USA and Korea (Park *et al.*, 2000).

Various explanations have been put forward as to why humans crave culinary pungency. In this context an interesting observation is that the bark of *Warburgia ugandensis*, which is used in Africa just like hot pepper, contains the pungent dialdehyde warburganal (**14a**), while another pungent dialdehyde, polygodial (**14b**) is contained in the European water pepper (*Polygonum hydropiper* L.), once used as a pepper substitute (Sterner and Szallasi, 1999). These unsaturated dialdehydes behave as biological analogues of capsaicin, targeting VR1 and evoking responses not unlike those elicited by capsaicin. Also piperine (**15**) from black pepper (*Piper nigrum* L.) shows vanilloid activity, suggesting a remarkable molecular convergence of pungency target between the hot cuisine of America, Africa, Europe and Asia. Capsaicin has gastroprotective activity, and chilli pepper has been shown to counteract the adverse gastric effects of aspirin and to display bactericidal activity against *Helicobacter pylori* (Szallasi and Blumberg, 1999). The beneficial effects of pungent compounds on the gastrointestinal tract, along with their effects on body temperature, have undoubtedly contributed to the popularity of hot cuisine.



14a OH
14b H



15

Dietary hot pepper increases energy expenditure and diminishes long-term excess energy intake, making capsaicin a potential slimming agent (Henry and Emery, 1986). This activity might be related to the presence of vanilloid receptors in the area postrema and the nucleus of the solitary tract, a zone involved in satiety (Szallasi and Blumberg, 1999). Though relatively unexplored, this area might receive increasing attention on account of the current social relevance of obesity.

Last but not least, capsaicin is also interesting for cancer research, curiously both as an anticancer and as a cancer-inducing agent. Capsaicin selectively inhibits plasma membrane NADH oxidase of cancer cells, binding to an enzyme (tNOX) which is expressed specifically on tumour cells (Morré *et al.*, 1995). Claims that hot pepper

consumption can induce tumour formation in the gastrointestinal tracts of rodents seem flawed by the exorbitant doses employed (150 mg/kg per day, over 100 times the average human consumption in tropical countries, where cuisine is hotter than in Western countries). Epidemiological investigations aimed at comparing the incidence of stomach cancer in hot pepper consumers and non-consumers gave conflicting results. Studies carried out in Mexico and India found a correlation between chilli pepper consumption and gastric cancer, while an Italian study reached an opposite conclusion, namely that regular eating of chilli pepper has a protective activity against gastric cancer (Szallasi and Blumberg, 1999).

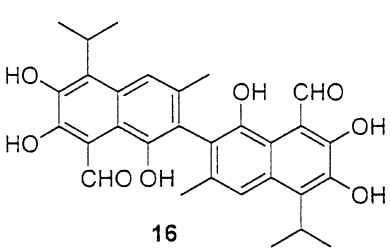
Mutagenesis studies have established that capsaicin is *per se* not mutagenic, but can produce mutagenic metabolites when activated by the liver and at very high concentrations, unlikely to occur in humans through dietary or medicinal exposure (Szallasi and Blumberg, 1999).

Small molecules of dietary origin under clinical development

A literature search, mainly based on the Journal *Drugs of the Future*, has evidenced several molecules of dietary origin which, over the past two decades, have proceeded from *in vitro* assays into clinical development. Most of these compounds are phenolics, and will now be reviewed, updating the literature up to March 2001.

Gossypol (Xian Oil and Fat Works, PRC; NY Hospital; Cornell University, USA)

In the 1950s, Chinese scientists discovered that the culinary use of crude cottonseed oil leads to infertility. In the following decades, further investigations revealed that males are much more sensitive than females to the 'cottonseed oil effect', and identified the antifertility principle in gossypol (**16**) (Leeson, 1979), a yellow dimeric cadinane sesquiterpene first reported in 1937 as the major pigment of the seeds of cotton, but present, in trace amounts, also in soybean and sunflower seeds (Adams *et al.*, 1960).



16

Due to its lipophylicity, gossypol is partly extracted during the oil production, making cottonseed oil unsuitable for human consumption unless processed, generally by simple heating. Owing to its multifunctional nature, gossypol is rather reactive. It is easily degraded by heating, and reacts spontaneously with the terminal amino groups of lysine-rich proteins, substantially decreasing the value of cotton oil cake. Though only mildly poisonous in assays of acute toxicity (oral LD_{50} = 2.6 g in rats), gossypol can cause trouble in non-ruminant animals, presumably due to accumulation.

The symptoms of poisoning are gastrointestinal distress and shortness of breath, seemingly due to a reduction of the oxygen-carrying capacity of blood. Severe poisoning results in cardiovascular effect and pulmonary oedema (Adams *et al.*, 1960).

Gossypol is contained in distinct pigment glands in the seeds and the root bark of cotton plants, at a level ranging from 0.3 per cent to 2 per cent of the dried plant material. Higher concentrations, up to 7 per cent on a dried weight basis, were found in other plants from the Malvaceae family. It was first synthesized in 1958, and a more recent synthesis was reported in 1989. Gossypol is axially chiral, and occurs in nature with various degrees of optical purity, from the almost pure (+) form as in the bark of *Thespia populnea* and *Montezuma speciosissima*, to extensively racemized mixtures, where the (+)-enantiomer prevails, as in most species and cultivars of cotton (Jaroszewski *et al.*, 1992). A remarkable exception is *Gossypium barbadense* L., one of the two cotton plants of the New World, which produces (−) gossypol of low optical purity (13 per cent) (Jaroszewski *et al.*, 1992). The natural (+) form has the *S* configuration (*P* according to the helicity rule), and the two atropisomeric forms are very stable, with theoretical studies showing a prohibiting barrier to rotational inversion, as expected from the presence of four substituents *ortho* to the bond connecting the two naphthyl moieties. Gossypol is obtained as a racemate when precipitated with acetic acid from plant extracts, since neither the (+) nor the (−) enantiomer forms a solvate with acetic acid. Gossypol is produced by oxidative phenolic dimerization of its monomeric form, hemigossypol, and it is not known what determines the occurrence of a particular ratio of the (+) and (−) forms. The enantiomeric enrichment might derive from either a biosynthetic process or from an enantiomeric cell turnover of the racemate. The two enantiomeric forms of gossypol show remarkable differences in biological activity (Joseph *et al.*, 1986), and it is regrettable that in many investigations the enantiomeric composition of the compound was not stated. Gossypol exists in solution in at least three different tautomeric forms, which have, however, been poorly characterized (Adams *et al*., 1960).

Clinical investigations on the antifertility activity of (±)-gossypol started in the 1970s and involved almost 9,000 healthy subjects. The administration schedule consisted of an attack oral dosage of 200 mg per day for two months, followed by a maintenance monthly dosage of 150–220 mg in divided doses for a period of 2–4 years (Leeson, 1979). After the two-month attack dose, semen analysis (necrospermia or sperm count below 4 million per mL), showed complete infertility in 99.9 per cent of all cases. Side effects were noticed, especially hypokalaemia and irreversible azospermia with consequent irreversible infertility once the treatment was ended. This eventually led to discontinuation of research on gossypol, with the World Health Organization declaring it unacceptable as an antifertility drug. This decision drew considerable criticism, since the data on the major side-effects (hypokalaemia and irreversible azospermia) were rather confusing (Yu and Chan, 1998). Furthermore, the toxic effects were mainly traced to the (+)-enantiomer, while most of the antifertility activity resides in the (−) form, and a chiral switch might lead to an improved clinical profile. Interestingly, the pure (−)-enantiomer is not a natural product – gossypol has never been reported to occur in this form alone (Jaroszewski *et al*., 1992).

Gossypol is absorbed relatively slowly after oral administration, with a half-life of more than 9 h in the gastrointestinal tract. Elimination is also slow, and occurs mainly via the intestinal route. The metabolism is oxidative, with formation of gossypolone, which retains part of the biological activity of the parent compound. The antifertility

activity of gossypol is due to prevention of implantation in females and to destruction of the seminiferous tubules with consequent oligospermia in males. The molecular mechanism is unknown. In *in vitro* assays, gossypol is a powerful inhibitor of lactate dehydrogenase, but it is not clear how this translates into the observed antifertility activity.

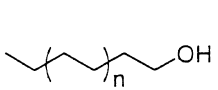
In the 1990s, interest in gossypol was rekindled by the discovery of its antiproliferative effects, selective for cancer cells, and more pronounced for the (−) form compared to the racemate (Joseph *et al.*, 1986). Clinical investigations were carried out in women with metastatic breast cancer resistant to doxorubicin and paclitaxel, and promising results were obtained. The dosages employed were 30–50 mg/day, considerably higher than those used in the antifertility trials (Seideman *et al.*, 1999). Similar encouraging results were also observed in patients with recurrent adult malignant gliomas and with metastatic adrenal tumours refractory to conventional chemotherapy. The mechanism of the anticancer activity has not yet been elucidated. In cultures of normal breast epithelial cells, gossypol does not cause cell death, though incorporation of ^{3}H-tymidine is inhibited in a dose-dependent way (Gilbert *et al.*, 1995). Biopsies from patients evidenced a reduction in cyclin D1 expression and an increase of the nuclear pRb expression. Blockage of cell-to-cell communication was also evidenced.

Gossypol is also a powerful inhibitor of prostate 5alpha-reductase, and has been considered for the treatment of benign prostate hyperplasia (BPH), also in consideration of its inhibiting activity on prostate epithelial cells' proliferation (Shidaifat *et al.*, 1996). Activity against the genital herpes and HIV viruses was also described. While the (−) enantiomer exhibited good anti-HIV activity, the (+) enantiomer was active only at cytotoxic concentration. The mechanism of anti-HIV activity is unknown.

Taken together, the biological studies show that gossypol is endowed with a pleiotropic pattern of activity, with a recent shift in clinical attention from the reproductive to the oncologic realm of applications. Gossypol seemingly interacts with specific biological target(s), whose identity is, however, still elusive. Gossypol is also a remarkable example of a chirally axial compound where remarkable differences in biological activity exist between the two enantiomeric forms.

Policosanol (Laboratorios Dalmer, Cuba)

Policosanol is a mixture of high-molecular-weight aliphatic alcohols obtained by saponification of sugarcane wax (*Saccharum officinale* L.). Simple extraction with organic solvents of the saponification mixture is sufficient to afford a material with a definite melting point (72–82 °C) and a relatively constant composition (Más, 2000). The very high batch-to-batch reproducibility is presumably due to the poor genetic variation of sugarcane and in general of crop plants. This is potentially dangerous from an agricultural standpoint, but is undoubtedly an important asset for the pharmaceutical exploitation of these plants. The composition of policosanol can be assessed by GC-analysis after silylation. The major constituent is 1-octacosanol (**17a**, 60–70 per cent), accompanied by its higher and lower homologues (1-triacontanol (**17b**, 10–115 per cent) and 1-hexadecanol (**17c**, 4.5–10 per cent).



	n
17a	12
17b	13
17c	11

Policosanol inhibits *in vitro* the biosynthesis of cholesterol, acting at a yet unidentified step between acetate consumption and mevalonate synthesis. It has no direct effect on HMG-CoA reductase, whose activity is nevertheless modulated by policosanol. Policosanol decreases the susceptibility of LDL to peroxidation, interferes with platelet aggregation and neointimal proliferation, and reduces the blood level of thromboxane B_2. All these activities are of great relevance for the prevention of arteriosclerosis and coronary heart disease. At least as regards the inhibition of cholesterol biosynthesis, the most active constituent of policosanol is hexacosanol, which, however, has an overall lower activity than the mixture, suggesting a certain level of synergy, either pharmacodynamic or pharmacokinetic. Overall, the pharmacological activity of policosanol is poorly understood at a molecular level. The bioavailability is, however, excellent, since [^{3}H]-octacosanol is rapidly (0.5–2 h) absorbed after oral administration. Elimination is, however, rather slowly, with a $t_{1/2}$ in humans of *c.*140 h.

Policosanol was discovered and developed in Cuba, where most of the clinical studies were carried out. These include randomized, double-blind, short- and long-term, placebo-controlled studies, as well as comparative, open and post-marketing surveillance investigations (Más, 2000). At doses of 5–20 mg/day, policosanol showed a significant reduction of LDL-cholesterol, total cholesterol and various atherogenic indices. The effect on triglycerides was, however, modest and erratic. This profile of anticholesterol activity is somewhat similar to that of statins, and comparative, randomized, double-blind studies showed that the decrease of LDL-cholesterol and total cholesterol obtained with 10 mg/day of policosanol was comparable to that observed with lovastatin at 20 mg/day. Remarkably, only policosanol could increase the HDL-cholesterol level (Más, 2000), and the superior activity of policosanol on HDL-cholesterol was also observed when this agent was compared to simvastatin and pravastatin (Ortensi *et al.*, 1997). Policosanol was also better tolerated than statins, and myalgya, the main side-effect of statins, was not observed. In a major post-marketing surveillance study on 3,609 patients who were followed for five years, weight loss was the most common cause for withdrawal of policosanol. It should be pointed out, however, that the number of patients involved in these studies was relatively small compared to the clinical studies of statins, and the conclusions should therefore be treated with caution.

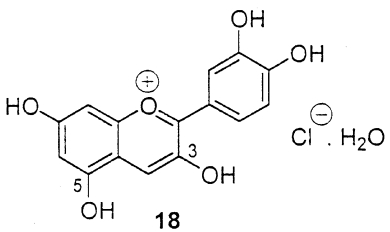
Policosanol can also be employed in combination with other cholesterol-lowering drugs. Of particular relevance is the combination with fibrates, which control serum triglycerides in a way complementary to statins and policosanol. The combined therapy with policosanol and gemfibrate gave synergistic effects, while the combination of policosanol and benzfibrate did not show any advantage over benzfibrate alone.

In conclusion, clinical studies have established a long-lasting cholesterol-lowering effect of policosanol, but a clearly identified molecular target has not yet been identified. The scientific isolation of Cuba, where the compound was discovered and developed and the troubled nature of its relationship with the USA, the major pharmaceutical market, have so far hampered the development of policosanol as a mainstream pharmaceutical.

Cyanidin (IdB-1027, Inverni della Beffa, Italy)

Cyanidin (18), a magenta-coloured phenolic, is the most widespread constituent of the anthocyanin fraction from berries and edible fruits, where it occurs in a range of

glycosidic forms. There is variation in the nature and location of the bound sugars as well as the presence of acyl residues (mainly derived from phenylpropanoid acids and dicarboxylic acids) on the sugar moieties. The most widespread derivatives of cyanidin are the 3-monoglucoside and the 3,5-diglucosides (Brouillard, 1988). Anthocyanins show a variety of useful properties *in vitro* (antioxidant, antiatherosclerotic, chemopreventive, anti-inflammatory), and their daily human consumption has been estimated in the range 180–215 mg (Kühnau, 1976). It is not known if these amounts can exert the beneficial effects observed in the *in vitro* experiments.



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The cyanidin-based anthocyanin fraction from bilberry shows remarkable anti-ulcer and ulcer-healing properties, and this observation prompted the investigation of cyanidin itself (Magistretti and Pifferi, 1989). In animal studies, cyanidin turned out to be effective in preserving gastric mucosal integrity, an effect mediated by an increased gastric luminal release of the protective factor PGE_2 (Mertznielsen *et al.*, 1990). The activity was confirmed in phase II clinical studies, which disclosed efficacy in the prevention of mucosal damage induced by aspirin (Barzaghi *et al.*, 1991). No effect on gastric acidity was evidenced, and the stimulation of PGE_2 secretion was confirmed at oral doses of 600 mg twice a day. Taken together, the animal and clinical studies show that cyanidin promotes the efficiency of the gastric mucosal barrier, and that this effect is mediated by a local release of PGE_2. The molecular mechanism underlying the increased secretion of PGE_2 is, however, unknown.

Toxicity studies in animals showed an excellent tolerance, with oral acute toxicity ranging from over 6 g/kg in rodents to over 3 g/kg in dogs. Doses of up to 2 g/kg per day were well tolerated in rats and monkeys, while no teratogenic or mutagenic effects were evidenced in rats and rabbits. In phase I studies, dosages up to 1.2 g per day were well tolerated in healthy volunteers (Magistretti and Pifferi, 1989). The lack of toxicity of cyanidin is not surprising, since the major industrial application of anthocyanins is as food additives.

Very little is known on the absorption and metabolism of cyanidin, a matter complicated by the instability of the product at physiological pH values and by its complex equilibria with hydrate and quinoid forms. Evidence for the human absorption of cyanidin glycosides has been provided (Miyazawa *et al.*, 1999), backing up an earlier report on the rat (Morazzoni *et al.*, 1991). Hydrolysis to cyanidin was not observed, and the plasmatic incorporation was low (*c.*1 per cent of the dose) but rapid, reaching a maximum 15 minutes after the ingestion. Intravenous administration of anthocyanins led to a rapid tissue distribution, while the flaviliun structure is apparently metabolically stable against bacterial hydrolysis and metabolic glucuronidation. Cyanidin is more conveniently synthesized than isolated. The commercial synthesis relies on the acid-catalysed condensation of ω-hydroxyresacetophenone triacetate and phloroglucinolcarboxaldehyde dibenzoate, followed by hydrolitic removal of the ben-

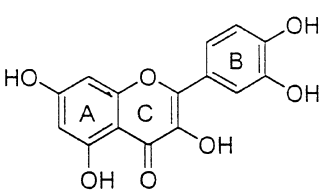
zoate groups under controlled basic conditions to avoid degradation of the alkali-labile anthocyanin core (Scheme 1). The product is obtained as the chloride salt monohydrate (Magistretti and Pifferi, 1989).

Recently, anthocyanins from red cherries made headline news for their powerful anti-inflammatory activity (Wang *et al.*, 1999). This was traced back to the inhibition of COX2 and, in part, COX1. This finding is surprising and difficult to reconciliate with the PG_2-promoting activity of cyanidin. On the other hand, the crude anthocianidin fraction from cherry showed a better anti-inflammatory activity than ibuprofen and aspirin, showing potential as a natural 'super-aspirin' on account of its selectivity towards COX1 (Anon., 1999).

Quercetin (Beijin Chemical Reagent Factory PRC, Taiyuan Second Pharmaceutical Factory, China)

Quercetin (19) is the archetypal dietary flavonoid. In glycosidic form it is almost ubiquitous in fruits and vegetables, with significant amounts occurring in onions, tomatoes and apples as well as in wine (Hertog *et al.*, 1992). With the exception of onions, quercetin generally occur in skin tissues. On average, the human diet contains almost 1 g flavonoid per day, and flavonoids have been implicated in the protective effect of the consumption of large amounts of fruits and vegetables against cancer and heart diseases. Evidence for this correlation is, at present, weak, especially as regards the chemopreventive activity (Hollman and Katan, 1999).

As the major dietary flavonoid, quercetin has been extensively investigated as a pharmacological agent (Jiyun *et al.*, 1997). Quercetin shows all the key structural elements underlying the radical-stabilizing properties of flavonoids, namely the 3-hydroxyl and the olefin bond in the heterocyclic ring and the catechol system on ring B. The presence of the 4-oxo-3,5-dihydroxy moiety also guarantees excellent metal-binding properties, and, unsurprisingly, quercetin shows very high antioxidant activity, with a TEAC index comparable to that of epigallocatechin gallate (Pietta, 2000). These properties are, however, attenuated by glycosidation of the 3-hydroxyl, as commonly encountered in the natural form of quercetin.



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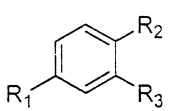


	R_1	R_2	R_3
20a	CH_2COOH	OH	OH
20b	CH_2COOH	OH	OMe
20c	COOH	OH	OMe
20d	COOH	OH	OH
20e	COgly	OH	OMe

The oxidative theory of atherosclerosis highlights the role of oxidized lipoproteins, and especially LDL, in the pathogenesis of this condition. Several studies have shown that quercetin has excellent preventive activity on the oxidation of LDL *in vitro*, and can also scavenge superoxide anion and prevent its formation by oxidative stress. Quercetin has therefore a remarkable cardiovascular potential, with applications in the realm of the prevention of cerebral ischemia, cardiac arrhythmias and myocardial infarction (Jiyun *et al.*, 1997).

Quercetin is also an inhibitor of PKC, the molecular target of tumour-promoting

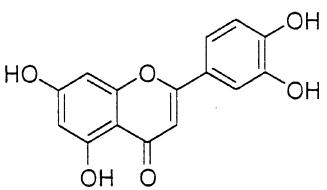
phorbol esters (Feri *et al.*, 1989), and can inhibit cell proliferation in a reversible and dose-dependent way. The anticancer activity has been linked to the presence of type II EBS receptors, for which quercetin shows selective affinity. The cytotoxicity is synergically magnified by the combination with genistein, and quercetin was also effective at reversing resistance to adriamicin. Most interestingly, quercetin can inhibit the expression of PSA and the androgen receptors on prostate cells, and holds potential for the prevention of prostate cancer.

Only limited data are available on the pharmacokinetics of quercetin. Its glycosidated forms were long assumed not to be absorbed after oral ingestion, but in 1995 a high intestinal absorption of onion quercetin rutinoside (17 per cent) as well as of quercetin itself (24 per cent) in ileostomized humans was demonstrated (Hollman *et al.*, 1995), showing that absorption in the small intestine is possible. Quercetin 3-rutinoside was also shown to be absorbed after eating tomato purée, and the sugar moiety was found to have an important role for the absorption, which was highest when the sugar residue was glucose (Hollman *et al.*, 1997). Flavonoids generally occur in food plants as β-glycosides. No general digestive enzyme capable of splitting the β-glycosidic bond is excreted into the gut or is present in the intestinal wall (Kühnau, 1976), but hydrolysis can only take place in the colon, where β-glycosidases of broad specificity are present in the bacterial flora. However, enzymes capable of degrading flavonoids are expressed, and the overall absorption is therefore poor (Kühnau, 1976). The phenolic acids resulting from the degradation of flavonoids can be absorbed and show a decreased, but still significant antioxidant activity. For quercetin itself, formation of the acids **20a–e** was evidenced (Pietta, 2000). The pharmacokinetic of quercetin by i.v. administration was investigated in rabbits, and a bioavailability of 43 per cent was found. Rapid metabolization was also observed, but the metabolites were not characterized (Zhao *et al.*, 1992). In humans, plasma levels $>1.0\ \mu M$ were detected after a quercetin-free diet and the ingestion of a single meal corresponding to 200 mg quercetin. The long half-life (*c.* 25 h) means that repeated intake leads to accumulation (Strick *et al.*, 2000). Taken together, these data show that substantial differences between the pharmacokinetics and the metabolism of quercetin (and presumably of other flavonoids as well) exist between humans and experimental animals.

Dietary flavonoids and especially quercetin have been associated with a decreased risk of adult malignancies. Yet they have also been related to infant leukaemia, a disease which is triggered by events occurring *in utero* (Ross, 2000). Thus, quercetin (and genistein as well) can inhibit topoisomerase II, causing abnormalities in a gene (MLL) related to infant leukaemia indistinguishable from those elicited by the chemotherapeutic agents etoposide and doxorubicin. Remarkably, the potency of quercetin in topo-II inhibitory assays was found similar to that of etoposide (Strick *et al.*, 2000). Bioflavonoids assumed during pregnancy from food or dietary supplement can cross the placenta and are found in fetal tissues, and have been identified as a major causative agent for infant, and possibly childhood, leukaemia (Ross, 2000). Given the ubiquity of the dietary exposure to flavonoids, it is unclear why infant leukaemia is so rare (less than 40 cases per million newborns). Other causes, like an impaired DNA repair, are probably involved, and ongoing molecular epidemiology studies will hopefully clarify this important matter.

Luteolin (Anhui Provincial Institute of Medical Sciences, PRC)

Luteolin (21) is, along with apigenin, the major flavonoid of cereal grains and aromatic herbs (parsley, rosemary, thyme) (Pietta, 2000). Owing to the lack of the 3-hydroxyl, it shows reduced antioxidant activity compared to quercetin. Luteolin is currently under development for the treatment of chronic obstructive bronchitis (Wang, 2000). The compound is available by synthesis from phloroglucinol or by semi-synthesis from hesperidin, a by-product of the *Citrus* industry. The activity of luteolin is quite different from that of the conventional drugs used to treat bronchitis. Thus, luteolin shows a combination of antibacterial, expectorant, antitussive and anti-inflammatory activity. This, coupled with a very low toxicity, makes luteolin an attractive drug to treat chronic bronchitis, and good efficacy was observed in preliminary clinical studies, with substantial improvement of all major symptoms, including cough (Anhui Medical University Study Group, 1985).



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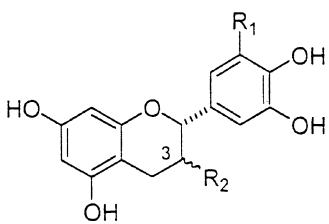


	R_1	R_2
22a	OH	α-galloyl
22b	OH	α-OH
22c	H	α–galloyl
22d	H	α-OH
22e	H	β-OH

Pharmacokinetic experiments in rats with the radiolabelled compound showed a surprisingly good and rapid oral absorption in rats, with a wide distribution and selective accumulation in liver and kidney. Luteolin shows a very low oral toxicity, with an LD_{50} value >400 mg/kg for the parenteral administration (Wang, 2000). Nevertheless, owing to its coagulant and vasodilatatory activity, luteolin should be administered with great care in patients with cardiovascular diseases. Luteolin shows selective cytotoxicity *in vitro*, but a study on azoxymethane-induced colon carcinogenesis in rats evidenced only a poor chemopreventive activity (Matsunaga *et al.*, 2000).

Epigallocathechin gallate (National Cancer Research Center Institute, Japan; Pharmanex, USA)

(−)-Epigallocatechin gallate (EGCG, **22a**) is the archetypal flavan-3-ol (catechin), an important group of polyphenols. These compounds can be isolated as individual monomers or as part of oligomeric assemblies formed by Friedel–Crafts alkylation of ring A by the C-4 cation of another unit. The process can be repeated several times, eventually affording high-weight oligomers known as non-hydrolysable tannins or pro(antho)cyanidins (Bombardelli and Morazzoni, 1995). Procyanidins are ubiquitous in the plant kingdom, and occur in high concentration in many edible plants (apples, grapes, persimmons, legumes) and products thereof (cocoa, malt, beer, wine). Monomeric catechins are of more limited distribution, but are found in substantial amounts in tea (100–400 mg/L) and red wine (30–100 mg/L, with exceptional levels – up to 300 mg/L – in those made from Pinot Noir grapes). Industrial processing can

lead to the almost complete destruction of monomeric catechins. Thus, both apples and grapes contain sizeable amounts of catechins, but the commercial products thereof are almost devoid of these compounds (Arts *et al.*, 2000). Pressing, storage and decoloration by treatment with activated carbon are probably responsible for the loss. Oligomeric catechins can be de-polymerized to catechins by the intestinal flora (Heilmann and Merfort, 1998), and therefore oligomeric and monomeric flavan-3-ols share many aspects of their clinical potential.

(−)-EGCG (**22a**) is the major constituent of the catechin fraction from green tea (Yoshizawa *et al.*, 1987). Accompanying compounds are (−)-epigallocatechin (**22b**), (−)-epicatechin-3-gallate (**22c**), (−)-epicatechin (**22d**) and (+)-catechin (**22e**). A mixture of these compounds is obtained from tea leaves with hot water, followed by partition with chloroform to remove caffeine, and re-extraction with ethyl acetate. EGCG is peculiar to tea, and gallic catechins are overall rare, being absent for instance in grapes and wine (Arts *et al.*, 2000). Because of its powerful antioxidant, anticarcinogen, antiviral and antibacterial properties, EGCG has attracted considerable interest within the biomedical community (Fujiki and Okuda, 1992). *In vitro* studies have suggested a strong potential to prevent chronic diseases, but not all epidemiological studies found a significant clinical efficacy, and the human epidemiology remains inconclusive (Woodward and Tunstall-Pedoe, 1999).

Interest in the Japanese green tea was sparked by epidemiological studies from the Japanese Ministry of Health which revealed a lower mortality rate from total cancers (particularly stomach cancer) in the prefectures where green tea was produced and consumed in large amounts. Tests of antimutagenic activity identified EGCG as the most active tea polyphenol (Fujiki and Okuda, 1992). EGCG is also the most powerful of all tea catechins in *in vivo* assays for antioxidant and anticancer activity, though epicatechin gallate (**22c**) outperforms EGCG in apoptotic assays (Chung *et al.*, 2001).

The twofold trihydroxyphenyl motif plays an important role in the antioxidant activity (Benzie and Szeto, 1999). EGCG presumably acts as a chain-breaking antioxidant, trapping peroxyl radicals and quenching the chain oxidation. The specific mechanism of antioxidant activity remains unclear. The structure of the major compounds formed by reaction with the peroxyl radical was elucidated, disclosing that ring B is the prime site of oxidation, but a host of multiple product-forming reactions was also evidenced (Valcic *et al.*, 2000) EGCG shows iron-chelating action, and this could contribute to the chemopreventive activity. When tested in Ames assays, EGCG exhibits a protective effect (63 per cent protection) against peroxide-induced DNA damage. The activity is 100-fold more potent compared to vitamin C, and superior by a factor of almost two to that of red wine.

The radical scavenging and protein binding activities are common, with different degrees of potency, to almost all polyphenols. Nevertheless, an impressive number of interactions with specific targets have been reported for EGCG. Thus, EGCG is seemingly the most potent polyphenol for the inhibition of tumour-promotion triggered by phorbol esters, can interact with the genomic expression and the release of tumour necrosis factor-α (TNF-α), and has been shown to inhibit urokinase, a proteolytic enzyme crucial for cancer growth (Jankun *et al.*, 1997). Last but not least, it potently inhibits the activity of protein kinase C (Stammler and Volm, 1997), downregulating the expression of certain proteins related to drug resistance.

Antiviral activity was also reported for EGCG, which can reduce the infectivity of

the influenza A and B viruses interfering with the absorption step. EGCG and other phenolics of green tea showed antibacterial activity against strains of *Helicobacter pylori* obtained from patients with gastric ulcers (Yamada *et al.*, 1997). Inhibition of the the glycosyl transferases of *Streptococcus mutans* blocks the elaboration of the sticky glucan employed by these caryogenic bacteria to adhere to the tooth enamel, suggesting a possible use of EGCG for the control of dental caries (Hattori *et al.*, 1990).

The isolation of pure epigallocatechin gallate from green tea is tedious, low-yielding and costly, making the daily consumption of green tea extracts as the most practical and cheapest way to benefit from the antimicrobial and cancer chemo-preventive activities of this compound. Furthermore, a synergistic activity with other constituents has been evidenced (Suganuma *et al.*, 1999). Thus, apoptosis and inhibition of TNF-α induced by EGCG on lung cancer cell line PC-9 are enhanced in a dose-dependent way by epicatechin, another constituent of green tea, which seemingly helps the cellular uptake of the other catechins. Studies on the induction of apoptosis by EGCG showed that this compound increases the formation of reactive oxygen species and leads to mitochondrial depolariziation (Chung *et al.*, 2001). This pro-oxidant activity was also observed in other cancer cells and is rather surprising on account of the efficiency by which EGCG quenches reactive oxygen species *in vitro*.

Because of the synergistic interaction within the catechin fraction, whole green tea might be more effective than EGCG alone for cancer prevention One cup of green tea infusion contains about 100–200 mg of polyphenols. These are rapidly absorbed, reaching a peak plasma level 1 h after oral administration. After oral ingestion, EGCG could be detected unchanged in blood and tissues (small intestine, stomach, liver, lung and skin, the preferential targets of chemoprevention). Toxicity studies in animals and phase I trials in humans have indicated the general safety of EGCG.

Consumption of tea in the world ranks second only to that of water. Its beneficial effects have been demonstrated, but, not unlike wine, synergistic interactions might be involved. This, coupled with the chemical instability of EGCG, poses formidable obstacles to its pharmaceutical development as a mainstream drug. Nevertheless, the natural product might be used as a lead for derivatives more easily amenable to drug development.

Curcumin (Central Drug Research Institute, India)

Curcumin (**23a**) is a yellow diarylheptanoid pigment found in turmeric, the dried rhizomes of *Curcuma longa* L. Turmeric is widely used as a spice and to flavour food and beverages, and, as a major ingredient of curry, is produced in very large amounts (>160,000 tonnes per year) (Govindarajan, 1980). It contains up to 2 per cent curcuminoids on a dry weight basis. Commercial curcumin is a mixture of three compounds, namely curcumin (**23a**) and its mono- and bis-demethyl derivatives (**23b** and **23c**, respectively). These compounds can be separated by chromatography (Osawa *et al.*, 1995; Anderson *et al.*, 2000) and presumably show differences in their biological properties. Nevertheless, commercial curcumin has been employed in many investigations, complicating the interpretation of the bioactivity data.

Curcumin is one of the most thoroughly investigated dietary secondary metabolites, and over 600 papers on its chemistry and bioactivity have appeared in the past five years. It shows powerful activity in *in vitro* assays of anticancer, antiviral and anti-inflammatory activity, presumably because of excellent antioxidant properties (Srimal, 1987). It can also lower plasma cholesterol, and shows bactericidal activity against clinically relevant human pathogens. This excellent pharmacological profile has led to clinical investigation to assess to what extent the data from the animal studies could actually be translated into the clinics. The US National Cancer Institute (NCI) is currently investigating its activity as a chemopreventive agent, while the compound is being developed as an anti-inflammatory agent in India.

The mechanism of antioxidant activity of curcumin has been well investigated. Curcumin can form an extensively conjugated phenol radical, which quenches the radical oxidation cycle by either dimerization (Masuda *et al.*, 1999) or reaction with a lipid peroxide (Masuda *et al.,* 2000). Both reactions involve the combination of free radicals, and are, therefore, irreversible.

In the acute carrageenan oedema test in rats and mice, curcumin shows anti-inflammatory activity comparable to that of cortisone and phenylbutazone, but with only modest analgesic and no antipyretic activity (Srimal and Dhawan, 1973). This activity was confirmed in clinical studies where curcumin was compared to phenylbutazone in arthritic patients. The activity was comparable, but side-effects were milder or absent (Deodhar *et al.*, 1980). The molecular mechanism underlying the anti-inflammatory activity is multifactorial, with activity on various pathways involved in the inflammatory process. As an anti-inflammatory and anti-arthritic agent, curcumin is not superior to current drugs in terms of potency, but it is much safer and devoid of gastric irritation (Srimal, 1987).

Interest in curcumin as a chemopreventive agent was sparked by the discovery that this compound shows remarkable differential cytotoxicity, unrelated to apoptosis or apoptosis-related genes (Ramchandran *et al.*, 1997). In experimental animals, a protective effect against diethylnitrosamine-induced liver cancer was observed, but no effect on colon cancer could be evidenced. Phase I chemopreventive clinical studies doses up to 8 g/day gave encouraging results, with histological improvement of pre-cancerous lesions of various types (Cheng *et al.*, 1998).

Contrasting data exist on the bioavailability of oral curcumin (Asai and Miyazawa, 2000). In rats, [^{3}H]-curcumin administered orally was essentially (89 per cent) excreted with the faeces and only 6 per cent with the urine. In another study, it was found that the majority of orally administered curcumin was conjugated to glucuronic acid in the intestinal mucosa, and next conjugated to sulphate in the liver, a somewhat surprising finding since the UDP-glucuronosyltransferase of the intestinal mucosa is much lower than that of the liver. Glucuronides of hydrogenated derivatives were reported as the major biliary metabolites of curcumin after parenteral administration, but could not be detected in the plasma of rat fed with curcumin.

Curcumin has a very low toxicity. In monkeys, oral doses up to 0.8 g/day for three months did not produce, apart from a reduction of appetite, any evidence of toxicity on growth and behaviour, while no changes were observed in histopathological and biochemical parameters. Curcumin is also non-mutagenic and non-teratogenic (Srimal, 1987).

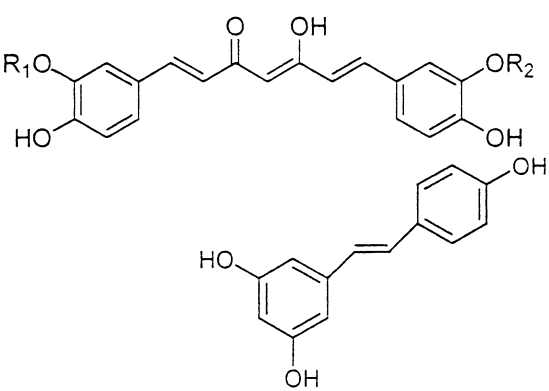
Turmeric is used for wound-healing in the Indian system of medicine, an activity documented also in Ayurvedic texts. Nevertheless, in 1995, two Indian-born

researchers from the University of Mississippi Medical Center in Jackson were granted a patent for the use of a turmeric extract for the treatment of surgical wounds and ulcers (Agarwal and Narain, 1996). The public outcry caused by this patent in India prompted the government in Delhi to file a case against the US Patent and Trademark Office (PTO) in Washington, ultimately leading to withdrawal of the patent in 1997 (Marshall and Bagla, 1997). This is the first case of the successful overturning of a US patent based on a traditional remedy of a developing country; previous attempts to cancel a US patent on products from the neem tree had failed.

The legal and medical matters surrounding the turmeric case are rather complex. An invention to be patented needs to be new and innovative over 'prior art', but considerable differences exist over the definition of 'prior art'. Thus, the American law considers 'prior art' only that available from printed publications and patents, making it eligible for patenting inventions which could not be patented in Europe and most other countries. Here, traditional knowledge and its obvious applications are not patentable, since 'prior art' includes everything which has been made available by means of written or oral description. Furthermore, without patent protection, pharmaceutical development is impossible. Turmeric might well be useful to heal untreatable leg ulcers, but solid science is needed to validate the observations from traditional medicine, and this requires considerable financial investments. Without the possibility of patenting, who is supposed to pay for this?

Resveratrol (Pharma Science, Canada)

Resveratrol (*trans*-3,5,4′-trihydroxystilbene, 24) is one of the most popular phytochemicals, and an impressive body of investigations has accumulated on its potential beneficial effects on pathological conditions ranging from inflammatory and cardiovascular diseases to cancer. Extensive studies on its occurrence in various food plants, and on its human pharmacokinetics have also been carried out.



	R_1	R_2
23a	Me	Me
23b	Me	H
23c	H	H

24

Resveratrol was first isolated from white hellebore (*Veratrum album* L.) but is better known as the phytoalexin of red grape skin, where it also occurs in oligomeric and glycosidic forms, and as a mixture of *cis*- and *trans*-isomers. Resveratrol and its derivatives have a broad distribution, and have been detected in almost one hundred plant species from over thirty genera, including edible plants such as mulberry and arachis. Red

grapes and red wine are the main source of resveratrol in the human diet. The high concentration in grapes (50–100 μg/g) seemingly underlies the notorious resistance of this plant to many fungal attacks. Resveratrol has also been isolated in relatively high amounts from medicinal plants traditionally used as anti-inflammatory and blood-thinning agents (Kimura *et al.*, 1995).

Resveratrol shows oestrogenic and antioxidant activity, as well as modulatory properties on hepatic lipoprotein synthesis and inhibitory activity on platelet aggregation, all of which are of potential relevance for cardiovascular diseases. The beneficial effects of resveratrol on the cardiovascular system might be due to inhibition of expression of the Cellular Adhesion Mediators ICAM-1 and VCAM-1 in endothelial cells. Interestingly, these factors are also implied in the development of tumour metastasis.

The anti-inflammatory activity of resveratrol can be mainly traced back to the inhibition of COX-1 and COX-2 expression as well as to the inhibition of protein kinase C (Jang *et al.*, 1997). Resveratrol can also interfere with the production and release of TNF-α and other cytokines involved in the inflammatory mechanism.

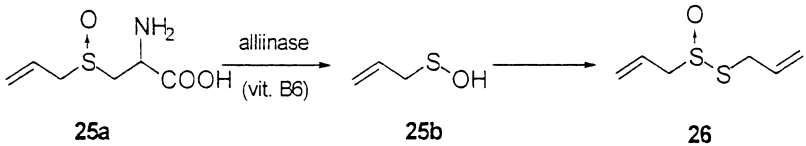
Current interest for resveratrol centres mainly on its multifactorial activity for the prevention of cancer and vascular diseases. The mechanism of this activity is not completely clear, and is seemingly related to its antioxidant and anti-inflammatory activities. Resveratrol reduces the oxidative stress induced by teradecanoyl phorbol acetate (TPA) (antioxidant activity) and the expression of pro-oncogens such as *c-fos* and *TGF-b1*. Inhibition of the expression of COX-2 (Subbaramaiah *et al.*, 1998), an enzyme overexpressed in some tumours such as colon cancer, decreases the tissue concentration of prostaglandins, which are known to stimulate cellular growth and to suppress the immune system. In addition, resveratrol binds in an antagonistic way to the Aromatic Hydrocarbon Receptor (AHR), which causes immunosuppression and carcinogenesis when activated by aromatic compounds (Casper *et al.*, 1999). Resveratrol also inhibits the expression of *CYP1A1*, which, when stimulated by aromatic hydrocarbons, produces enzymes acting as activators of carcinogenesis (Ciolino and Yeh, 1999).

Taken together, these data validate resveratrol as an antitumour and cardiovascular lead, but the clinical relevance of the wealth of *in vitro* experiments is difficult to evaluate, since the human pharmacokinetics and toxicology of resveratrol is not well known. The moderate consumption of red wine, an ancient tradition in the Mediterranean area, is currently considered not only as a way to better enjoy meals but also as a sound method to fend off heart disease and cancer. The concentration of resveratrol in red wine is generally lower than 5 mg/L, and seems unlikely to define this compound as the 'active ingredient' of a beverage which is so entrenched within the European eating habits.

Allicin (Tulane University, USA; Weizmann Institute, Israel)

Allicin (diallyl disulphide oxide, **26**) is a colourless, low-weight sulphur compound responsible for both the odour and much of the biological activity of garlic (*Allium sativum* L.). Garlic cloves have no smell until crushed, at which time allicin (**26**) is produced from the odourless *S*-allyl-*L*-cysteyne-*S*-oxide (alliin, **25a**) through the agency of alliinase, a C-S lyase released from vacuoles. This reaction initially generates a sulphenic acid (**25b**), which next condenses to allicin.

The content of allicin in crushed garlic is approximately 4 mg/g. Yet, because of its instability, allicin is practically absent in cooked garlic and also in aged commercial



25a **25b** **26**

garlic extracts. Allicin can be produced by treating synthetic alliin with purified alliinase. In order to get reproducible amounts of allicin, this process can be done immediately before administration (Rabinkov *et al.*, 1994).

The beneficial effects of garlic consumption are well documented in the traditional medicine of many cultures in the world, and especially in the Chinese system of medicine, where garlic is associated with longevity. Indeed, garlic extracts contain several biologically active compounds; and allicin and its analogues have been the most thoroughly investigated. Allicin shows *in vitro* and *in vivo* antimicrobial and anticancer activity, and has beneficial cardiovascular effects, lowering blood lipids and interfering with thrombogenesis. It also shows antibacterial activity, and freshly obtained aqueous garlic extracts as well as crude allicin (15 mg/kg) could counteract a lethal dose of intestinal *Shigella* in rabbits (Chowdhury *et al.*, 1991).

Both animal studies and epidemiological investigations have shown an inverse association between the consumption of garlic and stomach and colon cancers, presumably due to the inhibition of gastric nitrosation in the acidic stomach environment. Gastric nitrosation is responsible for the formation of carcinogenic *N*-nitroso compounds, and its inhibition might be related to the antibiotic activity of allicin on nitrate-reducing bacteria (Mei *et al.*, 1989). Allicin, along with other constituents, has also been related to the antitumour effects of garlic. Synergistic interaction with diallyl sulphide, which strengthens the detoxification activity of gluthathione; spirostane saponins (common to other *Allium* species), which show powerful cytotoxicity *in vitro*; as well as with the phytoalexin allixin have been proposed. Allicin might also be responsible for most of the serum lipid-lowering effects of garlic. Six major clinical trials with fresh garlic cloves (3–10 g) and almost thirty with garlic powder tablets (0.6–0.9 g) support a beneficial activity on serum triglycerides (approx. 12 per cent reduction) and cholesterol (approx. 11 per cent reduction). Amounts of allicin as low as 0.07 mg/kg can still have effects on serum lipids. The hypolipaemic activity of garlic supplements is apparently stronger in women than in men.

Allicin and allicin-derived garlic compounds (ajoene, diallyl trisulphide, vinyldithiins) are strong inhibitors of human platelet aggregation. *In vivo* clinical studies with fresh garlic (100 mg/kg, corresponding to *c.*0.4 mg allicin/kg) have shown a 50 per cent decrease of the blood clotting power, presumably mediated by the inhibition of c-AMP phosphodiesterase (Kiesewetter *et al.*, 1993).

Despite the excellent record of *in vitro* and *in vivo* activity, allicin is difficult to formulate for its instability, and it is not clear if it is superior to garlic extracts or fresh garlic in any of its potential pharmacological applications. Furthermore, nothing is known on the molecular mechanism of its various activities.

Further dietary constituents of pharmaceutical relevance

The number of secondary metabolites which occur in food plants and show biological activity is impressive, and Table 5.1 presents a list of some of these agents, along with

their molecular targets. Some of these compounds have become standard tools in molecular pharmacology and cell biology (genistein, phlorizin, apigenin). A full discussion on the pharmacological potential of all these agents is beyond the limits of this article, but reference to comprehensive reviews has been given whenever possible.

Table 5.1 Selection of biologically active dietary secondary metabolites of pharmacological relevance

Compound	*Occurrence*	*Molecular target*	*Pharmacological potential*
Apigenin[1]	Parsley	MAP kinase	Chemopreventive
β-Carotene[2]	Carrot, mango		Chemopreventive
Cynarin[3]	Artichoke		Choleretic Anti-hepatotoxic Hypocholesterolemic
Genistein[4]	Soy	β-Oestrogen receptor Tyrosine protein kinase MAP kinase DNA Topoisomerase II CCAAT binding factor	Chemopreventive Oestrogen-replacement therapy
Inositol Hexaphosphate[5]	Widespread		Chemopreventive
Limonine[6]	Citrus fruits		Chemopreventive
Lycopene[7]	Tomatoes		Chemopreventive
Oleanolic acid[8]	Olive oil		Anti-inflammatory Anti-ulcer
γ-Orizanol[9]	Rice		Anticholesterolemic
Persenones[10]	Avocado fruit	NO synthase	Anti-inflammatory
S-Perillyl alcohol[11]	Cherries	Isoprenylating enzymes	Chemopreventive
Phlorizin[12]	Apple	Renal Na^+/glucose cotransporters	Antidiabetic
Procyanidins[13]	Wine, cocoa, chocolate		Chemopreventive Cardiovascular prevention
Psoralene derivatives[14]	Citrus fruits Parsley	Cytochrome P450 enzymes DNA	Pharmacokinetic modifier Skin pigmentation
R-Limonene[11]	Citrus fruits	Isoprenylating enzymes	Chemoprevention
Rosmarinic acid[15]	Rosemary		Anti-inflammatory
Rutin[16]	Buckwheat	Antioxidant Aldose reductase	Vitamin PP activity Antidiabetic
Secoisolariciresinol[17]	Flax seed	Oestrogen receptors	Chemopreventive Oestrogen-replacement therapy
β-Sitosterol[18]	Widespread		Anticholesterolemic Cytotoxic
Sulphoraphane[19]	Broccoli	Cytochrome P450 enzymes	Chemopreventive
Tocotrienols[20]	Barley	HMG-CoA Reductase	Hypocholesterolemic

Notes
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Concluding remarks

Our diet contains a host of secondary metabolites which can interact with important pharmacological targets, and for which biological activity can be demonstrated in *in vitro* and *in vivo* assays. The role of these interactions for human homeostasis is unknown, and the translation of dietary constituents into pharmaceutical leads is undoubtedly fraught with difficulties. Nevertheless, it is not unconceivable that in the near future progress in biomedical sciences will put us in the position to better evaluate the physiological role and pharmacological potential of the multitude of dietary chemicals which have challenged our homeostasis over the course of evolution.

People in developed countries are expecting food to make them healthier, fending off the chronic diseases of ageing. This trend has led to the explosion of the functional food market, which is expected to constitute 10 per cent of the total food market in the USA (and presumably of other developed countries as well) by 2010 (Henry, 1999). The huge value of the functional food market will hopefully foster investigation of the scientific bases for its surge itself. On the other hand, while the protective effects of fruits and vegetables against various diseases are well established, evidence linking specific constituents to a specific disease is less convincing. Furthermore, if certain compounds in our diet are indeed magic bullets to fight diseases, reducing their natural dietary formulation to a pharmaceutical one will certainly raise eyebrows. Turning red wine into a pill in the search of better health might seem sacrilegious to many, but basic science will be learnt in the process, and we might all benefit from these advances.

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6 Biotechnology of plant-derived dietary supplements

Marco Mucciarelli

Introduction

'Biotechnology' is the name that has been given to a very wide range of agricultural, industrial and medical technologies that make use of living organisms (e.g. microbes, plants or animals) or parts of living organisms (e.g. isolated cells or proteins) to provide new products and services. These include enzymes for food and drink production processes, vitamins, amino acids and other useful chemicals, obtained via fermenter technology. Cell manipulation and genetic engineering techniques are at present the new frontier of biotechnology, especially in the field of plant product improvement.

About 80 per cent of current research in plant biotechnology is directed towards the improvement of food plants; the remaining work is concerned with non-food crops such as cotton, tobacco and ornamental and medicinal plants.

The latter are of obvious social and economic importance. It has been reported by Farnsworth (1985) that a quarter of all prescription drugs used in the USA still contain plant-derived substances isolated from or contained in plant sources. Europe has a very long tradition in phytomedicines, dating back to the first century. Fifty per cent of the world sales of herbal remedies occur in Europe, with a retail sales volume of over $6 billion (Gruenwald, 1998). Within Europe, Germany is the leading country for herbal drugs with annual sales of $2.5 billion, followed by France, Italy, the United Kingdom, Spain, the Netherlands and Belgium (Gruenwald, 1998).

Concerning the relationship existing between nutrition and human health, a further step in this direction is the idea that some foods of plant origin may supplement human diet by their natural chemical constituents, having therapeutic or at least preventive activity against various kinds of infectious diseases. As in the case of plant drugs, these botanical products and their derivatives are not consumed for a nutritional purpose and include processed or unprocessed plant parts (bark, leaves, flowers, fruits and stems) as well as active constituents, extracts and essential oils. The Dietary Supplement Health and Education Act (DSHEA) of 1994 included herbal products in its definition of 'dietary supplements', even though herbs have little or no nutritional value.

Cowan (1999) has noted that the use of and search for drugs and dietary supplements derived from plants have accelerated in recent years. Ethnopharmacologists, botanists, microbiologists and phytochemists are combining their efforts for phytochemicals and botanical preparations, which could be developed for treatment of chronic and infectious diseases.

Many of these scientific and socio-economic goals have not yet been completely achieved and biotechnology still remains a tool of choice for research application. Success in maintaining yield and efficiency while improving the ability of the botanical supply to provide good nutrition and health requires the joint efforts of nutrition, food, plant and animal scientists in order that the most effective strategies can be targeted (Scheemann, 2001).

Main techniques employed by plant biotechnology

Plant secondary metabolism is very important in determining flower colour, flavour of food, and plant resistance against pests and diseases. Moreover, it is the source of many useful chemicals such as drugs, dyes, flavours and fragrances (Verpoorte *et al*., 1999, 2000), which are the main active constituents or at least simple components of most medicinal plants and herbal dietary supplements. Therefore, it is of interest to be able to engineer the secondary metabolite production of the plant cell factory, e.g. to produce more of a useful chemical, to produce less of a toxic compound, or even to make new compounds or valuable herbal products. Our limited knowledge of secondary metabolite pathways and the genes involved is one of the main bottlenecks (Verpoorte *et al*., 2000).

Plant tissue culture techniques and genetic engineering of plants may be regarded as the main tools of plant biotechnology aimed at the production of plants with improved yield and agronomic traits, which means also improved products. The reader should keep in mind that each of these techniques has been derived from different biological disciplines, whose combination is essential for research advancement (Verpoorte *et al*., 1999).

Plant cell tissue cultures

Plant cell tissue culture (PCTC) is the cultivation of plant cells or tissues on specially formulated nutrient media. In appropriate conditions, an entire plant can be regenerated from each single cell, permitting the rapid production of many identical plants. This technique finds different applications, including:

- micropropagation of high-value plants for nurseries when a high standard of plant quality is requested, as in the case of varieties with elite agro-metabolic traits;
- production of cell suspension cultures to be maintained in fermenter vessels with the aim of producing valuable products such as phytochemicals, enzymes and natural food flavourings;
- conservation via shoot-tip culture as well as through cryoconservation of cells and tissues of those plant varieties that cannot be maintained in a normal seed bank;
- production of valuable hybrid plants through embryo rescue and plant recovery from crosses between genetically distant parents;
- regeneration of whole plants after genetic manipulation of cell or tissue cultures.

Genetic engineering

Genetic engineering is the controlled modification of genetic material (DNA) by artificial means. It relies upon the isolation of specific stretches of DNA using specialized

enzymes, which cut the DNA at precise locations. Selected DNA fragments can then be transferred into plant cells in several ways:

- *Agrobacterium* technology, the best-established gene-transfer method for plants, employs this pathogenic soil bacterium as a vector so that desirable genetic information rather than that which induces the plant disease is transferred into plants (Zambryski, 1992).
- Bio-ballistic methods involve sticking the DNA that is to be introduced into the plant on to minute gold or tungsten particles, then firing these (like bullets) into the plant tissue. A proportion of the plant cells treated in this way take up the DNA from the metal pellets. Whole plants are then regrown from the cells by tissue culture (Newell, 2000).
- Electroporation is a technique which works best with plant tissues that have no cell walls (protoplasts or pollen tubes). Micro- to millisecond pulses of a strong electric field cause minute pores to appear momentarily in the plant cells, allowing DNA to enter from a surrounding solution. Recently this technique has also been applied to intact plant tissues (Chowrira *et al*., 1995). A more recent yet similar method uses microscopic silicious fibres to puncture holes in the plant cells (Songstad *et al*., 1995).
- Microinjection is based on the use of glass micropipettes (internal diameter 0.5–10 μm) to effect the direct transfer of macromolecules into the cytoplasm or the nucleus of a recipient cell or cell structure (Draper and Scott, 1991).
- Somatic hybridization via protoplast fusion is a technique which is used to speed up the process and improve the precision of plant breeding, allowing *in vitro* cell fusion, producing hybrid plants between phylogenetically unrelated parent plants (Grosser *et al*., 2000).
- Somaclonal variation (Larkin and Scowcroft, 1983) is the genetic variation induced in plants regenerated via tissue culture methods. It can result in a range of genetically stable mutated clones, useful in crop improvement (Jain, 2001).

Plant cell tissue culture for the production of active compounds

For plant compounds one can consider several possibilities of biotechnological production: plant cell tissue cultures, transgenic plants or plant cells, transgenic microorganisms and isolated enzymes. For transgenic systems it is necessary to know the pathway of production involved and have the genes available. Isolated enzymes can be used only for bioconversions, that is going from a precursor to the next product. One application of PCTC is to provide an alternative method for producing food ingredients and medicinals that have traditionally been extracted from field-grown plants. As more is known about the biochemical and genetic regulation of plant secondary metabolism, and more advances are made in the development of yield improvement strategies and design of large-scale bioreactors, commercial application of PCTC-derived molecules is expected to increase. Therefore plant cell tissue culture seems the most interesting alternative to plant metabolite industrial production, provided that

- the technology is feasible,
- the process is economically competitive with existing production systems.

At present, many cell culture systems have demonstrated this feasibility, even if as reported by Verpoorte and colleagues (1999) the optimum production (0.3 g L^{-1}/14 days) of ajmalicine in *Catharantus roseus* cell cultures and that the 10-fold higher reported for berberine in *Coptis japonica* cells is still far below the productivity for antibiotics such as penicillin in culture of microorganisms (Verpoorte *et al*., 1999). Berberine, an isoquinoline alkaloid used as a remedy for intestinal disorders in the Orient is now produced by Mitsui Petrochemical group on a large scale (1.4 g L^{-1} in two weeks) (DiCosmo and Misawa, 1995).

Types of cultures and their applications

Several types of cell-organ culture are used to produce food ingredients and drugs of plant origin, including cell suspensions, organized tissues and transformed shoot and root cultures.

Shoot Cultures are employed as a source of axenic plant material and as a commercially viable method of plant micropropagation. Well-developed techniques are currently available to meet the demands of the pharmaceutical industry (Rout *et al*., 2000) and they are to be extended to dietary supplement production. These protocols of *in vitro* culture are designed to provide plant material with optimal levels of carbohydrates, organic compounds (vitamins), mineral nutrients, environmental factors (e.g. light, temperature and humidity) and growth regulators required to obtain high regeneration rates of many plant species. Moreover micropropagation enables cloning of the resulting plants, allows us to propagate plants that cannot form seeds because of adverse climatic conditions and assures the production of virus-free plants (Rout *et al*., 2000).

Cell Suspensions are plant cells freely suspended in a nutrient medium. They are the preferred type of culture for large-scale production because they are similar to microbial cultures and have rapid growth cycles. The existing microbial fermentation technology has been adapted to plant cell production, though some discrepancies in cell growth and yield between shake flask and fermenter cultures have been documented, as for antraquinones production in *Morinda elliptica* cells (Abdullah *et al*., 2000). Plant cells are totipotent; that is, cells in culture can produce the same metabolites as the whole plant. However, the product profiles of callus (undifferentiated cell mass) or cell suspension cultures often differ from those of parent plants. Effects of culture age and those following the application of conditioning factors are of great importance in stimulating metabolite production. Cell suspensions of strawberry, *Fragaria ananassa* L. cv. Shikinari, and those of *Ajuga pyramidalis* have been used to produce anthocyanins, which are useful as commercial dyes (Mori *et al*., 1994; Madhavi *et al*., 1996). The content of peonidin-3-glucoside, a major anthocyanin, increased as the number of days of cell growth proceeded, while that of cyanidin-3-glucoside decreased. The contents of these two major anthocyanins were also changed by adding a preculture broth as a conditioning factor (Mori *et al*., 1994).

Since the use of many synthetic food colourants has been restricted due to their potential risk for consumers' health, natural pigments and their biotechnological production have become of great interest in the food industry (Jimenez-Aparicio and Gutierrez-Lopez, 1999). This is the case for red-violet betacyanins and yellow betaxanthins; betacyanins particularly have been extracted as food colourant only from beetroot (*Beta vulgaris* L.). Valuable betacyanin production by cell cultures of this species

has been obtained by Akita and colleagues (2000). Here, as well in many other cell systems, the envisaged advantages coming from *in vitro* culture production are:

- no seasonal fluctuation of the product;
- reduced contamination by microorganisms;
- easy extraction of the product;
- sustainable production and conservation of natural resources in developing countries.

A recent article by Ekiert (2000) reviews the results of biotechnology applied to the family Apiaceae. These species are well-known sources of many important herbal products and drugs. The number of active compounds naturally contained by these plants and that can now be obtained *in vitro* is impressive: furanochromones, coumarin compounds (especially furanocoumarins), saponins, pigments (flavonoids, anthocyanins, carotenoids), phytosterols, phenolic acids and essential oils (Ekiert, 2000).

Furanocoumarins, and especially psoralen derivatives exhibiting antiproliferative and photosensitizing activity (psoralen drugs in oral and topical forms are routinely used in medicine for the treatment of vitiligo and psoriasis) (Turegun *et al*., 1999), have been synthesized *in vitro* from *Ammi majus* L., *Heracleum sphondylium* L., *Pastinaca sativa* L. and other plants (Ekiert, 2000). Many of these active substances advocated as natural remedies or dietary supplements (e.g. methoxy-psoralen) have been extensively studied because their consumption or topical contact (e.g. psoralens) has long been associated with adverse effects on humans (Clifford, 2000).

Carotenoids, a totally different class of plant pigments, have recently received considerable interest because of their potential in delaying or preventing degenerative conditions such as heart disease, cancer and ageing (Giugliano, 2000). Their role in the plant is to act as accessory pigments for light harvesting and in the prevention of photo-oxidative damage, as well as acting as attractants for pollinators. Their function as antioxidants in the plant shows interesting parallels with their potential role as antioxidants in foods and dietary supplements (van den Berg *et al*., 2000). β-Carotene, lycopene and γ-carotene among others have been produced in cell suspension cultures of *Daucus carota* L. with a yield range of 3–1,000 μg/g of cell dry weight (Ekiert, 2000).

A very important group of herbal products is the essential oils, useful in medicine and flavourings. Most articles on the biosynthesis of oil constituents have reported its failure in undifferentiated callus or suspension cultures and many authors have noticed that a high degree of cell differentiation is needed to obtain positive results. However, several papers reported the biosynthesis of essential oil components (e.g. terpenes) in callus or suspension cultures of *Apium graveolens* L., *Coriandrum sativum* L., *Foeniculum vulgare* Miller and many other Apiaceae (Hsu and Yang, 1996; Ekiert, 2000), in *Citrus* spp. among Rutaceae (Reil and Berger, 1996), in *Melissa officinalis* (Bolade and Lockwood, 1992) and in *Artemisia dracunculus* L. (Cotton *et al*., 1991).

Most of the economically important natural products are produced by cell cultures only at very low levels or not at all (e.g. quinine, morphine, vinblastine and vincristine). This lack of production is, first, due to the failure to find conditions in the culture to stimulate their production and, second, owing to the frequent instability of production, once it has been established (Walton *et al*., 1999). PCTC-derived products that have reached the market are shown in Table 6.1. Shikonin, a red naphthoquinone

Table 6.1 Economic processes for the production of secondary compounds by plant cell cultures (from Walton *et al*., 1999).

Product	*Plant species*	*Company*	*References*
Shikonin	*Lithospermum erythrorhizon*	Mitsui Petrochemical Ind. Ltd	Fujita *et al.*,1982
Ginsenosides	*Panax ginseng*	Nitto Denko Corp.	Ushiyama, 1991
Purpurin	*Rubia akane*	Mitsui Petrochemical Ind. Ltd	Walton, *et al.*, 1999

dye produced in Japan by plant cells of *Lithospermum erythrorhizon* and used to promote healing, can be mentioned as one of the few pure chemicals so far produced on an industrial scale (Verpoorte *et al*., 1999). This species can provide accumulation of the compound exceeding 20 per cent of the dry matter of the cell culture (Walton *et al*., 1999). Ginseng is the third best selling herbal supplement in the United States, both as a disease-healing drug and as a general tonic, and it is now also being used as a flavouring agent in foods. The predominant pharmacologically active constituents of *Panax* are ginsenosides, at least 25 of which have been identified and are present in variable amounts and ratios to one another; depending on the particular species, variety and conditions of growth (Carabin *et al*., 2000). Plant cell and tissue culture methods have been explored as potentially more efficient alternatives for the mass production of ginseng and its bioactive components. Research into ginseng cell and tissue cultures started in the early 1960s and commercial applications have developed since the late 1980s (Ushiyama, 1991; Wu and Zhong, 1999; Carabin *et al*., 2000).

Other PCTC-derived products are polysaccharide mixtures (Verpoorte *et al*., 1999), rosmarinic acid and sanguinarine and there has been recent success with the anti-tumoural taxol, though whether this expensive procedure will become commercially available is not clear yet.

Plant cell cultures play an additional important role during the industrial development of new plant-derived compounds as drugs, when agricultural production is not feasible or not yet available (Verpoorte *et al*., 1999), or when these valuable compounds have limited occurrence in plants. Such is the case of the furanochromones of *Ammi visnaga* (L.) Lam. which have interesting vasodilatation properties on coronary and renal vessels (Ekiert, 2000).

Another important application is the production of novel natural pharmaceutical and food products that cannot otherwise be found in nature, like paniculide from *Andrographis paniculata* Ness. (Allison *et al*., 1968; Mandal *et al*., 2001), pericine from *Picralina nitida* and podoverine from *Podophyllum versipelle* (Walton *et al*., 1999), two new substances active on the central nervous system. As reported by Walton and colleagues (1999), these new biologically active compounds are highly attractive to several companies since they can be patented. For such applications, an understanding of the manufacturing process will help to determine the intended or unintended modifications made to the product and thus help the safety evaluation of these novel products (Fu, 1998).

Strategies to improve cell productivity

The two most important determinants of the overall productivity of *in vitro* cell-based processes are the growth rate of the culture and the specific production rate of the compound in question.

Due to the lack of productivity of large-scale cell culture systems, there have been various approaches to increasing cell yields.

Medium optimization

In the past many strategies have been followed to improve the yield of plant cell cultures. The first approach was directed towards the selection of high-yielding cell lines, since not all the cells present in a suspension culture or in a callus are able to synthesize the metabolite. Meanwhile another key step in these procedures was the optimization of culture parameters, and principally of growth and production media (Rout *et al*., 2000). This approach was particularly useful; it consisted of a first step characterized by massive growth of cells followed by the transfer of biomass to a second medium where intensive metabolite production and release of the metabolite starts (Fujita *et al*., 1982). In general an improvement in productivity of about 10–20-fold is obtained with this strategy (Verpoorte *et al*., 1999). This has been shown in *Tabernaemontana divaricata* (Apocynaceae) after replacing the auxin of the medium (2,4-D) with NAA (naphthalene acetic acid), another auxin-like substance: alkaloid production showed a maximum in the fourth subculture and stabilized on a higher level than found in the original cell lines (Sierra *et al*., 1992).

Differentiated organ cultures

An overview of *in vitro* regeneration of medicinal plants by direct and indirect organogenesis and by somatic embryogenesis from various types of explants and their use to improve medicinal plants through somaclonal variation and genetic transformation is reviewed by Rout and colleagues (2000).

The use of differentiated cultures, namely *in vitro* shoot and root cultures instead of single cells, has offered a partial solution to some cases of lack of metabolite production.

In plants there is a clear correlation between cell differentiation and secondary metabolism, and the fact that most secondary metabolites are produced and stored in differentiated and specialized cell structures is a clear demonstration of this. In organ cultures, in contrast to dispersed cell cultures, cell differentiation and cell-to-cell contact allow the presence of endogenous control mechanisms that specify the production of secondary metabolites (Walton *et al*., 1999). During differentiation of tissues and organs, these regulatory mechanisms are genetically and functionally able to regulate the expression of the biosynthetic pathways to the products and the stable generation of them along with many cycles of the culture process (Walton *et al*., 1999). This phenomenon, which has been referred to as cell competence to metabolite synthesis, depends on differences in gene expression between disorganized and organized cultures. Although the appropriate genes may be present in each cell, they are frequently not expressed in the absence of differentiating processes (Walton *et al*., 1999; Memelink *et al*., 2001).

The product profiles of these organized tissue cultures are similar to those of field-grown plants. Genetic stability in these tissue cultures is much higher than in cell suspension or callus cultures (Wysokinska and Chimiel, 1997); moreover stable growth and consistent secondary metabolite production have been observed in shoot and root cultures of many species (Ekiert *et al*., 2000).

Good examples of root cultures are those of *Senecio* and *Hyosciamus* spp. for the production of pyrrolizidine and tropane alkaloid biosynthesis respectively (Hashimoto *et al*., 1986; Hartmann *et al*., 1989). *H. alba* and *H. niger* root cultures allow high growth rates and root morphology conservation over extended periods of culture and represent good examples of the importance of optimal concentration of exogenous auxin to obtain a good level of growth without inhibiting alkaloid production (Walton *et al*., 1999). The successful establishment of these organ cultures is also of great value as a sterile source material for the isolation and cloning of the main genes involved in the biosynthetic process, as happened for the gene encoding hyoscyamine 6β-hydroxylase in *H. niger* (Matsuda *et al*., 1991) whose function is discussed in a later section.

Sugimoto and colleagues (Sugimoto *et al*., 1993, and references therein) found that the alkaloid levels of cultured roots of *Stephania cepharantha* were much higher than those of the plant and that aromoline, an antiplasmodial and antiamoebic compound, which was not present in the original plant, reached >1 per cent of the cell dry weight. Khanam and colleagues (2001) demonstrated the presence of hyoscyamine and scopolamine at different stages of shoot regeneration from non-organogenic and organogenic calli of *Duboisia myoporoides* R. Br., stressing the regulatory importance of plant morphogenesis on the metabolic productivity of these cultures.

Rosmarinic acid and related phenolics are natural antioxidants found as secondary metabolites in spearmint (*Mentha spicata*) and other plant species. These phenolic secondary metabolites have diverse food-processing and nutraceutical applications. Since natural cross-pollination results in plant-to-plant variation in the level of phenolic metabolites, tissue-culture-based techniques are essential to isolate elite antioxidant-producing clonal lines. Tissue-culture-based selection techniques to isolate high rosmarinic acid and phenolic-producing clonal lines from a heterogeneous bulk seed population of spearmint have been developed by Al-Amier and colleagues (1999).

One approach that has raised a lot of interest is the use of *Agrobacterium rhizogenes*, a soil bacterium able to stimulate the proliferating growth of roots, named 'hairy roots', via plasmid (Ri T-DNA) insertion into the plant genome (Chilton *et al*., 1982). The DNA-transforming plasmid of *A. rhizogenes* has the ability to insert into the plant DNA, thus transforming it. Hairy roots have production rates very similar to those of normal roots, and can grow *in vitro* without the addition of exogenous growth regulators, thus solving the problem of possible inhibitorial activity of these phytohormones. Third, these cultures exhibit a high level of genetic and biochemical stability. Table 6.2 shows the wide range of plant species and hairy root products that are currently under study (Walton *et al*., 1999).

A clear example of success in obtaining high productivity is that of tropane alkaloids (Verpoorte *et al*., 1999). These are a well-recognized group of structurally related natural products and have long been known to have anticholinergic, antiemetic, parasympatholytic, anaesthetic, and many other actions. This class of alkaloid includes such important medicinal alkaloids as cocaine, scopolamine and atropine. The genera *Atropa*, *Datura*, *Duboisia*, *Hyoscyamus* and *Scopolia*, which belong to the Solanaceae, are especially rich sources of tropane alkaloids.

Despite the higher stability of transformed tissue cultures, substantial clone-to-clone variation has been observed, as for growth rate, opine production and tropane alkaloid content among 29 clones of *Scopolia japonica* hairy-root cultures (Shimomura *et al*., 1991). Marked clone-to-clone variation has also been observed in *Duboisia leich-*

Table 6.2 Examples of secondary products obtained from hairy-root cultures (from Walton *et al*., 1999)

Plant species	*Product*
Artemisia annua	Artemisinin
Astragalus mongholicus	Cycloartane
Catharanthus roseus	Tabersonine, serpentine, 19(S)-epimisiline
Coleus forskohlii	Forskolin
Datura stramonium	Polyamines, tropanes
Datura candida × *D. aurea*	Hyoscyamine
Digitalis lanata	Antraquinones, flavones
Glycyrrhiza glabra	Isoprenylated flavonoids
Hyssopus officinalis	Rosmarinic acid
Hyioscyamus muticus	Hyoscyamine, lubimin, solvavetivone
Lawsonia inermis	Lawsone, tannins
Lithospermum erythrorhizon	Hydroxyechinofuran
Lotus corniculatus	Tannins
Pauwlonia tomentosa	Verbascoside
Pimpinella anisum	Essential oils
Scutellaria baicalensis	Flavonoids glycosides
Solanum aviculare	Solasidine
Trachelium coeruleum	Polyacetylenes
Trigonella foenum-graecum	Diosgenin
Valeriana wallichi	Valepotriates
Wahlenbergia marginata	Polyacetylenes

hardtii hairy-root cultures, where it was attributed to the differences in the length, copy number and chromosomal location of the Ri T-DNA fragments (Fu, 1998). However, in *D. leichhardtii*, the selection of a high-yield hairy-root clone has allowed the continuous production of scopolamine via a bioreactor with interesting application on a commercial scale (Muranaka *et al*., 1993).

Other typical organ cultures for the production of plant metabolites are *in vitro* cultures of shoots, developed from axillary buds and grown in solid or liquid media. These green cultures have been employed for the synthesis of rosmarinic acid from both shoot and green callus cultures (Komali and Shetty, 1998), cardenolides in *Digitalis* spp. and terpenoids in *Pelargonium* spp. (Walton *et al*., 1999, and references cited therein). Due to the weakness of shoot tips, especially when grown in bioreactors and owing to their short duration in this kind of culture, an attempt has been made to produce transformed shoot cultures, as in the case of hairy roots. This has been achieved in *Mentha* spp., where Spencer and colleagues (1990, 1993) induced shooty teratoma able to produce essential oils. The essential oils resembled those of the parent plant, with linalool and linalyl acetate as the major components in the case of *M. citrata*, but diverged to a great extent from the parent plant in the case of *M. piperita* (Spencer *et al*., 1993; Maffei *et al*., in press).

Similar success has been obtained in two other cases, namely the production of 18 mg/100 g dry weight of artemisinin *Artemisia annua* transformed shoot cultures and in *Pimpinella anisum* oil production (Walton *et al*., 1999, and references cited therein).

The underground organs of members of the genus *Valeriana* (Valerianaceae), as well as related genera such as *Nardostachys* and *Patrinia*, are used in the traditional medicine

of many cultures as sedatives and tranquillizers. *V. officinalis* is the species most commonly used in northern Europe and still retains its official pharmacopoeial status although it is most commonly encountered as an ingredient of herbal medicines (Houghton, 1999; Salles *et al*., 2000). This plant is still the subject of considerable research aimed at establishing the chemical and pharmacological basis of the activity which has been clearly shown in a number of animal and clinical studies (DiCosmo and Misawa, 1995; Houghton, 1999). The non-volatile monoterpenes first isolated in 1966, and collectively known as valepotriates, contribute to the overall *Valeriana* activity by possessing sedative activity based on the CNS (Houghton, 1999).

Valerian is a good example of both the negative and positive aspects of herbal drugs. The considerable variation in its composition and content as well as the instability of some of its constituents pose serious problems for standardization but the range of components which contribute to its overall activity suggest its great value for the treatment of many diseases. A biotechnological approach to this genus has led to the establishment of *in vitro* propagation of *Valeriana jatamansi*, a wild herb exploited for its roots and rhizomes which contain valepotriates highly effective against leprosy (Kaur *et al*., 1999). Cell suspension and *in vitro* plant regeneration have been established in *Valeriana edulis* (Castillo *et al*., 2000). The biotechnological production of valepotriates from transformed roots has been obtained in the genus *Valerianella* (Caetano *et al*., 1999; Kittipongpatana *et al*., 2002).

Transformed organ cultures

The use of *Agrobacterium rhizogenes* is also of great interest since it can be used as a natural vector to transfer new genes to plant cells. *Agrobacterium* cells can act as a gene vector after genetic manipulation of their plasmids. In practical terms, transformed roots may be developed by infecting sterile plant material, leaves or petioles, excised from the explant and transferred to the growth medium (Walton *et al*., 1999). This technique has been applied to *Atropa belladonna* hairy-root cultures in which the enzyme that converts hyosciamine into scopolamine was overexpressed. The engineered belladonna hairy roots showed increased amounts and enzyme activities of the hydroxylase, and contained up to five-fold higher concentrations of scopolamine and 6,β-hydroxyhyoscyamine than wild-type hairy roots (Yun *et al*., 1992; Hashimoto *et al*., 1993). Another application allowed increasing productivity of *Cinchona* hairy roots. *Cinchona officinalis* Ledgeriana hairy roots were initiated containing constitutive-expression constructs of cDNAs encoding the enzymes tryptophan decarboxylase (TDC) and strictosidine synthase (STR) from *Catharanthus roseus*; these are two key enzymes in terpenoid indole and quinoline alkaloid biosynthesis (Geerlings *et al*., 1999).

Other bioactive compounds with important implications as botanical supplements, obtained from transformed root cultures are the alkamides of *Echinacea purpurea* (Trypsteen *et al*., 1991) and the sesquiterpene hernandulcin from *Lippia dulcis*, an intensely sweet herb endemic to tropical America (Sauerwein *et al*., 1991).

Agrobacterium rhizogenes technology has also been applied for essential oil production in *Pimpinella anisum* L., *Anethum graveolens* L. and *Coriandrum sativum* L. and for the production of furanochromones and furanocumarins in *Ammi* spp. (Ekiert, 2000).

A major disadvantage of differentiated organ cultures is that they are difficult to grow on a large scale (Verpoorte *et al*., 1999) since they are not amenable to stirred

culture systems. Specialized bioreactors have been devised having a capacity of up to 500 L and allowing the culture of 40–50 kg of root within 40 days as obtained in *Datura stramonium*.

Immobilized cells

Cell immobilization techniques are well-developed commercial systems for the production of metabolites used in the food industry, such as enzymes, amino acids, organic acids, alcohols, aroma compounds, polysaccharides and pigments. Biotechnological production of flavours and fragrances using microorganisms, plant cells or isolated enzymes has been reviewed by Krings and Berger (1998).

Recently, an alternative approach to microbial production of bioflavours, eliminating the need for lengthy purification, has been the co-immobilization of precursors for bioflavour generation by microbial cells, traditionally employed for food and beverage processing, within beads made of food-grade gel matrix additive (Kogan and Freeman, 1994). Following incubation under controlled conditions the bioflavour or bioflavour mixture is generated and accumulated within the beads. The flavour-retaining bead may then be employed as a food additive (Kogan and Freeman, 1994).

At present, one of the flavouring products under extensive biotechnological exploitation is vanillin, whose production by PCTC, microbial biotransformation and molecular approaches has been reviewed by Rao and Ravishankar (2000).

The immobilization of plant cells leads to the formation of large cell aggregates layered and embedded into alginate or artificial foam supports. Cell disposition within this artificial layer resembles that of *in vivo* plant tissues (i.e. outer cell layer of stems or roots), thus favouring cell contact and differentiation, as observed in the case of *Cinchona* immobilized cells for the production of quinoline alkaloids (quinine) (Hoekstra *et al*., 1990). This technique has the advantage of increasing cell-to-cell contact, thus stimulating cell metabolism activation, and allows media refreshing and metabolite recovery. This is possible only when the plant metabolites are actively secreted into the medium; otherwise they must be forced in this sense by sonication (ultrasounds applied to plant cells) or through treatment with organic solvent, which is time-consuming and economically disadvantageous (Verpoorte *et al*., 1999).

The interest in immobilizing plant cells originated from processing advantages such as protecting the cells from mechanical stress and facilitating product recovery. Cell immobilization often had positive effects on secondary metabolite production. The production of capsaicin, the major pungent principle of chilli pepper fruit, was increased 1,000-fold after *Capsicum frutescens* cells were immobilized in the pores of a polyurethane foam matrix. Immobilization of placental tissues of *Capsicum frutescens*, the site of synthesis of capsaicin, has been used to enhance yields of metabolites *in vitro* using a combined precursor treatment. To increase yields of capsaicin and dihydrocapsaicin, cells were fed with intermediate metabolites of the capsaicinoid pathway (Johnson and Ravishankar, 1998).

Prolonged biosynthesis and cell viability have been demonstrated in many immobilized cell culture systems. Alfermann and Petersen (1995) showed that the biotransformation ability of immobilized *Digitalis lanata* cells remains constant for more than 60 days. Nakajima and colleagues (1990) used alginate-entrapped cells of *Lavandula vera* to produce pigment for more than seven months.

Many studies compared the productivity levels of immobilized cells and suspended

cell cultures; however, few studies compared the product profiles of immobilized cell cultures with those of intact plants. The physiological state and consequently the secondary metabolite production of plant cells is sensitive to the surrounding microenvironment. To ensure batch-to-batch product consistency, the same microenvironment must be created during each immobilization preparation. It is not clear whether this is possible with current cell immobilization techniques (Fu, 1998).

Elicitation

Considering the role as defensive compounds played by most of the secondary metabolites, the addition to the culture medium of molecules able to induce the defence response against infection may result in a prompt stimulation of the secondary metabolic apparatus of the cell. Therefore many elicitor molecules have been tested to induce such a response *in vitro*. Elicitors are usually cell-wall constituents of different microorganisms, plant cell-wall-degrading enzymes, pathogenic proteins, oligosaccharides or lipids. Among the plant molecules shown to function as signal transductors, jasmonates can mimic the effect of elicitation as demonstrated in *Rauwolfia canescens* and *Eschscholtzia californica* (Gundlach *et al*., 1992).

Several studies have shown that elicitation increased the production yield of many food ingredients and pharmaceuticals in various types of plant cell and tissue cultures as well as capsaicin, phenols, benzophenanthridine alkaloids (sanguinarine, chelerythrine and macarpine), rosmarinic acid, taxol (Johnson *et al*., 1991; Dornenburg and Knorr, 1996; Villegas *et al*., 1999; Hui and Feng, 2000; Wu *et al*., 2001).

The induction, however, is often restricted to certain biochemical pathways and the elicited released molecule may not be the one the researcher is interested in. This is especially true for those compounds constitutively produced by the healthy plant, such as quinine, morphine and vinblastine (Verpoorte *et al*., 1999). Among metabolites affected by elicitation are antraquinone from *Cinchona* spp., sanguinarine in *Eschscholtzia californica* under an elicitor derived from yeast extract (Piatti *et al*., 1991) and alkaloids from papaver (Verpoorte *et al*., 1999, and references cited therein).

The mechanism of elicitor action is not well understood. Multiple enzymes in different biosynthetic pathways can be up- or down-regulated on elicitation. It is now clear that elicitation ultimately affects the transcription rates of genes encoding enzymes involved in plant defence pathways. There seems to be a regulatory mechanism for coordinating the activation or inactivation of these genes; however, a detailed understanding of this regulation is still missing (Fu, 1998).

Many phytoalexins are toxic to humans. Because elicitation activates phytoalexin production, a concern exists that elicitation may increase production of these toxic compounds, expecially when the final cell-culture-derived product is not going to be subjected to a complete purification process, as is the case for plant-derived food supplements (Fu, 1998).

A detailed review of elicitors of various natures aimed at the stimulation of flavonoids, coumarins and volatiles in plant-cell suspension cultures among the Apiaceae family, has been prepared by Ekiert (2000).

An interesting semi-empirical approach to manipulating or stimulating culture yield is the establishment of co-culture experiments, in which the plant product may be directly elicited by another organism or further metabolized by it up to the desired molecule. Co-culture in a dual-bioreactor system of transformed hairy roots of *Atropa*

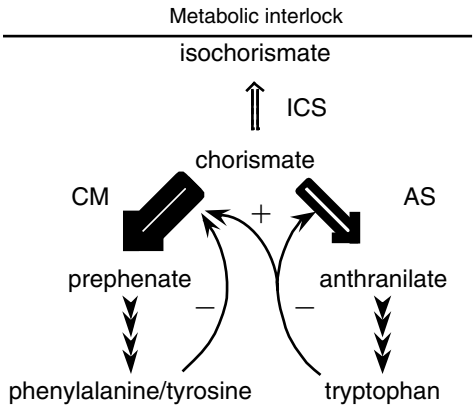

Figure 6.1 The biosynthesis of the aromatic amino acids, phenylalanine, tyrosine and tryptophan is an example of how the feedback mechanism can play a role in the regulation of the fluxes to secondary metabolites (from Verpoorte *et al*., 1999).

belladonna and *Duboisia* hybrid shooty teratomas led to a scopolamine content in the shoot cultures of up to 4.8 mg g^{-1} dry weight, many times the average concentration normally found in leaves and in hairy roots cultured alone (Mahagamasekera and Doran, 1998).

We have obtained essential oil stimulation of *Mentha piperita* shoots and menthol enrichment of its chemical profile after inoculation with a plant-growth-promoting endophytic mycelium isolated from wild peppermint plants (Figure 6.2) (Mucciarelli *et al*., 1997). In the case of peppermint, as well in other species, such an *in vitro* culture system might offer the advantage of developing plant nurseries exploitable for commercial propagation of yield-elicited plants.

In situ *product removal*

In situ product removal is another strategy that involves the addition to the cell culture of a second phase that has a preferential affinity towards the product of interest. The process is referred to as *in situ* extraction or *in situ* absorption, depending on whether the second phase is liquid or solid, respectively. *In situ* product removal has been applied to various cell culture systems – in combination with elicitation and cell immobilization – as part of an integrated approach for plant yield improvement. Application of *in situ* product removal not only makes the recovery of product easier, it often leads to increased productivity. This increased productivity may be the result of reduction of feedback inhibition, protection of the product from degradation, or an enhanced product excretion.

Tagetes spp. produce thiophenes, heterocyclic sulphurous compounds with biocidal activity. Buitelaar and colleagues (1993) studied the effect of the presence of an organic phase, namely the Amberlite™ resin XAD-7, on the growth and thiophene production of *Tagetes patula* hairy-root cultures. They showed that the excretion of the thiophenes was strongly influenced by the addition of XAD-7 to the cultures. Without this resin there was no measurable excretion at all, whereas the presence of

XAD-7 raised the excretion to 50 per cent of the total thiophenes produced. When these experiments were extended to bubble columns, about 20 per cent of the total production was excreted (Buitelaar *et al*., 1993). Similarly, since caffeine suppresses purine alkaloid (caffeine and theobromine) production by *Coffea arabica* cells, the effect of removing caffeine from the medium was investigated using the hydrophobic resin Amberlite™ XAD-4 (Kuruta and Furusaki, 1993; Kurata *et al*., 1994). This method increased alkaloid production to 1.4 times that for the culture with no removal treatment. In *Vetiveria zizanioides* Stapf., a tropical grass producing essential oils in root tissues, we have obtained cell suspensions (Figure 6.3) actively growing after medium optimization and addition to the culture of XAD-4 (Mucciarelli and Leupin, 2002). Addition of charcoal to adsorb vanillin produced from ferulic acid by detached, cultured aerial roots of *Vanilla* and of hydrophobic Amberlite™ to stimulate antraquinone production in cell culture of *Cinchona* has been documented (Walton *et al*., 1999).

Biotransformation

The nearly unlimited enzymatic potential of cultured plant cells can be employed for bioconversion purposes. Plant enzymes are able to catalyse (from regio, 'direction' in Latin) and stereospecific (from στερεφξ, a solid body) reactions, highly selective chemical reactions that can therefore be applied to the production of specifically active compounds of pharmaceutical interest. Naturally occurring as well as related synthetic compounds may be used as precursors by freely suspended and immobilized plant cells or enzyme preparations. Many review papers dealing with bioconversions of added precursors of natural or synthetic origin by several biocatalytic systems are available (Pras, 1992; Pras *et al*., 1995; Cosson, 1997; Verpoorte *et al*., 1999).

A good example of how some of these culture techniques may be assembled for an industrial application is given by the work of Hong and colleagues (1998) on *Digitalis lanata*. Cardiac glycosides or cardenolides are used in treatment of heart diseases. Cultivation of *Digitalis* plants to produce commercial clinical drugs involves several countries, among which are the Netherlands, Hungary and Argentina. Lanoxin is the Burroughs Wellcome digoxin product that has major markets in the United States and Italy (DiCosmo and Misawa, 1995). For the enhanced production of the cardiac glycoside, digoxin, Hong and colleagues (1998) used *in situ* adsorption by biotransformation from digitoxin in plant-cell suspension cultures of *D. lanata*. Culture conditions and digoxin production were optimized when Amberlite™ XAD-8 was added as adsorbing material 36 h from the beginning of biotransformation. This process consists in the ability of *Digitalis* leaves as well as cultured cells to use digitoxin as a substrate to produce digoxin via a 12 β-hydroxylation. In addition, to prevent the cells from having direct contact with resin particles, the authors adopted the immobilization technique which further improved the advantages of *in situ* adsorption, resulting in enhanced digoxin production (Hong *et al*., 1998).

L-3,4-dihydroxyphenilalanine (L-dopa) is an important intermediate in plant metabolism as precursor of alkaloids, betalayns, melanin etc. and it is used as a potent drug in the treatment of Parkinson's disease. Cells of *Mucuna pruriens* immobilized with alginate beads produced and secreted into the medium L-dopa from tyrosine in yields of up to 2 per cent of the dry cell weight (DiCosmo and Misawa, 1995). More recently, this drug has been produced via biotransformation in cell suspension cultures

Figure 6.2 Plant growth elicitation in *Mentha piperita* micropropagated plants by *in vitro* application of an endophytic mycelium. On the right: two 30-day-old peppermint plants inoculated with the fungus. On the left: two control plants (Mucciarelli *et al*., 1997).

of *Stizolobium hassjoo* (Huang and Chou, 2000). An alternative route to L-dopa *in vitro* production is represented by transformed hairy roots (Sung and Huang, 2000).

Other examples of actively biotransforming plant cell cultures are given in Ekiert (2000) for the family Apiaceae.

Metabolic engineering

As mentioned above, plant scientists possess a vast array of genetic tools to insert new genes into plants. Making use of knowledge of the biochemical and molecular–genetic regulation of secondary metabolite pathways and the fact that a number of plant genes have been cloned enables us to alter the yield and chemical profile of plants. The objective may be to enhance the formation of an existing compound, diverging the plant carbon flux towards the desired product, or to introduce a biosynthetic pathway leading to novel compounds or to modify the spectrum of end-products by controlling ancillary or competitive pathways. Catabolism or competitive pathways can be blocked by antisense genes (Verpoorte *et al*., 1999). At present, the majority of the relevant work has been done with whole plants rather than with cell or organ cultures (Walton *et al*., 1999).

These introductory and general considerations must not create the disappointing idea that such genetic approaches are easy. Secondary metabolites generally are not products of single genes but are the results of multistep, multienzyme processes. Changes in the activity of one enzyme often result in the simultaneous up-regulation of some enzymes and down-regulation of other enzymes in the same and parallel pathways. For example, Berlin and co-authors (1994) showed that only one of the two active tryptamine-using pathways was affected by the overexpression of a tryptophan decarboxylase gene in *Peganum harmala* root cultures for the production of harmalol, harmol and harmine, tryptophan-derived beta-carboline alkaloids having anthelminthic and oxytoxic activity.

At the same time, increasing the activity of one or a few enzymes by genetic manipulation may leave overall metabolic flux to the end-product unaffected (Walton *et al*., 1999). Burtin and Michael (1997) obtained the overexpression of the *adc* gene, encoding arginine decarboxylase involved in the synthesis of pyrrolidine alkaloids in *Nicotiana* plants. The sole apparent effect they obtained was an increase of the amino decarboxylation of arginine; there was no effect on the alkaloid content, probably as a result of the intervention of other rate-limiting processes buffering completely the overexpression of the *adc* gene (Walton *et al*., 1999).

Instead of increasing the activity of existing enzymes, one can introduce new enzymes through heterologous gene expression, to make new compounds. The Rubiaceae family includes many species having roots with medicinal and tincturing properties (Paris and Moyse, 1971). The expression of a bacterial gene encoding isochorismate synthase in *Rubia peregrina* hairy-root cultures, increased the biosynthesis of antraquinones (Lodhi *et al*., 1996).

The first target to achieve in engineering plant secondary metabolism is the identification of key enzymes involved in the pathway, though some other factors are of great relevance in determining final success. Regulatory aspects such as feedback mechanisms must not be neglected. An example is given in Figure 6.1 for the biosynthesis of tryptophan. This is one of the main precursors of indole alkaloid substances having pharmacological activities, among them those produced by *Catharantus roseus*.

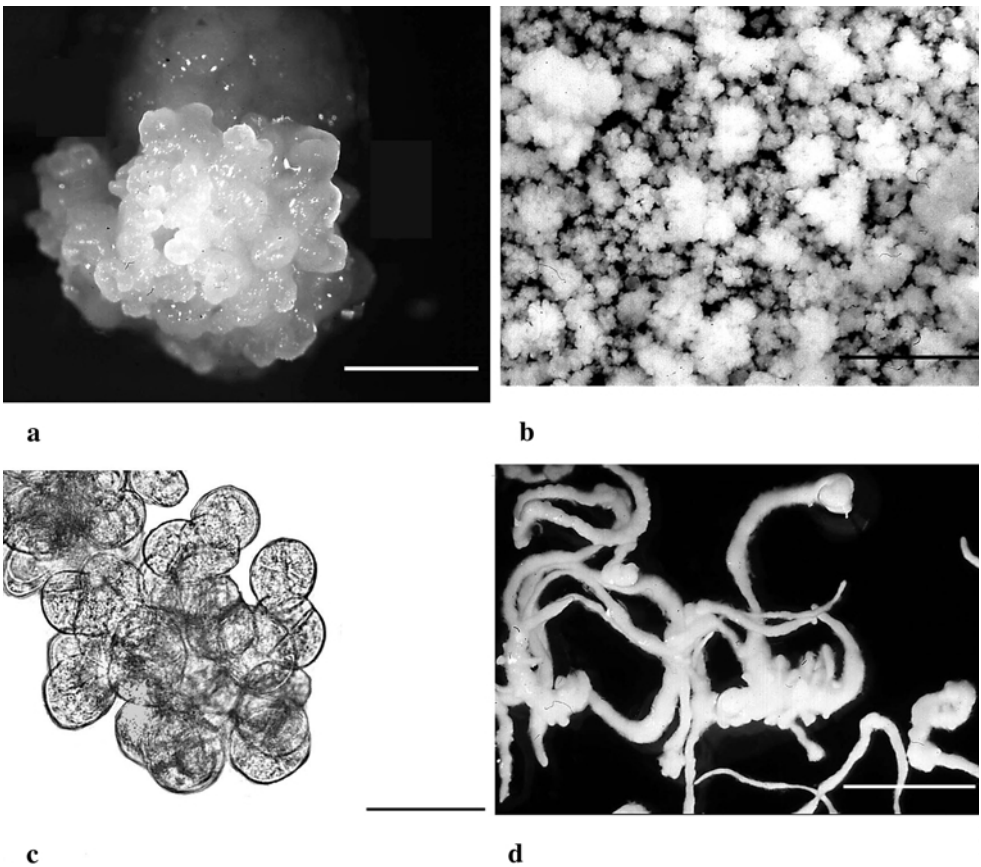

Figure 6.3 Liquid culture induction in *Vetiveria zizanioides*: (a) compact callus as starting material for liquid culture induction (bar = 2 mm); (b) liquid cultures with compact clumps after about one year on mN6 medium (bar = 5 mm); (c) a typical cell aggregate proliferating in mN6 liquid medium (bar = 50 μm); (d) root-like structures from mN60.5D0.5B liquid medium (bar = 5 mm) (Mucciarelli and Leupin, 2002).

The overexpression of anthranilate synthase (AS) determines a feedback inhibition by tryptophan, the end-product of interest. Even if a gene encoding a non-feedback-regulated AS is used, the overproduction of the end-product tryptophan will result in the activation of a second enzyme, the chorismate mutase (CM) which can divert the carbon flux into a competitive pathway leading to phenylalanine and tyrosine (Verpoorte *et al*., 1999). This cell metabolic pathway is involved, among many others, in the biosynthesis of the *Catharanthus* indole alkaloids vincristine and vinblastine which have antitumoural properties. Vinblastine is employed in the treatment of severe Hodgkin syndrome cases. The biotechnological production of these substances has been exploited. As underlined by Verpoorte and colleagues (1997, 1999) the biosynthesis of these alkaloids requires at least three different cell compartments, that is the plastid for the production of the terpenoid moiety and tryptophan, the cytosol for tryptophan decarboxylation and the vacuole for coupling tryptamine with secologanin

(Verpoorte *et al*., 1997). The transport of these alkaloids and precursors within cell compartments is therefore a key step in the regulation of their synthesis, for both initial and terminal enzymatic processes (De Luca *et al*., 1986). Cell suspension cultures of *Catharanthus roseus* led to the production of ajmalicine, serpentine and chatarantyne, all of which have important pharmacological properties and applications such as the cure of hypertension. But, attempts to produce vindoline and vinblastine failed, probably as a consequence of low knowledge of the genetic regulation of such complex metabolic pathways. What is more, cell suspensions are often unable to reach a level of cytodifferentiation sufficient to allow enzyme expression and may show gene expression dependency on genotypic effects.

Many different parameters have been considered for the regulation of the *Catharanthus* metabolic pathway, such as media composition, temperature of incubation, photoperiod, and the application of exogenous stimuli such as UV irradiation (Moreno *et al*., 1995). But at present metabolic engineering seems to be the future direction if we are to succeed in activating the enzymes directly involved in the synthesis of the antitumoural molecules.

Following a different methodological approach we have obtained a stimulatory effect on the root content of ajmalicine through *C. roseus* inoculation with fungal endophytic mycelia. Working with two different high-yield cultivars, we observed that only in cv. Little Delicata did fungal treatment elicit cell primary metabolic pathways leading to increased chlorophyll leaf content and expression of a differential protein pattern (Figure 6.4). This was accompanied by an 83 per cent increase of ajmalicine in roots. On the other hand, plants of cv. Bright Eye responded to fungal elicitation with catalase activation, ajmalicine root content reduction and increased phenolic compound accumulation (Maffei and Mucciarelli, unpublished data) (Figure 6.4). Therefore, the two *Catharanthus* sp. cultivars responded differently towards fungal elicitation: in Little Delicata, cytological and biochemical amelioration of plant photosynthetic apparatus (plastid differentiation and chlorophyll synthesis) resulted in ajmalicine overexpression, while in Little Bright Eye fungal inoculation resulted in plant defence responses directed to phenol accumulation. Two different pathways may have been elicited in this case: one directed to the synthesis of tryptophan, a putative ajmalicine precursor, and the second starting from phenylalanine/tyrosine and leading to phenol synthesis (Mucciarelli and Maffei, unpublished).

The use of new plant gene introgression to alter secondary metabolism has found some interesting applications such as the case of transfer of *Hyosciamus niger* hyosciamine-6-hydroxylase gene into plants of *Atropa belladonna* by the use of an *Agrobacterium*-mediated transformation leading to oversynthesis of scopolamine (Yun *et al*., 1992). This tropane alkaloid is a medicinally important anticholinergic drug that is present in several solanaceous plants. In the primary belladonna transformant and its self-crossed progeny that inherited the transgene, the alkaloid content of the leaf and stem was almost exclusively scopolamine. Such metabolically engineered plants should prove useful as breeding materials for obtaining improved medicinal components (Yun *et al*., 1992).

Stilbene synthase gene has been isolated from *Vitis vinifera* and introduced in other species, resulting in the production of the stilbene-type phytoalexin resveratrol. Resveratrol is a phenolic compound important in the defence of plants against fungal infection and has gained much interest as a dietary phenol with health-promoting effects (Hain *et al*., 1993). There is growing recognition that many phenolic secondary

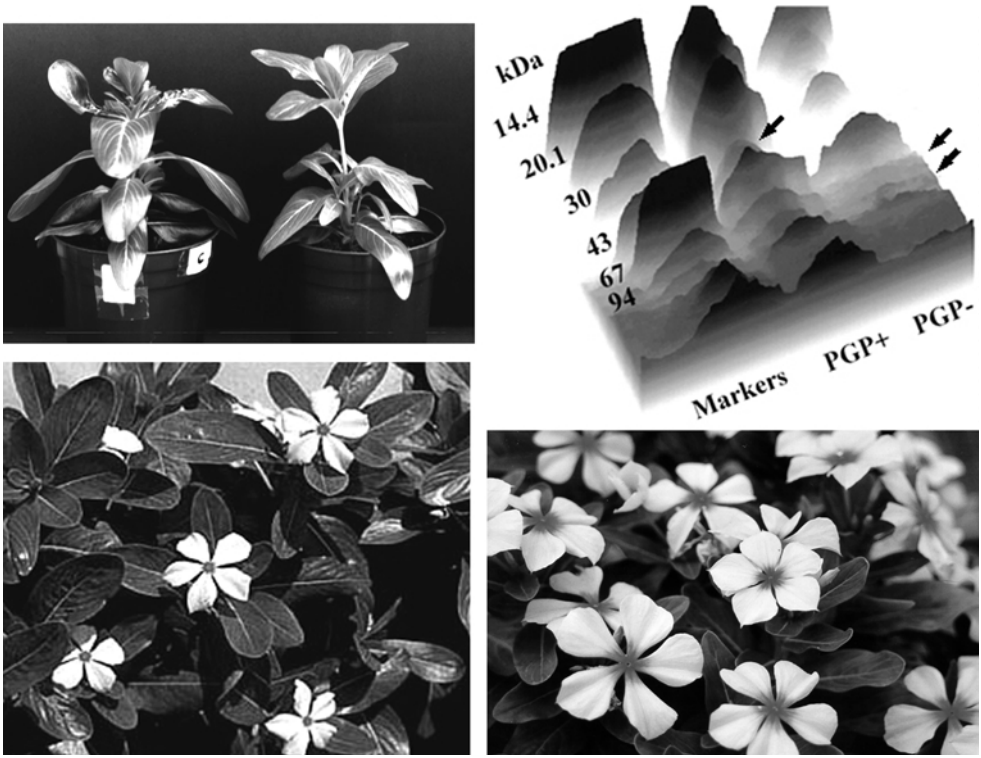

Figure 6.4 Catharanthus roseus cv. Little Delicata plants employed for fungal inoculation experiments (upper left). On the upper right the 3D SDS-PAGE electrophoretic profile of cv. Little Delicata; arrows indicate the three new protein peaks (41, 73 and 96 kDa) present in plants elicited by the fungus (PGP+). In the bottom of the figure, cv. Little Bright Eye (left) and cv. Little Delicata (right) plants in full bloom (Maffei and Mucciarelli, unpublished).

metabolites present in foodstuffs may exert beneficial effects on human health. This may to some degree be mediated via antioxidant actions, but a range of more specific pharmacological effects have also been proposed. Resveratrol has also been postulated to be implicated in the cardioprotective effect of red wine and the low incidence of breast and prostate cancers among vegetarians and Orientals respectively (Rodrigue *et al*., 2001).

There is a growing interest in the West in improving general health by optimizing the phenolic content of food crops, using either conventional plant breeding along with manipulation of agronomic practices, or the more targeted approaches of modern biotechnology (Parr and Bolwell, 2000).

Phytoalexin production in *Vitis vinifera* cell suspension cultures has been obtained by optimizing the carbon source (Larronde *et al*., 1998) and through methyl jasmonate elicitation (Krisa *et al*., 1999). A resveratrol (3,5,4′-trihydroxystilbene) 3-O-beta-glucoside has been isolated from these types of cell cultures (Teguo *et al*., 1996).

Gene transfer from microbial cells

The already mentioned naphthoquinone shikonin from *Lithospermum erythrorhizon* (Boraginaceae) was the first plant secondary metabolite produced on an industrial scale from plant cell cultures. It is used as a dye for food and cosmetics. Boehm and colleagues (2000) have recently manipulated the biosynthetic pathway leading to shikonin in *L. erythrorhizon* by introduction of the bacterial gene ubiA. This gene of *Escherichia coli* encodes 4-hydroxybenzoate-3-polyprenyltransferase, a membrane-bound enzyme that catalyses a key step in ubiquinone biosynthesis (Boehm *et al*., 2000). Despite the ubiA-transformed lines showing an average 22 per cent increase of shikonin production in comparison to the control lines, there was no significant correlation of UbiA enzyme activity and shikonin accumulation. This suggests that further enzymes are involved in the regulation of this pathway (Boehm *et al*., 2000).

Plant genes in microorganisms

Most plant secondary substances having pharmaceutical or phytoterapical application have been overexpressed in microorganisms. Few of these conversions have been commercially scaled up since it is too expensive (Verpoorte *et al*., 1999).

This route is commercially available only when the chemical produced is of great economic value. There has been an attempt to produce terpenoid indole alkaloids in transgenic yeasts, through the introduction of the gene encoding strictosidine synthase and strictosidine glucosidase. The yeast culture after feeding with the two precursors tryptamine and secologanin produced and excreted stictosidine into the culture medium (Geerlings *et al*., 1998; Verpoorte *et al*., 1999).

Transgenic crops for improved medicinal plants and pharmaceutical products

Phytochemicals of plant origin are being widely examined for their ability to provide health benefits as bioactive compounds and as potential nutritionally active ingredients (Cowan, 1999). As discussed by Dillard and German (2000), the number of possible physiological and biochemical ways through which they could provide health benefits is enormous. Such phytochemicals include terpenoids, phenolic compounds and alkaloids.

Even though the number of synthetically produced drugs has increased enormously during the past hundred years, more than 25 per cent of all prescriptions still contain active ingredients of plant origin (Oksman-Caldentey and Hiltunen, 1996). The amount of natural products is expected to increase, due to the above-mentioned limitations of synthetic industrial processes and also as a consequence of the increasing scientific and public interest in traditional and herbal medicine, e.g. homeopathy and so-called functional foods.

Growing scientific evidence suggests that specific food components can promote human health by reducing disease risk and that these components can be incorporated into food products by the newer methods of molecular genetics. The development of these compositionally enhanced food products has had positive effects on health outcomes beyond the satisfaction of basic nutritional requirements (Young and Jones, 1996).

There is therefore great interest in obtaining plants with desired agronomic traits such as improved resistance to pathogens, increased biomass or improved product quality (Oksman-Caldentey and Hiltunen, 1996).

As discussed earlier, secondary metabolite pathways are complicated, since they require multiple enzymatic steps to the desired end-product used as therapeutic substance or dietary supplement and since these enzymatic pathways are under the control of multifactorial regulatory processes.

Efficient methods of gene characterization and gene cloning are now available, and the expression in regenerated plants of useful genes under the specific control of appropriate tissue-organ-specific promoters is regarded as the most promising technique to obtain plants expressing the desired agronomic traits in the desired plant organ.

Transgenic plants therefore have been obtained from a number of species (Galun and Breiman, 1997), but only few of them from medicinal species (Oksman-Caldentey and Hiltunen, 1996).

Expression of foreign genes in medicinal plants

Hairy-root cultures represent naturally genetically changed organ cultures able to regenerate complete plants. Phenotypical alteration of transformed plants regenerated through *in vitro* systems might confer new valuable characters for increasing shoot or root yield.

Yang and Choi (2000) obtained transgenic plants from hairy roots of *Panax ginseng*. Starting from three culture lines, they obtained embryogenic callus induction, somatic embryo formation and their complete germination into adult plants. The transformed ginseng plantlets showed an actively growing root system with abundant lateral roots (Yang and Choi, 2000).

The T-DNA, namely the piece of *Agrobacterium rhizogenes* plasmid which is transferred from the bacterium and incorporated into host-plant nuclear DNA, can be properly disarmed in order to limit bacterium virulence and reconstructed via a binary vector (Saito *et al*., 1992a; Saito, 1994) with foreign genes capable of changing plant metabolic activity (Tepfer, 1990; Oksman-Caldentey and Hiltunen, 1996; Giri and Narasu, 2000).

As for most genetically engineered crop species, medicinal plants have been tested for their suitability for gene transfer. The introduction of chimeric reporter genes, whose expression in the plant can be easily assessed by measuring enzymatic activities of gene products, allows the estimation of transformation efficiency (Table 6.3) (Oksman-Caldentey and Hiltunen, 1996).

Transgenic tobacco (*Nicotiana tabacum* L.), liquorice (*Glycyrrhiza uralensis* Fisher) and foxglove (*Digitalis purpurea* L.) plants were obtained with binary vector systems based on a disarmed *Agrobacterium tumefaciens* or on a virulent *A. rhizogenes*. The chimeric neomycin phosphotransferase II (NPT-II) gene (kan) and the β-glucuronidase (GUS) gene (uidA) were introduced and expressed by transgenic plants (Table 6.3) (Saito *et al*., 1991).

Although reporter genes were not expected to influence expression of plant secondary metabolites, these experiments demonstrated the feasibility of obtaining regenerated plants from hairy-root cultures and laid the technical basis for the development of metabolically engineered medicinal plants.

Attempts have already been made for the transfer and expression *in planta* of specific

Table 6.3 Transfer and expression of chimeric reporter genes in medicinal plants (from Oksman-Caldentey and Hiltunen, 1996)

Plant species	*Reporter gene*[a]	*Transfer system*[b]	*Effect*
Nicotiana rustica	hyg, kan	Ri-plasmid	Nicotine production
Nicotiana tabacum	neo, gus	Ri-plasmid	Increased production of nicotine, nornicotine, anabasine and anatabine
Beta vulgaris	hyg, kan	Ri-plasmid	Betalain production
Glycyrrhiza glabra	neo, gus	Ri-plasmid	Inhibited glycyrrhizin production
Glycyrrhiza uralensis	neo, gus	Ri-plasmid	Inhibited glycyrrhizin production
Digitalis purpurea	neo, gus	Ri-plasmid	Cardenolide production
Datura innoxia	neo, hyg	PEG-mediated	Not determined

Notes

a hyg: gene encoding hygromycin B phosphotransferase; kan: gene encoding kanamycin resistance; neo: gene encoding neophosphotransferase II; gus: gene encoding β-glucuronidase

b PEG: polyethylene glycol; Ri: A. rhizogenes.

genes encoding enzymes involved in key steps of the metabolic pathway (Table 6.4) (Oksman-Caldentey and Hiltunen, 1996). Some examples are represented by the expression of resistance to the herbicides phosphinothricin and bialophos in *Scuparia dulcis* (Yamazaki *et al*., 1996) and in *Atropa belladonna* regenerated plants (Saito *et al*., 1992b; Saito, 1994) enhanced production of nicotine in *Nicotiana rustica* plants overexpressing the ornithine decarboxylase (ODC) gene (Hamill *et al*., 1990), resveratrol biosynthesis in *N. tabacum* obtained by stilbene synthase direct gene transfer (Hain *et al*., 1990), and the earlier mentioned successful transfer of *H. niger* hyoscyamine-6,β-hydroxylase in *A. belladonna* leading to scopolamine-overexpressing transgenic plants (Table 6.4) (Yun *et al*., 1992).

In China, molecular cloning of key enzymes regulating the biosynthetic pathway and introduction of genes into hairy-root cultures for a number of medicinal plant species have been partially obtained, as in the species *Artemisia annua* L. that produces the antimalarial component artemisinin (Chen and Xu, 1996). Geng and colleagues (2001) have found a direct correlation between the content of endogenous plant growth regulators, such as cytokinins, chlorophyll and artemisinin in *A. annua* L. They have transferred the isopentenyl transferase gene (ipt) coding for cytokinins (iPA and iP) into *A. annua* L. via *A. tumefaciens*. In the resultant 19 transgenic shoot lines chlorophyll and artemisinin contents were found increased to different degrees. Content of cytokinins (iPA and iP) was elevated 2- to 3-fold, chlorophyll increased 20–60 per cent and artemisinin increased 30–70 per cent compared with the control plants, respectively (Geng *et al*., 2001).

Recently, genetic transformation via particle bombardment has been obtained in *Catharanthus roseus* through plant regeneration from adventitious organogenetic buds (Zarate *et al*., 1999).

Molecular techniques should also be useful for the identification and neutralization of toxic compounds that remain present in botanical and herbal products.

Something like this has already been done for food crops such as potatoes. Steroidal glycoalkaloids in potatoes are among the most prominent naturally occurring food toxicants. Engel and colleagues (1996) have developed a technique to mathematically correlate tuber size and alkaloid content, which allows the assessment of different potato varieties in terms of glycoalkaloid content. Potatoes modified by means of

Table 6.4 The effect of transfer and expression of some gene constructs on secondary metabolites in medicinal plants (from Oksman-Caldentey and Hiltunen, 1996)

Plant species	*Gene construct*[a]	*Transfer system*	*Effect*
Atropa belladonna	35S-*bar* (pARK5)	Ri-binary plasmid	Herbicide resistance, decrease tropane alkaloid production[c]
Atropa belladonna	35S-H6H	Ti, Ri-binary plasmid	Scopolamine production[c]
Nicotiana rustica	35S-yeast ODC	Ri binary	2-fold enhanced nicotine production[b]
Nicotiana tabacum	TR2′-*rlc* P450	Ti cointegration	Decreased nicotine production, nicotine degradation products[c]
Nicotiana tabacum	TR-*ldc*, rbcS-*ldc*	Ti binary	Cadaverine biosynthesis[c]
Nicotiana tabacum	35S-*ldc*	Ti binary	Increased cadaverine and anabasine production[c]
Nicotiana tabacum	Stilbene synthase gene	Direct gene transfer	Resveratrol biosynthesis[c]
Scoparia dulcis	35S-*bar*	Ti binary	Herbicide resistance, decreased scopadulcic acid B production

Notes
a ODC: ornithine decarboxylase; *rlc*: rabbit liver cytochrome; *ldc*: lysine decarboxylase; *rbc*: ribulose-1,5-biphosphate carboxylase and H6H: hyoscyamine 6, β-hydroxylase
b Secondary metabolites determined from transgenic roots/calli
c Secondary metabolites determined from transgenic plants

recombinant DNA techniques have been investigated with this method. Inhibition of amylose biosynthesis by antisense RNA expression had no effect on the glycoalkaloid content. However, insertion of an invertase gene from yeast caused a reduction of the concentrations of these critical food toxicants. This technique has been proposed by these authors as suitable for the safety evaluation of transgenic food plants (Engel *et al*., 1996) and could be extended to medicinal plants and their PCTC-derived products, in order to satisfy the principle of 'substantial equivalence'.

Final considerations

Even if progress in plant biotechnologies aimed at increased levels of fine chemicals has not yet found many commercial applications it has allowed the screening for biological activities of new compounds and new food colours, tastes or fragrances.

These characters are largely determined by the presence of secondary metabolites, produced by plants. All these compounds are commonly assumed by the human body either indirectly through the use of different botanical preparations or directly following isolation from plant or cell cultures.

In this second case the growing interest in the application of PCTC for food supplements and phytochemical production has prompted considerations from international regulatory agencies to look at the safety of PCTC-derived products (Fu, 1998).

At the moment, the licensing of herbal drugs differs in the EU from country to country. Germany and France each have a list of plants and indications. In other member states phytomedicines are still non-registered dietary supplements. The

situation should change in the future since European laws declare all herbal preparations to be drugs which have to be licensed on the basis of their quality, safety and efficacy (Gruenwald, 1998).

Similarly to the European situation, many botanical products are used widely in the United States and are often marketed as dietary supplements. A recent survey by the US FDA regarding the number of submissions related to botanical drug products over the past ten years and the resultant regulatory actions has stressed the need for more official evaluation of efficacy and safety of all these products (Wu *et al*., 2000).

Advances in molecular biology and recombinat DNA have allowed the development of molecular techniques useful in identifying the presence of different plant species, herbal contaminants and toxicants in botanical products as well as in adulterated foods. Such techniques have been recently developed for herbal supplements too, as in the case of the identification of commercial samples of the herbal compound ginseng (Mihalov *et al*., 2000). Expert botanical identification can now be substituted by assays based on the DNA sequence analysis of ginseng supplements aimed at identifying specific nuclear ribosomal regions (like the ITS region). Other genetic tests suitable for safety control of herbal products include sequence analysis of the chloroplast ribulose-1,5-bisphosphate carboxylase large subunit gene and DNA fingerprinting by the rapid amplification of polymorphic DNA technique (RAPD analysis) (Mihalov *et al*., 2000).

Genetic engineering of valuable crops is the science that involves deliberate modification of the genetic material of plants. As noted by Uzogara (2000) it is an old agricultural practice dating back to early historical times, but recently improved by technology. Many plants consumed today are either genetically modified (GM) whole plants, or contain ingredients derived from gene modification technology. Despite applications available at present being confined to agronomic traits of crop and industrial plants, rather than medicinal plants, in the near future GM technology's benefits are expected to consider many other species of interest for human and animal health, including medicinal plants and their herbal products.

Potential toxicity and allergenicity from consuming GM foods together with more general concerns such as environmental pollution, unintentional gene transfer to wild plants and possible creation of new viruses and toxins are possible risks of this new technology. Because crops with improved output traits means health benefits to millions of people who suffer from malnutrition, the benefits of GM foods apparently far outweigh these risks. It is therefore extremely important to increase public awareness of this technology to enhance worldwide acceptability (Uzogara, 2000) and gain regulatory approval of genetically modified plants and plant products, medicinal plants included.

In Europe, efforts are under way to develop a safe policy, able to determine whether the whole of or a part of the transgenic plant or only its products are used.

Various methods for isolation, identification and characterization of plant constituents altered by biotechnology are already available (Takeoka *et al*., 1996) and will be applied to the safety and efficacy control of medicinal plants and their products.

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7 Biochemistry, physiology and bioengineering of bioactive compounds from plants used as dietary supplements

Massimo Maffei

Introduction

Plants are a rich source of bioactive compounds. From ancient times humans have been aware of the importance of use and exploitation of plants as sources of both food and medicaments. For thousands of years, humans have used extracts and eaten plants to relieve aches and pains. Humanity's transition from fisher–gatherer–hunter to herder–farmer (the so-called agricultural revolution) did not only relate to food, but also to the use of plants as sources of chemicals for herbal medicine. The great progress in chemistry during the 19th century took us from traditional use to pharmaceutical use of natural remedies, with an evident increase in global health and longevity. During the 20th century, with technological progress and alongside high-input agroindustry, herbal medicine has changed from some sort of quackery to a global business, rediscovering traditional medicine, involving biotechnology, making large use of Internet facilities and encouraging awareness of the sustainable use of natural resources. This last example has been the object of international strategies, plans and programmes for the conservation of biological diversity, following the awareness of the huge undermined and endangered potential of plant resources to produce bioactive natural compounds. Unfortunately, of the estimated total of almost 300,000 plant species, only 5,000 have been examined for their possible medical application. Losing plant biodiversity means losing a biological treasure that took millions of years to evolve; at the same time the loss of herbalists who understand the medicinal use of plants leads to the impoverishment of a cultural treasure that took almost a million years to accumulate. Losing biodiversity eventually leads to loss of bioactive natural compounds.

Bioactivity is often defined in terms of human health; however, it is in fact the activity of a natural product with respect to a particular biological process in a living cell or organism. This concept is thus applicable to all living cells and organisms, plants included. The point is that bioactive compounds produced by plants are the results of an evolutionary process which started millions of years ago in the continuous struggle of plants to survive adverse environmental conditions, pathogen and herbivore attack, nutritional competition with other plants and so on. The appearance of humans in this complex framework of biotic and abiotic interactions has created a further element of disturbance in the eco-biosphere. Co-evolutionary processes between plants and their environment have been hampered by the turn of humans from gatherers to growers, with the ensuing control of cultivated areas, the need for new arable land and the consequent deforestation. On the one hand, the selection of

genomes and the making of plants more suitable for food use has led to genetic erosion and the loss of pathways for the production of bioactive compounds; on the other hand, the need for land has eliminated apparently worthless plant species with the consequent loss of biodiversity and the identification and exploitation of bioactive compounds.

The aim of this chapter is to introduce the reader to the 'hidden' world of natural bioactive compounds. In the following pages we will try to answer some basic questions, e.g. where, when, how and why plants produce bioactive compounds which are used as a source of dietary supplements. Furthermore, we will discuss the technological and ethical issues raised by the use of recombinant DNA for the production of the so-called genetically modified (GM) plants, potentially used as a source of powerful bioactive compound production and use.

Our journey will start from general consideration of where bioactive compounds are produced, and then we will enter the world of plant physiology and biochemistry to understand when and why these compounds are produced. We will continue to answer questions on how bioactive compounds are synthesized by exploring some of the main biochemical pathways that lead to the various molecules that accumulate in the sites of synthesis.

Finally, we will analyse how humans intervene with the chemistry of natural compounds and we will face the ethical and biomolecular problems arising from the use of molecular biology and bioengineering as tools to improve the ability of plants to produce bioactive compounds. We will try to understand the gains and losses, the risks and the advantages, the fear of the unknown and the faith in science in the gamble to increase life span and, thus, improve quality of life.

Sites of production of bioactive plant compounds

Bioactive plant compounds can be present in every part of the plant and, in some cases, can accumulate in particular structures called 'secretory tissues'. These aggregates of specialized cells are able to produce large amounts of specific compounds that can be of various chemical natures. However, accumulation of bioactive compounds depends on a series of important factors such as the presence of particular cell compartments, the occurrence of specialized secretory tissues, the effect of environmental pressures, and attack by pathogens and herbivores. In order to understand better the general picture of how bioactive compounds are produced by plants we will try to answer three basic questions: where, when and why bioactive compounds are produced.

Where are bioactive plant compounds produced?

Before entering the fascinating world of plant structure we need to recall some basic characteristics of plants. Plants are static organisms, anchored to the ground by a complex root system and continuously exposed to the atmosphere, to changing environmental conditions and to pathogen and herbivore attack. This dramatic scenario helps us to enter the complex world of plant responses to external factors – conditions that allowed the evolution of a wide array of structural, chemical and physiological adaptations aimed to increase fitness. Bioactive compounds are a component of this scenario and represent the way plants have evolved in order to defend themselves from the attack of other organisms. Modern ecology calls this strategy

'chemical defence'. The success of bioactive compounds is surely chemodiversity. Hundreds of thousands of molecules are produced by plants and most of them accumulate in specialized structures called secretory tissues. The secretion of synthesized substances together with water and solutes supplied by the plant vascular system emphasizes the difficulty in the classification of secretory tissues, along with the fact that secreted substances often contain more than one chemical component (Fahn, 1988). However, in general, two major classes of compounds accumulate in secretory tissues: lipophilic and non-lipophilic substances.

Secretion of lipophilic substances

Plants secrete a wide array of lipophilic substances. Among these are isoprenoids, waxes, flavonoid and isoflavonoid aglycones and fats. Bioactive lipophilic compounds interfere not only with the biomembranes of microbes and herbivores but also with those of the producing plants. For this reason these molecules are sequestered in the cuticle, in dead resin ducts or cavities and in specialized glandular trichomes, which are lined not by a biomembrane but by an impermeable solid barrier (Wink, 1999). As we will discuss below, isoprenoids are a major class of compounds and include important bioactive molecules such as carotenoids, phytosterols, essential oils, phorbols and bitter compounds such as sesquiterpene lactones. At the same time, flavonoids and isoflavonoids, fats and waxes are all components of food and are important because of several medicinal properties.

Lipophilic substances are produced by a variety of anatomical structures, from simple idioblasts to epidermal cell aggregates up to highly specialized tissues such as resin ducts, secreting cavities and glandular trichomes. These tissues can be present either inside the plant body or on plant surfaces.

Surface structures such as glandular trichomes show a spectacular variability of form and function. The use of scanning electron microscopy offers the possibility to understand better how bioactive compounds are stored as chemical weapons to be used in case of attack. Figure 7.1 shows some developmental stages of glandular trichomes present on mint (*Mentha* spp.) leaves. These structures generate from epidermal cells and through a series of anticlinal and periclinal cell divisions (Maffei *et al*., 1989). More recently, Turner *et al*. (2000) have shown that new glandular trichomes are continually produced during leaf growth and that newly initiated glands do occur together with mature glands in growing regions, such that neighbouring glands within the same leaf zone are often of different ages. The secreted lipophilic substances accumulate inside the glandular trichomes and represent a constitutive way of defending the underlying tissues. At full maturation, glands are filled with bioactive molecules and the rupturing of the cuticle releases them to the environment. This is what happens when we crush between our fingers a leaf of peppermint, basil, rosemary, sage, thyme, lavender, yarrow, *Cannabis*, valerian or *Atropa* or any other plant species that has glandular trichomes on its leaf surfaces. This is also what happens when these leaves are boiled or distilled as well as when a herbivore bites one of them.

Other secreting structures are less visible because they are hidden in the deep tissues of the plant. These are secreting ducts and cavities that consist of relatively large intercellular spaces lined by an epithelium of secretory cells (Fahn, 1988). In this case also, bioactive compounds are accumulated and represent a constitutive defence ready to be delivered when tissues are ruptured. Resin ducts are typical of evergreens

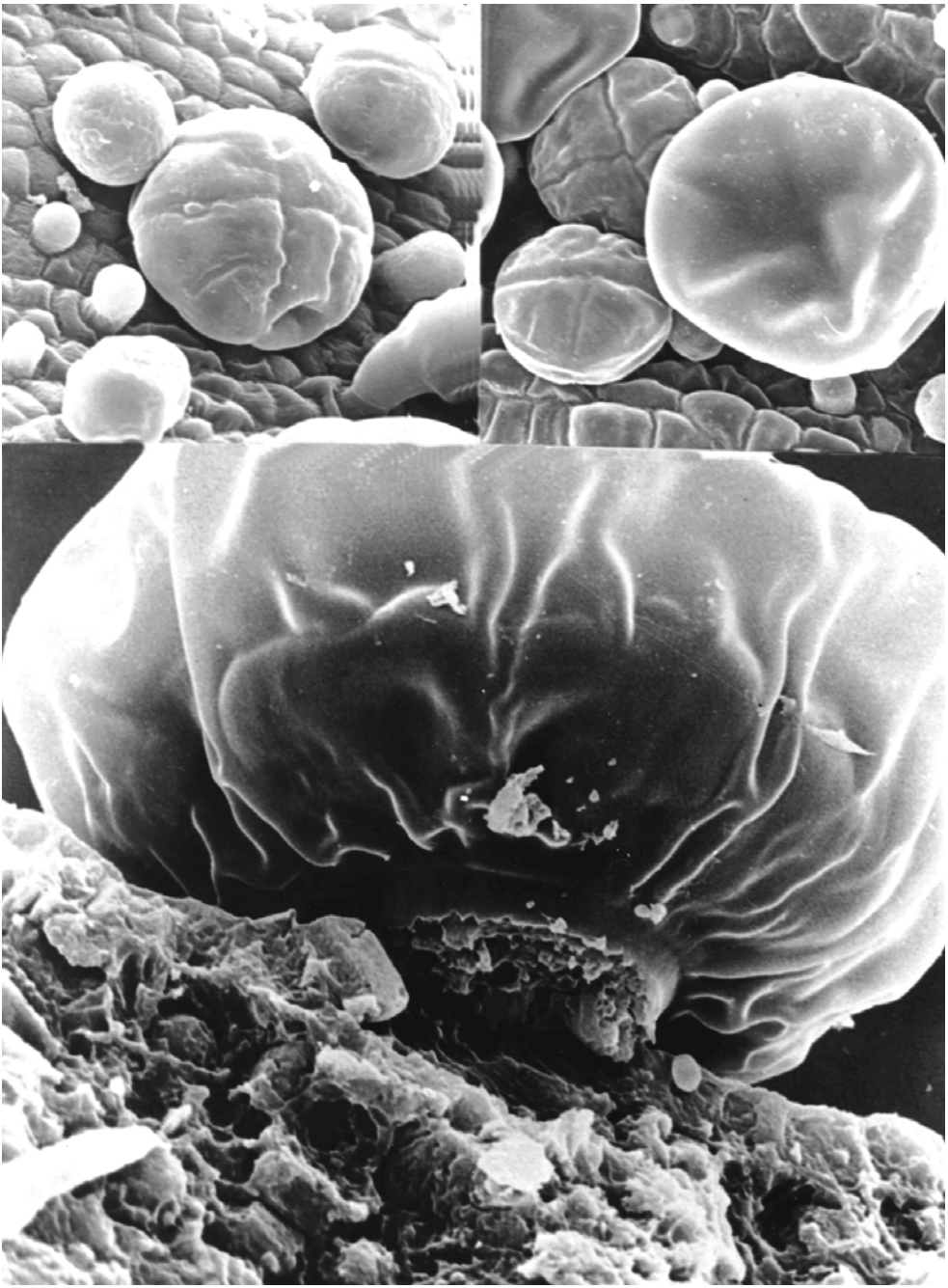

Figure 7.1 Distribution of peltate and capitate glandular trichomes over a mint leaf. In the upper part are young emerging peltate trichomes showing division between secretory cells below the cuticle. A fully differentiated trichome (lower part) shows the enlarged subcuticular space where essential oils accumulate. (Photo: M. Maffei.)

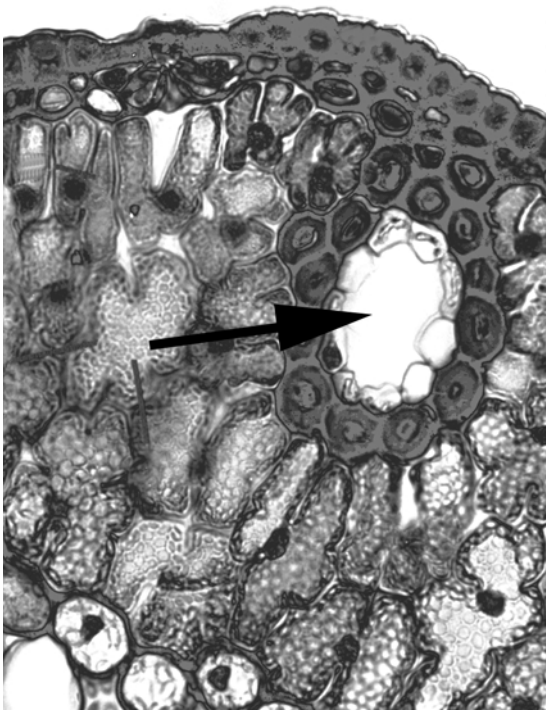

Figure 7.2 The smell of terpenes in a pine wood is due to the presence of resin ducts which are present all over the plant body. The picture shows a cross-section of a pine needle in which a resin duct (arrow) differentiates inside the leaf parenchyma. (Photo: M. Maffei.)

such as the Pinaceae, but are also present in several other plant families such as the eucalyptus family, the Compositae (the yarrow family), the Umbelliferae (the celery family), the Leguminosae (the soybean family) and others (Fahn, 1988). These tissues generate by the progressive separation of cells (schizogeny) with the creation of a large intercellular space inside which secretion accumulates. Figure 7.2 shows a cross-section of a Scots pine needle. In this family resin ducts are present all over the plant body, from leaves to roots, and they accumulate isoprenoids which are used as a chemical weapon against herbivore and pathogen attack.

Secretory cavities are typical of families such as the Rutaceae (the lemon family), the Hypericaceae (the St John's wort family) and the Myrthaceae (the myrtle family) (Fahn, 1988). Unlike resin ducts, secretory cavities originate both by schizogeny and lysigeny (disruption – lysis of cell walls and mixing of protoplasts). Typical structures are those present in the skin of citrus fruits (Figure 7.3). Compression of surrounding tissues forces the secretion to get out and the ensuing release of compounds into the environment represents, in this case also, a constitutive chemical defence.

Other tissues able to produce lipophilic substances are represented by secretory cells that accumulate the secreted products inside their vacuoles. This is the case of essential oils produced by the odorous roots of the species *Vetiveria zizanioides* (vetiver). Secretion of essential oil in *V. zizanioides* occurs within the cortical layer and close to the

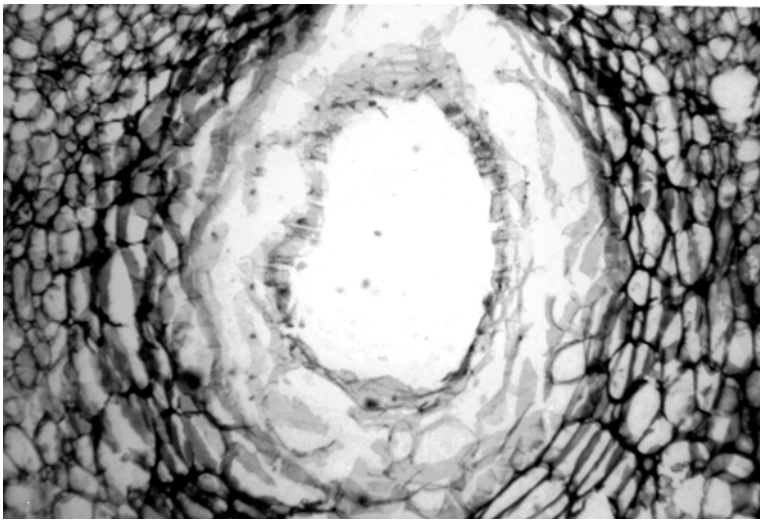

Figure 7.3 Lemons, oranges, tangerines, limes and many other citrus fruits possess secretory cavities filled with mixtures of terpenoids and other secondary metabolites. The picture illustrates a secretory cavity present in the flavedo of a citrus fruit peel. (Photo: M. Maffei.)

endodermis. Figure 7.4 shows a cross-section of vetiver root, where the essential oil-producing cells are evidenced by treatment with Sudan Black B (Maffei, 2002). These results are in accordance with those obtained by Viano *et al*., (1991a, b) who analysed root ultrastructure using electron transmission microscopy and detected essential oil crystals in the inner cortical layer close to the endodermis. According to these authors the secretion of the essential oil occurs in this region and successively reaches all parts of the cortex. The oil density increases as a function of the age of the root, becoming more and more viscous and forming crystal structures in old roots. Other lipophilic molecules such as ubiquinones and tetraterpenes may accumulate in plastid membranes, from which they are released by food intake.

Secretion of non-lipophilic substances

Plants secrete complex acidic or neutral polysaccharide polymers known as mucilages. These compounds are used by plants as reserve food sources, for water retention, as lubricants of the growing root tips, as adhesive in seed dispersal and for the capture of insects by carnivorous plants (Fahn, 1988). However, many polysaccharides contained in these secretions are also used as bioactive compounds. The source of mucilage is in the mucilage cells which are present in several plant tissues like the okra fruit and vegetative organs. Other important species producing mucilages are hibiscus, prickly pear, *Lycopodium* spp., *Rheum*, *Rumex*, *Pharbitis nil* and many others.

Other tissues secreting non-lipophilic substances are the stinging trichomes of nettle. These stinging structures are able to inflict pain by release of toxins and they usually consist of an elongated tapered stinging cell and a varying number of cells

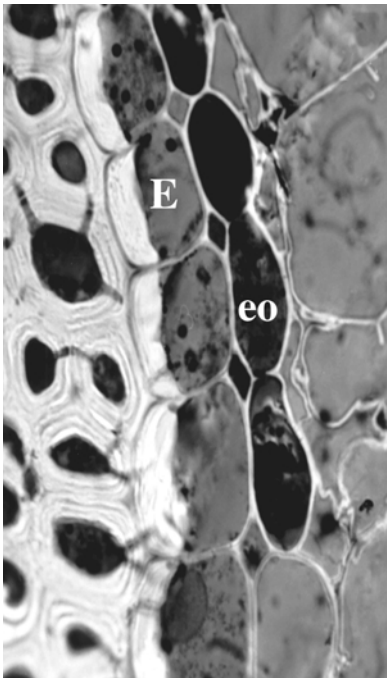

Figure 7.4 The odorous roots of the grass *Vetiveria zizanioides* possess secretory cells that accumulate sesquiterpenoids in the essential oil (eo). These cells are close to the endodermis (E). (Photo: M. Maffei.)

sheathing its lower part (Fahn, 1988). The toxic reaction caused in animals and humans ranges, according to the plant species, from mild skin irritation to the death of some animals. Little is known of the cellular and molecular mechanism and findings indicate that part of the immediate reaction to nettle stings is due to histamine introduced by the needle. However, the presence of other substances toxic to nerves or capable of secondary release of other mediators must not be excluded (Oliver *et al*., 1991).

Between tissues producing lipophilic and non-lipophilic substances are the laticifers. These specialized tissues produce the latex, a suspension, or in certain cases an emulsion, of many small particles in a liquid with a different refractive index (Fahn, 1988). Laticifers may contain alkaloids (in plant species such as *Lobelia*, *Papaver*, *Chelidonium*), cyanogens, NPAAs (non-polar amino acids) and cardiac glycosides, but they can also contain lipophilic substances such as polyterpenes, diterpenes (phorbol esters), lipophilic flavonoids and quinones (Wink, 1999). Latex occurs in more than 12,000 species belonging to about 20 families, mostly of the Dicotyledonae. Among these are the Apocynaceae (the periwinkle family), the Compositae (the dandelion family), the Euphorbiaceae and the Papaveraceae. Laticifers are also present in some monocotyledons such as the Liliaceae (the lily family) and the Musaceae (the banana family). One of the most famous examples of laticifers is present in opium poppy (*Papaver somniferum*). This plant accumulates more than 40 alkaloids in a milky exudate which can be obtained by incising its unripe capsules. The latex tubes of the capsules are

associated with the phloem of all organs of the plant; when the capsules are incised with a knife the latex tubes open into one another. The white milky latex oozes out, but rapidly turns brown and coagulates. This material, called raw opium, is then removed and processed to yield a series of pharmacological products (Dewick, 1998). Humans learned from nature to exploit this constitutive defence and through breeding increased the amount and quality of alkaloid produced. Another example of laticifer is in the Para rubber tree, *Hevea brasiliensis*. In the bark of this plant, and in many other tissues of the whole plant body, are present laticifers that, when injured, extrude a mixture of polyisoprenes which condenses to yield rubber. In the tropical species *Carica papaya*, damaging of the tree inevitably severs its laticifers, eliciting an abrupt release of latex which is a rich source of cysteine proteinase, papain, chymopapain, caricain, glycyl endopeptidase and other enzymes. Together, these enzymes could provide an important contribution to plant defence mechanisms (El Moussaoui *et al*., 2001). Finally, the latex of some plants in the genus *Euphorbia* is toxic, and can cause poisoning in humans and animals, skin dermatitis, cell proliferation and tumour promotion. For this reason many species of Euphorbiaceae are regarded as potentially toxic. Most of the bilogical effects are due to diterpenic esters, e.g. esters of phorbol, which are potent activators of protein kinase C (Dewick, 1998). The rupture of tissues or the ingestion of plant parts causes the release of latex with the consequent toxic effects. Even in this case, latex under pressure inside the laticifers represents a constitutive chemical defence mechanism.

When are bioactive plant compounds produced?

Factors affecting quality and quantity of bioactive compounds

Secondary plant metabolites acting as bioactive molecules are produced at different stages of plant development. Since most of these molecules are defence compounds, they are often accumulated in the early stages of plant development and, in some cases, are already present in the seed long before the embryo develops into a seedling. As we noted above, constitutive defence compounds are ready-to-use molecules that accumulate in specialized tissues and this, though costly and energy-consuming, is a winning strategy against most external attacks. However, the cost of defence is a trade-off with growth and reproduction and, at present, much discussion concerns whether a plant is optimizing growth or reproductive capability with respect to the cost of chemical defence (Kimball, 1996). It has been predicted that fast-growing trees would invest in the cheapest type of defence while slow-growing trees would benefit from investment in more expensive defence strategies (De Jung, 1995). A further distinction can be made between quantitative and qualitative defences. The former involve dose-dependent compounds which are considered to be costly to produce (feeding deterrents such as tannins, flavonoids, terpenoids); the latter are toxins which are lethal or highly effective in small concentrations and thus less costly to produce (e.g. cardenolides of milkweeds, glucosinolates of annual mustard, furanocoumarins, cyanogenic glycosides, alkaloids) (Feeny, 1976; Louda and Mole, 1991; Bryant *et al*., 1992).

Besides constitutive defences, an important role is played by induced chemical defence. The latter consists of a relatively rapid response to external factors via activation of the gene machinery, with eventual production of specific secondary metabo-

lites. Examples are phytoalexins, which are low-molecular-weight and lipophilic compounds that accumulate rapidly around sites of infections and in response to an external array of biotic and abiotic elicitors (Smith, 1996). Induction of secondary plant metabolites can be triggered also by changing environmental and growth conditions. Plants, in response to alteration in their environment, may change the morphological and physiological characteristics in order to maintain or improve fitness. Phenotypic plasticity consists of specific responses, each of them caused by an environmental factor, and the differences may differ between different genotypes (Bradshaw, 1965).

Water stress and nutrient stress induce severe changes in secondary metabolite production. Terpenoid, flavonoid and alkaloid chemical composition can be qualitatively and quantitatively changed by different light conditions as well as by temperature, humidity and wind changes. Light is obviously a key factor in the ultimate production of bioactive compounds because it supplies the energy needed to fix carbon through photosynthesis. But light also catalyses the synthesis of several compounds and by its action on gene activation regulates the amount and the quality of synthesized molecules. Considering the terpenoids produced by some *Mentha* species, the essential oil concentration and biogenesis was maximal in short-day plants, and changes in the photoperiodic treatment affected the oil composition, as has been demonstrated in a number of papers (Ruminska and Nieweglowska, 1965; Burbott and Loomis, 1967; Clark and Menary, 1980; White *et al*., 1987; Maffei *et al*., 1989; Farooqi *et al*., 1999). As well as photoperiod, light quality has been demonstrated to affect mint oil composition. The response of peppermint essential oil to blue light plus white light (W) was a 40 per cent reduction in the total content, caused by a general decrease in p-menthanes, with particular reference to the main compound, menthol (Maffei and Scannerini, 1999). In UV-A plus W irradiated peppermint plants, the increased synthesis of menthofuran was responsible for the total terpenoid increase and appeared to depend on activation of specific genes such as those related to the cytochrome P450 pulegone hydroxylase, which appears to be involved in menthofuran synthesis (see section on biochemistry below) (Maffei *et al*., 1999). Total oil content was significantly increased in peppermint when it was irradiated with UV-B, and no qualitative differences were observed in the direct comparison of irradiated plants and controls (Maffei and Scannerini, 2000).

These results show that in peppermint the essential oil composition is affected in a differential way by different wavelengths. The finding that UV-A (360 nm) stimulates the biosynthesis of some monoterpenes (e.g. menthol), which are suppressed by UV-B (280–320 nm) and blue light (450 nm) indicates that some of the reported light effect on terpene biosynthesis may be wavelength-dependent (Maffei and Scannerini, 2000). Table 7.1 summarizes the effects of blue light, UV-A and UV-B irradiations on peppermint.

Light intensity also plays an important role in the biosynthesis of many other medicinally important metabolites. An excellent example is the tree of joy (*Camptotheca acuminata*), where levels of the anti-prostate-cancer drug, camptothecin, significantly increase as the amount of light impinging on its leaves decreases (Cseke and Kaufman, 1999).

Temperature changes may drastically change the production of bioactive compounds by directly affecting the enzyme machinery responsible for metabolite production. At reduced temperatures around 0 °C, most enzymes are inactive but as the

Table 7.1 Oil chemical composition of essential oils distilled from *Mentha piperita* plants irradiated with white light (W), blue light (B), UV-A and UV-B. Data are expressed as mg kg^{-1} fresh weight ± s.e.m. ( ).

Compound	*Control (W)*	*Light irradiation*		
		B[a]	*UV-A*[b]	*UV-B*[c]
α-pinene	10.27 (1.25)	18.61 (1.66)	3.80 (0.77)	17.61 (2.04)
β-pinene	11.33 (1.04)	20.91 (1.22)	5.60 (0.49)	16.20 (1.42)
sabinene	7.44 (0.25)	13.90 (0.84)	4.05 (0.82)	12.20 (1.14)
myrcene	11.79 (0.35)	30.53 (1.03)	6.21 (1.02)	20.75 (0.89)
limonene	22.28 (3.49)	54.28 (2.63)	11.55 (1.09)	28.74 (1.34)
1,8-cineole	76.67 (4.63)	120.11 (4.58)	29.89 (1.08)	68.89 (5.24)
γ-terpinene	8.31 (2.73)	9.47 (2.20)	3.32 (0.28)	9.47 (1.07)
(E)-β-ocimene	1.66 (0.14)	3.30 (0.81)	0.92 (0.14)	3.51 (1.17)
menthone	365.46 (46.87)	424.74 (34.73)	127.42 (6.63)	445.97 (41.19)
menthofuran	150.65 (67.98)	341.54 (19.56)	212.14 (12.10)	369.25 (22.21)
isomenthone	39.40 (9.10)	51.61 (4.18)	18.12 (1.57)	43.38 (1.12)
menthyl acetate	24.75 (1.67)	49.36 (2.51)	14.11 (0.79)	392.67 (33.27)
α-terpineol	11.91 (3.97)	20.17 (1.24)	6.94 (0.58)	15.84 (2.51)
β-caryophyllene	60.75 (16.98)	51.80 (6.52)	38.27 (2.06)	90.94 (4.45)
menthol	780.88 (89.77)	350.94 (15.51)	518.98 (43.52)	173.30 (12.58)
germacrene D	32.78 (5.73)	74.08 (12.28)	14.90 (1.24)	62.28 (5.65)
piperitone	22.48 (5.52)	29.67 (2.72)	22.29 (3.52)	49.75 (8.91)
TOTAL	**1668.81 (78.88)**	**1665.02 (92.51)**	**1038.51 (52.49)**	**1820.75 (91.87)**

Sources:
a Maffei and Scannerini, 1999;
b Maffei *et al.*, 1999;
c Maffei and Scannerini, 2000.

temperature increases the rate of enzyme activity increases up to 40–50 °C, according to the enzyme. Above 50 °C most plant enzymes are inactivated and even permanently damaged (Cseke and Kaufman, 1999). During seasonal changes, temperature may play a major role in the quality and quantity of bioactive compound produced. In temperate regions, a hot summer may not be accompanied by a suitable rain regime, thus leading to water stress and growth reduction. On the other hand, high temperatures and heavy rain generate high humidity which may trigger pathogen growth and disease spread.

Developmental stages are another important factor involved in bioactive compound quality and quantity. Some compounds such as terpenoids accumulate in secretory structures (see above) which differentiate in the early stages of leaf development. The density of these structures is very high in young leaves and tends to decrease with leaf development (Maffei *et al*., 1989). In many species, anthocyanin production is often more evident in young leaves than in old ones. Capsules of opium poppy produce more alkaloid than any other part of the plant, whereas roots of some medicinal plants produce metabolites that are present only in small amounts in leaves. The number of investigations related to changes of bioactive compounds with regard to plant part and development is extraordinarily great (Bernath, 1986). Functional relationships exist between ontogenetic change and development and between development and accumulation of bioactive compounds. Morphogenesis (the acquisition of shape or form) and photomorphogenesis (light-dependent morphogenesis) are the sum of patterns of both

cell division and cell expansion, which in turn differentiate owing to the turning on or off of genes (gene expression) and the ensuing alteration of the cell's complement of enzymes and structural protein.

Temperature, light, water, nutrients, plant development, pathogen and herbivore attack, all affect the quantity and quality of bioactive compounds and this has to be taken into consideration when secondary metabolites are present in dietary supplements of plant origin. The growth conditions of a certain herb (place of growth, fertilizers used, environmental conditions, etc.) are important factors affecting the presence and the content of a compound.

Standardization of dietary supplements of plant origin

The approach for drug development from plant resources depends on the aim. Different strategies will result in a herbal medicine or in an isolated active compound. However, apart from this consideration, the selection of a suitable plant for a pharmacological study and the standardization of the process are very important and decisive steps (Rates, 2001). There are several ways in which this can be done, including chemical content, toxicity, randomized selection, biological and physiological studies or a combination of several criteria (Ferry and Baltassat-Millet, 1977; Soejarto, 1996; Williamson *et al*., 1996).

Phytomedicines are freely marketed and, in underdeveloped or developing countries, the use of medicinal plants is widely accepted. This can result in toxic accidents from the use of plants as food or for therapy or from accidental ingestion by children or animals. Toxicity can result from highly concentrated doses or from the state of conservation of plants and the form of use (Rates, 2001). Among the various types of registered cases, we can find: (A) accidents due to mistakes of botanical identification (use of a wrongly identified plant is common, as is the substitution of different plants for the same indication) (Pereira, 1992); (B) intoxication by popular remedies (popular remedies, made without legal authorization and sold by herbalists or even prescribed by religious leaders for use in rituals, have often resulted in toxic symptoms immediately after ingestion or later) (Rates, 2001); (C) accidents with cardiotonic plants: plants with a high content of cardiac glycosides, such as *Nerium oleander*, *Thevetia peruviana*, *Gomphocarpus fruticosos* and *Calotropis procera*, are used as decorative plants and have caused a number of domestic accidents involving children and animals (Rates, 2001); (D) plants that interfere with conventional pharmacological therapy (see chapter by Cott); (E) other toxic plants.

Food contains nutrients which are not only capable of meeting caloric and general metabolic needs but can also affect specific metabolic functions which can either initiate or retard the process of degeneration of the cells and tissues of the human body (Burn and Kishore, 2000). Endangering consumers' (patients') health by changing the product's quality, whether deliberately or otherwise, is a major concern of all regulating authorities. Therefore, any change to the process has to be proved to yield a comparable product. If the product's characteristics have been modified, the resulting product would be considered to be a new product and have to be evaluated fully (Doblhoff-Dier and Bliem, 1999).

The above issues clearly point to the fact that one of the major problems in dealing with dietary supplements of plant origin is the definition of reference standards. Different types of standards have been proposed; among these are: (a) authenticated

reference plant material (ARPM); (b) powdered plant material; (c) purified 'active' chemical constituents; and (d) plant extract (powdered or semi-solid).

a ARPM requires a positive identification that has to be at different levels such as histological and taxonomical; to this end, plant organs or tissues have to be certified by specialists such as taxonomists, botanists, histologists and pharmacologists. ARPM requires reference material to enable us to detect adulteration with other plant species of the same genus or other foreign organic matter. In order to establish ARPM there is the need for a careful selection of seeds, planting and irrigation as well as harvesting at the right time. Furthermore, the preparation of a herbarium is required, as is the consequent identification, certification and storage of plants.
b Powdered plant material is prepared from dried plant parts (e.g. leaves, flowers, stems, roots). Positive identification is done by comparison with the sample under test by using analytical techniques such as TLC (thin-layer chromatography), HPLS (high-performance liquid chromatography), LC-MS (liquid chromatography – mass spectrometry) and GC-MS (gas chromatography – mass spectrometry).
c Issues related to purified chemical constituents are mainly: purity of compounds, their stability, their availability or replaceability, the quantity supplied and the relative cost.
d Plant extracts must be stable, the quality of raw material must be high, and the solvent(s) used and the ratio of solvent(s) to plant material has to be known, as well as the consistency in composition.

According to the Consumer Healthcare Products Association (the national trade association representing US manufacturers and distributors of non-prescription, over-the-counter medicines and dietary supplement products), dietary supplements have been attacked for not meeting quality standards. However, while some of these allegations may seem accurate, there has been a concerted effort of the industry and governmental organizations to establish standards for dietary supplements. Surely, the physiology and biology of the living plant cannot be ignored when quality and quantity of products are to be certified and standardized in herbal remedies and dietary supplements of plant origin. Through partnership(s) with private, professional, academic and governmental groups one can achieve the goal of establishing quality standards for dietary supplements of plant origin.

Based on results of national opinion surveys, including the views of both users and non-users of supplements, Blendon and co-workers (2001) found that a substantial percentage of Americans surveyed reported that they regularly take dietary supplements as a part of their routine health regimen. Usually they do not discuss the use of dietary supplements with their physicians because they believe that the physicians know little or nothing about these products and may be biased against them. Many users felt so strongly about the potential health benefits of some of these products that they reported that they would continue to take them even if they were shown to be ineffective in scientifically conducted clinical studies. The majority of Americans surveyed want the Food and Drug Administration to review the safety of new dietary supplements prior to their sale, to provide increased authority to remove from sale those products shown to be unsafe, and to increase government regulation to ensure

that advertising claims about the health benefits of dietary supplements are true (Blendon *et al*., 2001).

The extent of concern about safety and efficacy of herbal remedies is widened by recent consideration of the healing properties not only of herbs but of plant foods in general. Burn and Kishore (2000) propose a new name – 'vitalins' – to those molecules of dietary origin which help preserve vital functions and, thus, vitality of the body. 'Anti-vitalins' are those molecules that have the opposite effect, i.e. one of accelerating the degenerative process. Vitalins are therefore a class of nutrients and anti-vitalins are a class of anti-nutrients. Functional foods are foods containing one or more of these vitalins or foods depleted of anti-vitalins. They are more functional in their ability to meet metabolic needs of either the whole body or specific organs of the human body, in a context which includes vitality in addition to general metabolic activities (Burn and Kishore, 2000) (see Chapter five for further details).

While scientists continue to dissect food and determine its molecular components that deliver nutritional and health benefits at an unprecedented pace, there is a growing need for science-based regulations that will help to distinguish science-based nutrition and disease prevention from quackery and anecdotal stories (Tyler, 1999). Thus, standardization is not just an analytical operation and does not end with the identification and assay of the main active principles. Standardization signifies the body of information necessary to guarantee not only a constant chemical composition of herbal medicines, but also an equal efficacy (Capasso *et al*., 2000).

Why are bioactive plant compounds produced?

The last question to be answered in this section is why plants produce bioactive molecules. Our anthropocentric view of herbal remedies leads us to think that plants evolved their ability to produce secondary metabolites not for their own use, but to be used by animals and humans to heal pains and diseases. However, plants appeared more than 400 million years ago, long before humans, during the Silurian Period. During the Permian, plants and animals slowly changed and gymnosperms and Cycadaceae took the place of Lycopodales and tree-ferns. At that time, probably half of all insects were feeding on plants. Most herbivorous insects were present in the Palaeozoic as well as during the Mesozoic. Over the ages plants and their predators have evolved several strategies to survive. The tolerance of plants to herbivory reflects the degree to which they can regrow and reproduce after damage from herbivores. Autecological factors, as well as the influence of competitors and mutualists, affect the level of plant tolerance. Recent work indicates that there is a heritable basis for tolerance and that it can evolve in natural plant populations (Strauss and Agrawal, 1999). Although tolerance is probably not a strict alternative to plant resistance, there could be inter- and intraspecific tradeoffs between these defensive strategies.

In general, the success of plants derives in part from their highly evolved defence systems, which have allowed them to withstand interspecific competition as well as herbivore and pathogen predation (Phillips and Croteau, 1999). From a biochemical point of view, because multiple pathways are elicited during attack by either herbivores or pathogens, a clear dichotomy between pathogen- and herbivore-specific defence pathways does not always exist (Felton and Korth, 2000). The contemporary evolution of plants in producing chemical defences and herbivores in counterattacking them has been termed co-evolution, as we will discuss in the next subsection.

Co-evolution

Ehlrich and Raven (1964) were among the first researchers to introduce the theory of biochemical co-evolution, in which bioactive plant metabolites are biosynthesized as a consequence of host-plant utilization by herbivores. The advent of this theory, along with the suggestions already made half a century ago by Fraenkel (1959), have changed the view of secondary metabolites from waste products to bioactive molecules. The core of the theory is centred upon the concept that a systematic evaluation of plants fed upon by herbivores leads to the conclusion that bioactive plant metabolites play the leading role in determining patterns of utilization. This concept allows us to explain the vast and irregular distribution of secondary plant metabolites in the plant kingdom. Plants, via occasional mutations and recombinations, have produced an array of chemical compounds not necessarily linked to primary metabolism. Some of these compounds have been successful in reducing or deterring insect and herbivore feeding and for this reason have been 'conserved', whereas others did not find a metabolic and functional 'place' in the evolutionary path of some species and for this reason have been genetically deleted. On the other hand, herbivores have faced up to plant defences and have evolved ways to detoxify them. Individuals able to feed chemically defended plants have survived, whereas others, unable to feed or deterred by plant compounds, had to move to other plants in search of food or die. This animal–plant co-evolutionary behaviour eventually led to insect discrimination between plants in feeding, with many herbivores feeding on a small number of related species belonging to the same genus, tribe or family. The toxicity of some compounds to insects may be exerted by individuals sharing the same biochemical machinery but with different anatomy and morphology, while plants sharing the same morphology or anatomy may produce different chemical weapons. As we observed above, plants can use constitutive and/or induced bioactive compounds, depending on the environmental pressure imposed by herbivores, predators and abiotic factors (Harborne, 1993).

The continuous pressure of herbivores upon plants and the ensuing plant responses and herbivore counterattacks have generated the sea of metabolites that Mother Nature offers and that humans and animals have learned to use. To this picture we may add the environmental pressures caused by changing weather conditions, water and nutrient availability, light intensity and quality (UV-A and UV-B irradiation) and, more recently, air, soil and water pollution caused by human activity.

Another way plants have evolved bioactive compound synthesis is through multiple interactions with herbivores. Plants respond to an initial attack by herbivores and pathogens by increasing their levels of defence (Agrawal, 1998). For example, volatile compounds that are attractive to natural enemies of herbivores are hypothesized to be an evolved response to herbivory. Although the net costs or benefits in plant performance of such induced volatile responses have not been identified, intricate and highly specific interactions between constituents of herbivore saliva and plant responses provide circumstantial evidence of their importance. This kind of interaction of plants, herbivores and predators is called tri-trophic interaction. Studies of tri-trophic interactions aim to identify them, understand their mechanistic basis and document their consequences. Ultimately manipulating these interactions may result in better pest control and the reduced use of pesticides (Agrawal, 2000). In terrestrial environments, strong trophic interactions are modified by the chemistry, morphology and behaviour of each organism involved. Plants recruit natural enemies of herbivores using volatiles.

Plants must discriminate between different environmental challenges in order to optimize the allocation of their resources to growth, defence and reproduction. Phytophagous insects display a great diversity of feeding modes and life histories, and it is important for plants to distinguish between insects that have different fitness consequences for the plant (Baldwin *et al*., 2001). The essential constituents of these interactions, ranging from herbivore saliva to plant hormones and regulatory enzymes, are now being isolated, and their genes cloned. It is not known whether plants that are infested with microbial diseases or nematodes attract or facilitate natural enemies of these plant parasites. Such interactions are probably abundant, yet their natural history and potential application have not been explored (Agrawal, 2000).

Paths of production of bioactive plant compounds

Our discussion about bioactive molecules produced by plants cannot avoid the important answer to the general question on *how* these metabolites are produced. Biochemical studies on medicinal natural products have their roots in the continuous human search for healing remedies and stimulant and medicinal extracts. When eighteenth-century chemists turned from alchemy to modern science, the true properties of plant extracts began to reach their proper place in the search for bioactive molecules. When scientists began to separate, isolate, identify, purify and finally analyse compounds produced by plants, they laid the foundations of the study of living organisms to build these molecules. When radiolabelled precursors were available, plant biochemistry took off towards the new frontiers of secondary metabolites that eventually led to biotechnology and gene manipulation. The recognition of biosynthetic principles is the most significant development in bioactive plant product research. Biosynthetic studies on terpenoids, flavonoids and alkaloids opened a door into the fascinating synthetic workshop of living cells, leading to the deciphering of various biosynthetic pathways, mapping out compounds and identifying precursors and intermediates (Torssell, 1997).

Today, biosynthetic principles are fundamental in the process of structural determination of natural products, allowing us to easily discern human-made compounds from natural ones.

In this section we will explore the biosynthetic world of natural compounds by considering the three main classes of bioactive plant molecules: flavonoids, terpenoids and nitrogen-containing compounds. Plants use an integrated network of enzyme-mediated and carefully regulated chemical reactions in order to produce the hundreds of thousands of secondary metabolites. This is achieved through use of intermediary metabolism involving biosynthetic routes termed 'metabolic pathways'.

For obvious reasons I will not describe all pathways nor consider all bioactive molecules. Nevertheless the aim of this section is to provide the general reader with an exhaustive overview of what plants are able to do in order to produce bioactive molecules; at the same time the specialist will find up-to-date details of plant biosynthetic machinery.

Much of the research on plant natural-product biosynthetic pathways is still at the level of gene discovery. The recent studies reviewed here have increased our understanding of how biosynthetic diversity is generated using relatively conserved enzymatic mechanisms. This emerging knowledge will provide the bridge to the next era of natural-product gene discovery utilizing functional genomics (Dixon, 1999a).

In all classes of metabolites what is common is the presence of metabolic building blocks through which more complex molecules are constructed. Usually, these building blocks derive from primary metabolites, molecules produced by all plant species, and the surprising thing is that the number of building blocks needed to produce bioactive compounds is so low (Dewick, 1998). Another common feature to all bioactive compound biosynthesis is that reactions are catalysed by enzymes that catalyse several reactions such as: (a) alkylation reactions (nucleophilic substitution, electrophilic addition); (b) carbon–carbon bond formation; (c) carbon–nitrogen bond formation; (d) transamination; (e) decarboxylation; (f) oxidation and reduction reactions (dehydrogenases, oxidases, monoxygenase, dioxygenase, amine oxidase, phenol oxidases); (g) glycosylation reactions (Dewick, 1998).

In this section we will try to answer the fourth question: 'How are bioactive compounds produced?' We will start our biochemical journey from the most common secondary metabolites, the phenolic compounds.

Phenolic compounds

Phenolic compounds are defined chemically by the presence of at least one aromatic ring bearing one (phenols) or more (polyphenols) hydroxyl substituents, including their functional derivatives (e.g. esters and glycosides). Phenolic compounds can be further divided into three groups: (1) low-molecular-weight compounds, (2) complex compounds of relatively high molecular weight, and (3) polymeric compounds (Hättenschwiler and Vitousek, 2000).

Low-molecular-weight phenolic compounds

Low-molecular-weight phenolics (LMWP) occur universally in higher plants; some of them are common in a variety of plant species and others are species-specific. The pathway leading to the biosynthesis of LMWP is better known as the shikimate pathway. In the first step (Figure 7.5), the glycolytic intermediate phosphoenol pyruvate and the penthose phosphate pathway intermediate erythrose-4-phosphate are condensed to yield the seven-carbon compound 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP), a reaction catalysed by the enzyme DAHP synthase. In the second step (Figure 7.6), the heterocyclic ring of DAHP is changed to form a highly substituted cyclohexane derivative, 3-dehydroquinate. The following steps start the process of transforming the cyclohexane to a benzene ring. The process starts from dehydration and reduction of 3-dehydroquinate to yield shikimate, which in turn is transformed via a simple ATP-dependent phosphorylation reaction to shikimate-3-

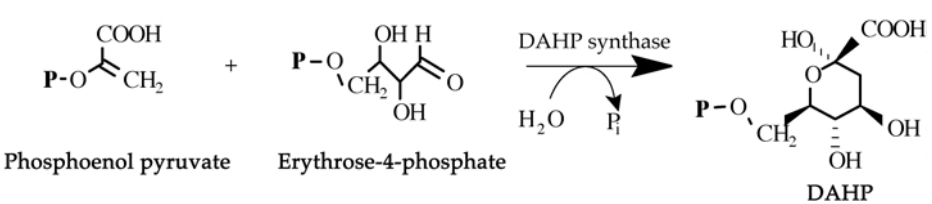

Figure 7.5 The first step in the shikimate pathway. Primary metabolites are condensed by the enzyme 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase to give DAHP.

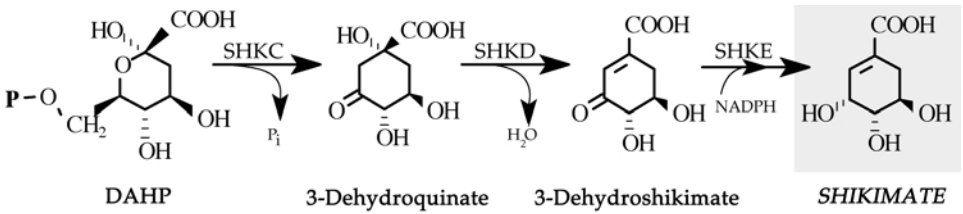

Figure 7.6 Formation of the shikimate. Enzymes involved are: SHKC, 3-dehydroquinate synthase; SHKD, 3-dehydroquinate dehydratase; SHKE, shikimate dehydrogenase.

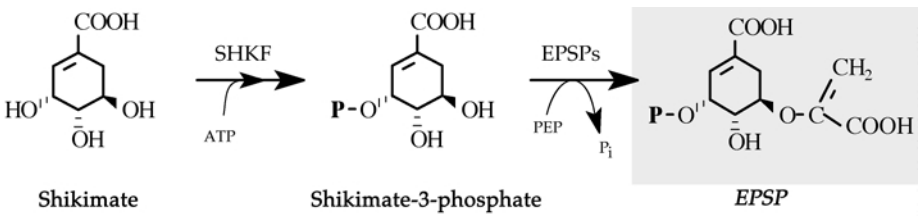

Figure 7.7 Formation of 5-enolpyruvylshikimic acid 3-phosphate (EPSP) by the action of the two enzymes shikimate kinase (SHKF) and EPSP synthase.

phosphate (Figure 7.7). This combines with PEP (phosphoenol pyruvate), giving 5-enolpyruvylshikimic acid 3-phosphate (EPSP). Glyphosate, a broad-spectrum herbicide, causes death of plants by acting as a powerful inhibitor of the enzyme responsible for EPSP synthesis (EPSP synthase) (Dewick, 1998). The transformations of EPSP to chorismate (a Greek term that means bifurcation) allow formation of prephenic acid, which is used for tyrosine and phenylalanine synthesis, and anthranilic acid, the precursor of tryptophan (Figure 7.8). Transamination of prephenic acid leads to arogenate formation (Figure 7.9); the latter, following decarboxylation and oxidation, is transformed to the amino acid tyrosine; on the other hand, decarboxylation and dehydration of arogenate yields the amino acid phenylalanine (Figure 7.10). Finally, the deamination of the two aromatic amino acids leads to formation of phenylpropanoids (Figure 7.11), the building block for flavonoid formation.

A large body of evidence suggests that plants, through the action of an important LMWP, salicylic acid, confer disease resistance. This important molecule, whose synthetic derivative is aspirin, derives from the building block cinnamate that is con-

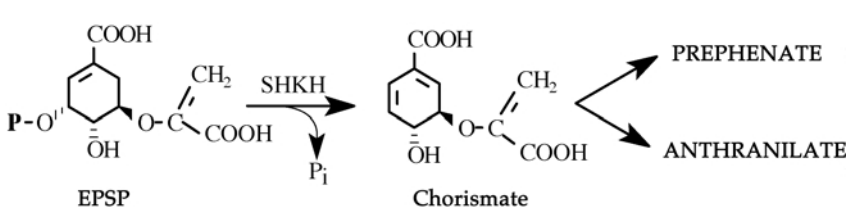

Figure 7.8 Formation of prephenate (the precursor of phenylalanine and tyrosine) and anthranilate (the precursor of tryptophan) from chorismate. SHKH, chorismate synthase.

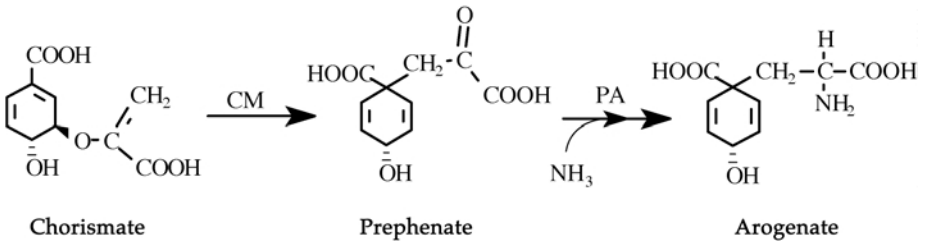

Figure 7.9 Chorismate mutase (CM) transforms chorismate to prephenate, which is aminated to arogenate. PA, prephenate aminotransferase.

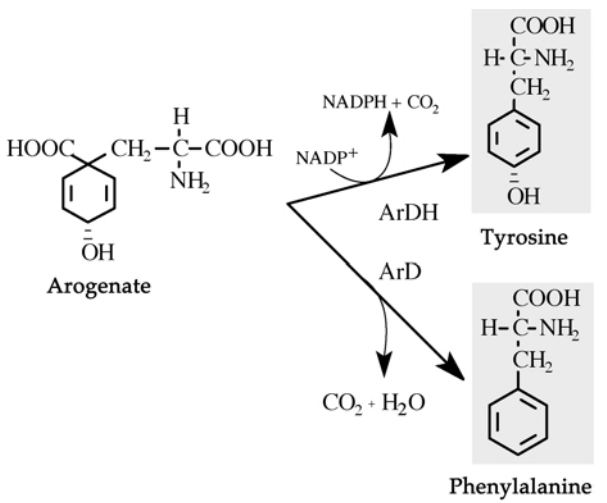

Figure 7.10 Formation of aromatic amino acids tyrosine (by the action of the enzyme arogenate dehydrogenase, ArDH) and phenylalanine (by the action of the enzyme arogenate dehydratase, ArD).

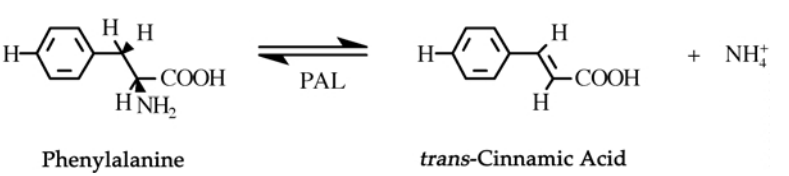

Figure 7.11 The building block molecule *trans*-cinnamic acid is biosynthesized by deamination of phenylalanine, a reaction catalysed by phenylalanine ammonia lyase (PAL).

verted to benzoic acid, which in turn is hydroxylated to salicylic acid (SA). The latter is then conjugated to glucose to form the glycoside that accumulates after tissue infection (Figure 7.12) (Crozier *et al*., 2000). Recent findings have suggested that the rate-limiting step in SA biosynthesis is the conversion of cinnamate to benzoate, and that this involves a β-oxidation pathway. Endogenous benzaldehyde can also be converted to benzoate and SA, but it is not the main endogenous precursor.

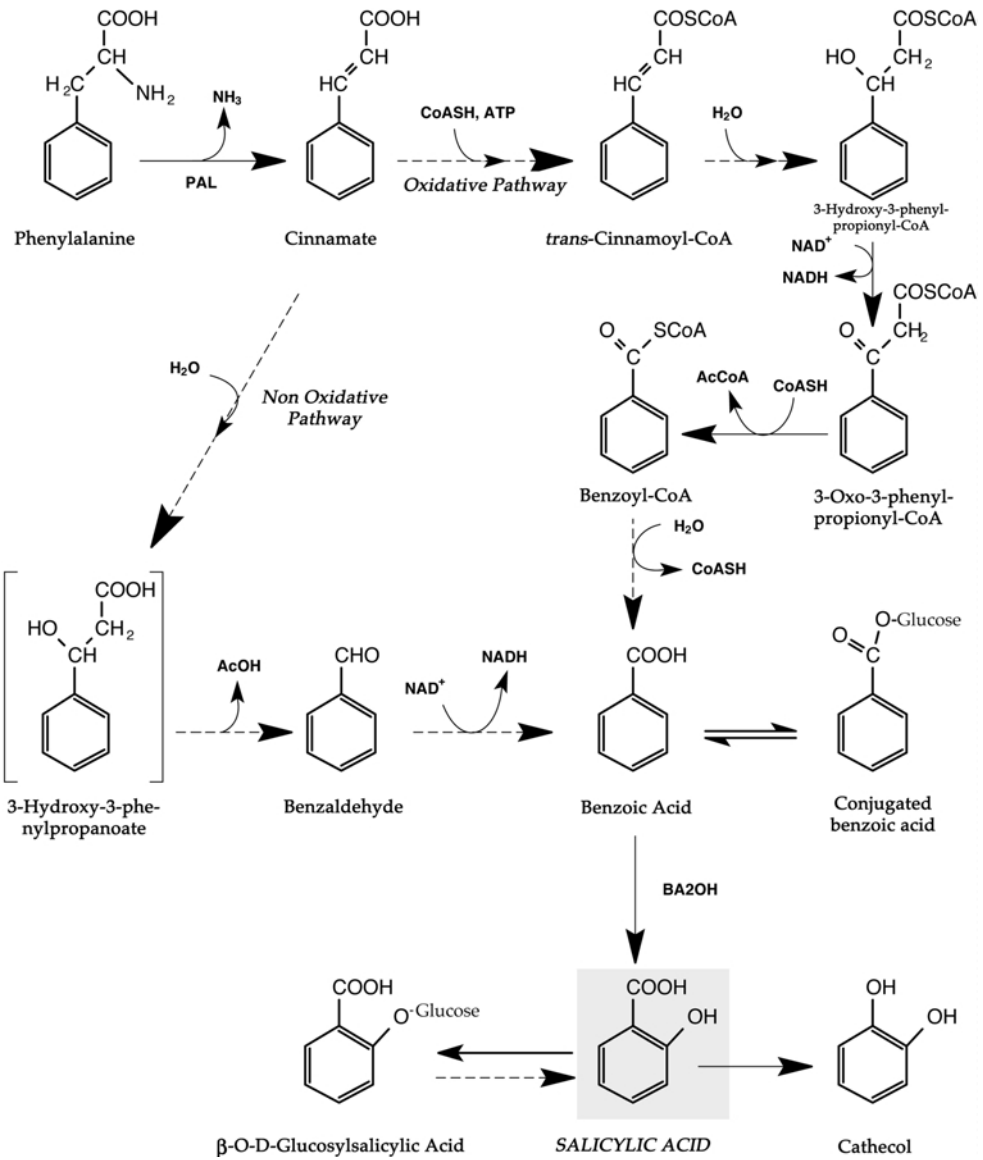

Figure 7.12 Biosynthesis of salicylic acid and related compounds. See text for details. (From Crozier *et al.*, 2000.)

The hydroxylation of cinnamate to form SA is a crucial step in the formation of a group of LMWP lactone derivatives, the coumarins. These compounds are widely distributed in plants, particularly in the Umbelliferae and Rutaceae families (Torssell, 1997), and they make up the sweet-smelling volatile material that is released from newly mown hay. The biosynthesis of these bioactive compounds requires some hydroxylation steps to yield 2,4-dihydroxycinnamic acid. Glycolysation of the latter compound triggers lactonization, giving rise to the coumarin umbelliferone (Figure 7.13).

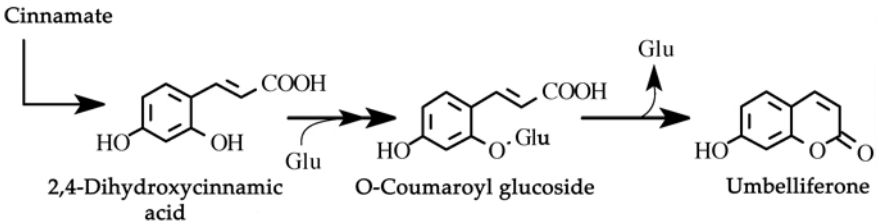

Figure 7.13 Biosynthesis of the coumarin umbelliferone. Glu, glucose.

Celery contains bergapten, a calcium antagonist compound. This compound belongs to the group of LMWP-defined psoralens. These are linear furanocoumarins which are widely distributed in plants and, when used internally and externally, they promote skin pigmentation and sun-tanning. The psoralens, because of their extended chromophore, absorb in the near-UV and allow this radiation to stimulate formation of melanin (Dewick, 1998). The biosynthesis of bergapten starts from the prenylation of umbelliferone to yield 7-demethylsuberosin. The latter compound goes towards cyclization into the furane ring of marmesin by the action of a cytochrome P450-dependent mono-oxygenase. Marmesin is converted to psoralen by cleavage of the hydroxyisopropyl fragment. Hydroxylation of psoralen yields bergaptol, the precursor of the methylated derivative bergapten (Figure 7.14). Psoralen can act as a precursor for other substituted furanocoumarins such as xanthoxin and isopimpinellin. Some furanocoumarins are troublesome for humans since they lead to photosensitization towards UV light, resulting in sunburn or serious blistering (Dewick, 1998).

Complex phenolic compounds of relatively high molecular weight

Cinnamate derivatives and their CoA (co-enzyme A) esters may function as building blocks for the construction of higher-complexity phenols. In general, three units of malonyl CoA are added, giving a polyketide which is transformed by the action of two enzmes, chalcone synthase (CHS) and chalcone isomerase (CHI), to chalcone and the flavanone naringenin, respectively (Figure 7.15). Compounds consisting of two substituted benzene rings joined by a heterocyclic ring containing oxygen are called flavonoids. Flavonoids are a diverse group of plant natural products synthesized from phenylpropanoids and acetate-derived precursors, and play important roles in growth and development, and in defence against microorganisms and pests. These compounds often possess antioxidant activity, and the potential health benefits of fruit, vegetables, green tea and red wine might partly be because of this property of flavonoids and other phytochemicals (Rice-Evans *et al*., 1997; Hollman and Katan, 1998; Khalsa, 1999). In addition, the isoflavonoids, which are limited primarily to the Leguminosae, exhibit oestrogenic and anticancer activity (Adlercreutz and Mazur, 1997; Dixon, 1999b), and, in common with the flavonoids, are receiving considerable attention as health-promoting 'vitalins' or nutraceuticals. Some isoflavonoids may exert oestrogenic activity. Different phyto-oestrogens have different mechanisms of action based on oestrogen-receptor subtypes, endogenous oestrogen concentrations, and cellular genetic make-up. Effects of phyto-oestrogens on the reproductive system have been known for decades, following several *in vitro* and animal studies; however, their roles

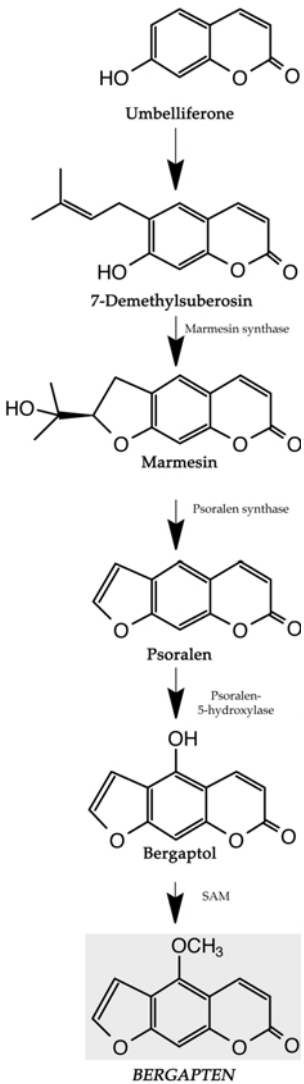

Figure 7.14 Transformation of the coumarin umbelliferone gives rise to marmesin, which is transformed to psoralen by the action of psoralen synthase. Hydroxylation of psoralen and the following esterification give rise to the furanocoumarins bergaptol and bergapten, respectively.

in humans remain unclear. Several beneficial health effects in adults have been associated with phyto-oestrogens, such as a protective role against the development of breast and prostate cancers (Haddad and Fuqua, 2001).

The biosynthesis of the major classes of flavonoid derivatives is depicted in Figure 7.16. The enzymes involved in the main flavonoid transformations are: CHS, chalcone synthase; CHR, chalcone reductase; CHI, chalcone isomerase; FSI, flavone synthase I; FSII, flavone synthase II; FLS, flavonol synthase; IFS, isoflavone synthase, consisting of

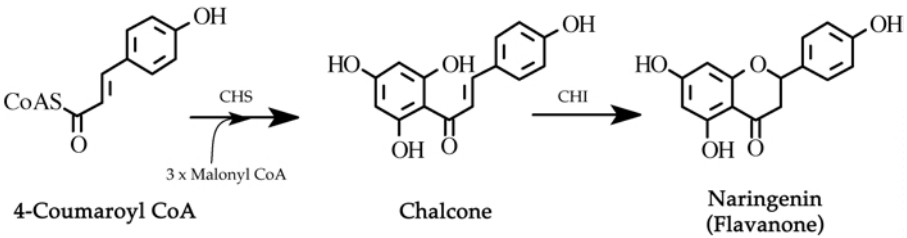

Figure 7.15 Early steps in the biosynthesis of flavonoids by the action of the two key enzymes chalcone synthase (CHS) and chalcone isomerase (CHI).

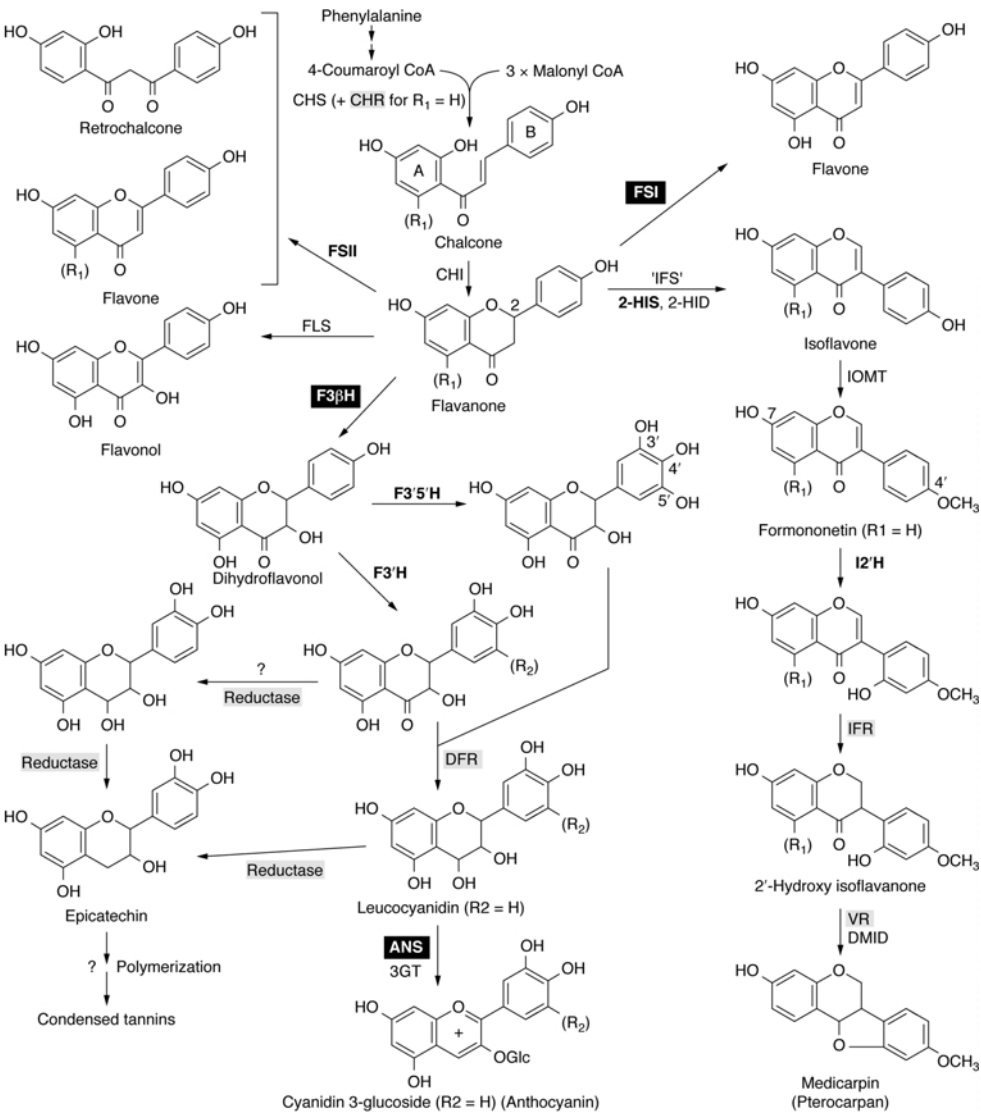

Figure 7.16 Biosynthesis of the major flavonoids: flavonols, isoflavones and anthocyanins. See text for details. (Reprinted from *Trends in Plant Science*, Dixon and Steele, 1999, with permission from Elsevier Science.)

2-hydroxy-isoflavanone synthase (2-HIS) and 2-hydroxyisoflavanone dehydratase (2-HID); F3βH, flavanone 3β hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase; 3GT, anthocyanidin 3-glucosyltransferase; IOMT, isoflavone O-methyltransferase; IFR, isoflavone reductase; VR, vestitone reductase; DMID, 7,2′-dihydroxy, 4′-methoxyisoflavanol dehydratase. Three types of enzymes are involved: 2-oxoglutarate-dependent dioxygenases (in white), cytochrome P450s (in black bold), and NADPH-dependent reductases (highlighted in grey). Parallel pathways function in the formation of anthocyanins with mono- and tri substituted B-rings. In the latter, F3′5′H can act at the level of the dihydroflavonol with a mono- or disubstituted B-ring. The pathway to epicatechin from a dihydroflavonol has been shown to follow two routes, both via leucocyanidin. The 4′-O-methylation of the B-ring of isoflavones occurs in alfalfa, pea and other legumes, but not in soy or other beans (Dixon and Steele, 1999).

Soybeans are a unique dietary source of the isoflavone genistein, which possesses weak oestrogenic activity and has been shown to act in animal models as an anti-oestrogen. Genistein is also a specific inhibitor of protein tyrosine kinases; it also inhibits DNA topoisomerases and other critical enzymes involved in signal transduction (Messina *et al*., 1994). Genistein is highly bioavailable in rats because its enterohepatic circulation may accumulate within the gastrointestinal tract (Sfakianos *et al*., 1997). The branch pathway for the formation of isoflavonoids shares several mechanistic features with the anthocyanin pathway. However, the first reaction specific for isoflavonoid biosynthesis is unique. It comprises 2-hydroxylation coupled to aryl migration of the B-ring of a flavanone (naringenin or daidzein) to yield, after a dehydration reaction that might be spontaneous or enzyme-catalysed (Hakamatsuka *et al*., 1998), the corresponding isoflavone (genistein or daidzein, respectively). The aryl migration enzyme (2-hydroxyisoflavanone synthase, 2-HIS) has been cloned recently from soybean, which accumulates isoflavone phyto-oestrogens in the seed and more complex isoflavonoid-derived phytoalexins, such as glyceollin, in pathogen-infected tissues (Figure 7.17). The cloning of 2-HIS opens up the possibility of introducing isoflavone nutraceuticals into a range of food crops (Dixon and Steele, 1999).

Recently, a renewed interest in flavonoids has been fuelled by the antioxidant and oestrogenic effects ascribed to them. This has led to their proposed use as anticarcinogens and cardioprotective agents, prompting a dramatic increase in their consumption as dietary supplements. Unfortunately, the potentially toxic effects of excessive flavonoid intake are largely ignored. At higher doses, flavonoids may act as mutagens, pro-oxidants that generate free radicals, and as inhibitors of key enzymes involved in hormone metabolism. Thus, in high doses, the adverse effects of flavonoids may outweigh their beneficial ones, and caution should be exercised in ingesting them at levels above that which would be obtained from a typical vegetarian diet. The unborn fetus may be especially at risk, since flavonoids readily cross the placenta. More research on the toxicological properties of flavonoids is warranted, given their increasing levels of consumption (Skibola and Smith, 2000).

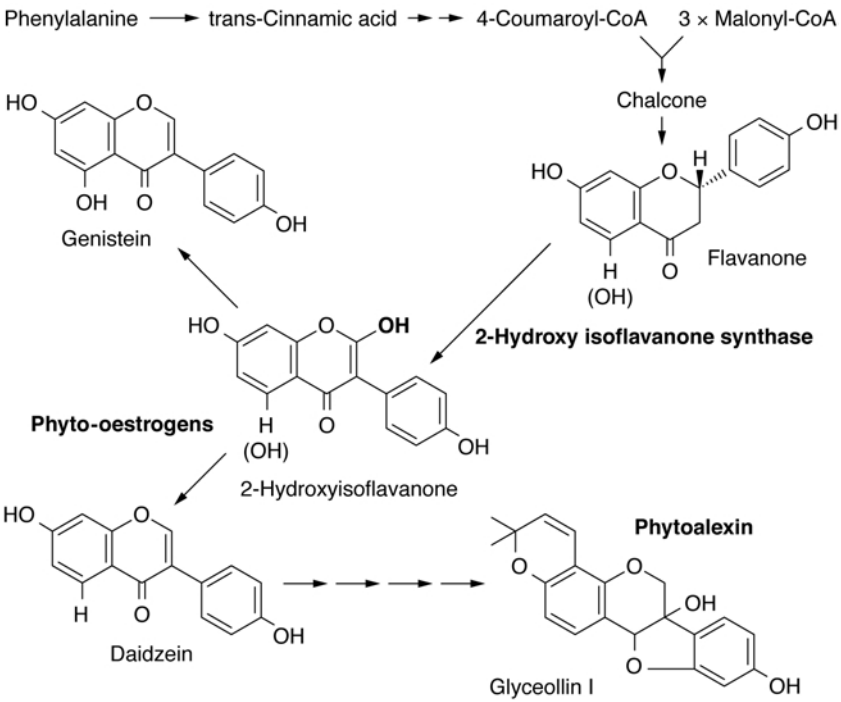

Figure 7.17 Metabolism of isoflavonoids. Biosynthesis of the bioactive isoflavonoid daidzein and of the natural phytoalexin glyceollin I. (Reprinted from *Trends in Plant Science*, Dixon and Steele, 1999, with permission from Elsevier Science.)

Polymeric phenolic compounds

Plants consumed by humans also contain thousands of phenolic compounds which are present as polymeric molecules, the polyphenols. Interest has been rekindled with the recognition that many polyphenols, although non-nutrients, show antioxidant, anti-inflammatory, anti-oestrogenic, anti-mutagenic and/or anti-carcinogenic effects, at least in *in vitro* or in animal systems (Bravo, 1998). A popular belief is that dietary polyphenols are anticarcinogens because they are antioxidants, but direct evidence for this supposition is lacking. Polyphenols may inhibit carcinogenesis by affecting the molecular events in the initiation, promotion and progression stages. Isoflavones and some other polyphenols have weak affinity for the oestrogen receptor and may be referred to as phyto-oestrogens. Understanding the bioavailability and blood and tissue levels of polyphenols is important in extrapolating results from studies in cell lines to animal models and humans. Epidemiological studies concerning polyphenol consumption and human cancer risk suggest the protective effects of certain food items and polyphenols, but more studies are needed for clear-cut conclusions (Yang *et al*., 2001).

Compounds of interest as dietary supplements include: (a) ellagic acid, a dicoumarin derivative found commonly in various fruits, nuts and vegetables; (b) curcumin, a diarylheptane that forms the yellow pigment in turmeric (*Curcuma longa*); (c) resveratrol, a stilbene (3,5,4-O-trihydroxystilbene), the parent compound of a family

of molecules found in a narrow range of plants including grapes (*Vitis vitifera*) (see below); (d) silybin, also known as silymarin, a flavonoid derived from milk thistle (*Silybum marianum*); (e) epigallocatechin gallate (EGCG), a flavonoid considered to be the major antioxidative green tea flavonoid. The likely biological consequences of polyphenols taken either as dietary supplements or in food are also determined by various factors governing uptake and retention in the body tissue. These include their basic structure, the degree of acylation and/or glycosylation, conjugation with other phenolics and degree of polymerization. This information is often not available for compounds or mixtures sold as dietary supplements (Ferguson, 2001). Figure 7.18 depicts the structure of some phenolic compounds commonly sold or being developed as dietary supplements.

Although most of our information on polyphenols comes from analyses of live tissues, the relative composition and activity of polyphenols can change considerably during plant tissue senescence. Substantial decreases in the number and concentration of LMWP (low-molecular-weight protein), and large increases in the protein-binding capacity of polyphenols, have been observed in leaf litter compared with green leaves (Gallet and Lebreton, 1995). According to Ferguson (2001), given the possibility that some polyphenol supplements and sources could be detrimental, there is an urgent need to perform controlled trials that incorporate estimates not only of antioxidant effects, but also of chromosomal damage in a relevant human tissue. Despite literally thousands of studies on plant polyphenols, it is still an open question as to whether or not they may provide a genuine beneficial effect in human populations, and if so, which should be consumed and at what level.

Terpenoids

Plants produce a wide array of lipidic compounds that are used for a series of metabolic and functional roles. We must remember that lipids are the main constituents of biological membranes, which are made by fatty acids. There are many other categories of lipids that do not contain fatty acids and the most important among them is the category of isoprenoids or terpenoids. More than 25,000 representatives with a variety of biological functions have been reported in the plant kingdom (Sacchettini and Poulter, 1997). To give just a few examples, volatile mono- and sesquiterpenes are highly involved in chemical defence and signalling, carotenoids serve as light-harvesting and light-protecting pigments, sterols play important roles as modulators of membrane properties, the phytol side-chain of chlorophyll (the most abundant organic pigment) is of terpenoid origin, and a wide variety of other plant terpenoids function as insect attractants or repellents. Various terpenoids have attracted commercial interest as pharmaceuticals and/or nutraceuticals. Thus, paclitaxel (Taxol), a diterpene from yew, has been established as a major cytostatic agent. Lycopene and lutein were recently registered as oncopreventive agents (Eisenreich *et al*., 2001).

All terpenoids are derived by repetitive fusion of branched five-carbon units based on an isopentane skeleton. These monomers are generally referred to as isoprene units because thermal decomposition of many terpenoid substances yields the alkene gas isoprene as a product and because suitable chemical conditions can induce isoprene to polymerize in multiples of five carbons, generating numerous terpenoid skeletons. For these reasons, the terpenoids are often called isoprenoids, although researchers have known for well over 100 years that isoprene itself is not the biological precursor of this

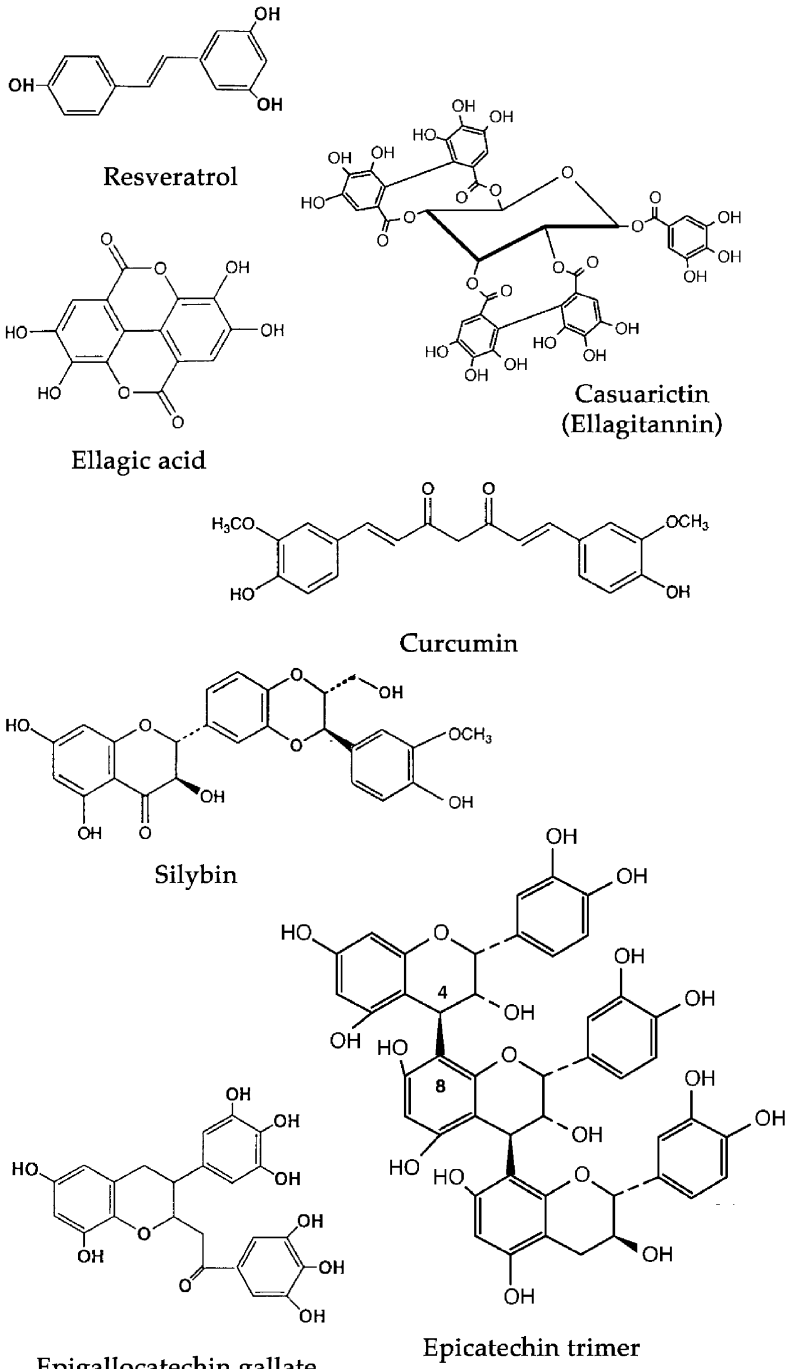

Figure 7.18 Representative phenolic compounds sold or used as dietary supplements. (See text for details.)

family of metabolites. The major subclasses of terpenoids are biosynthesized from the basic five-carbon unit, isopenthenyl pyrophosphate (IPP), and from the initial prenyl (allylic) diphosphate, dimethylallyl diphosphate (DMAPP), which is formed by isomerization of IPP. In reactions catalysed by a class of enzymes known as the prenyltransferases, monoterpenes (C_{10}), sesquiterpenes (C_{15}) and diterpenes (C_{20}) are derived from the corresponding intermediates by sequential head-to-tail addition of C_5 units. Triterpenes (C_{30}) are formed from two C_{15} (farnesyl) units joined head-to-head, and tetraterpenes (C_{40}) are formed from two C_{20} (geranylgeranyl) units joined head-to-head (Figure 7.19) (Croteau *et al*., 2000). During a period of several decades, the mevalonate pathway was considered as the universal source of the biosynthetic precursors DMAPP and IPP. The existence of a second isoprenoid pathway was discovered relatively recently by the research groups of Rohmer and Arigoni in the course of stable isotope incorporation studies using various eubacteria and plants (Rohmer *et al*., 1993). These

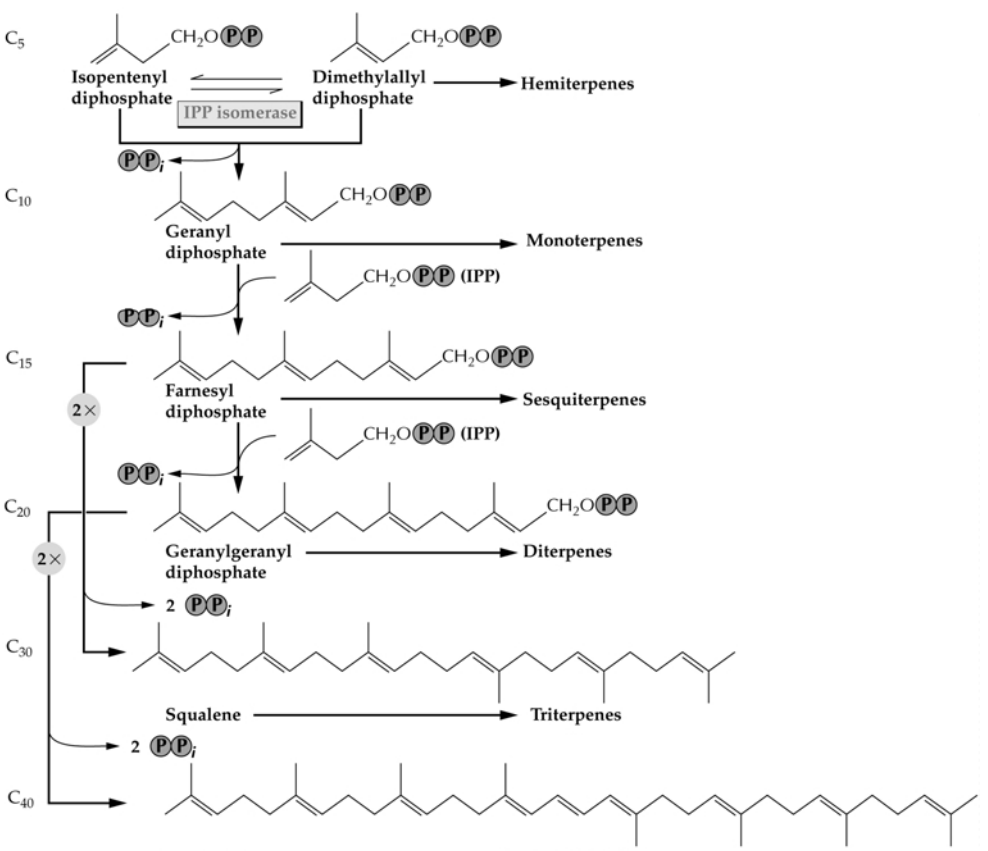

Figure 7.19 The biosynthesis of terpenoids starts from the building block molecules isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Monoterpenes, sesquiterpenes and diterpenes are synthesized by addition of IPP units, whereas triterpenes and tetraterpenes result from the condensation of two units of farnesyl pyrophosphate and geranylgeranyl pyrophosphate, respectively. (From Croteau *et al*., 2000, copyright of the American Society of Plant Biologists, reproduced with permission.)

data suggested that pyruvate and a triose phosphate can serve as precursors for the formation of IPP and DMAPP by an alternative pathway (Arigoni and Schwarz, 1999; Rohmer, 1999).

An important feature of the organization of terpenoid metabolism exists at the subcellular level. The sesquiterpenes (C_{15}), triterpenes (C_{30}) and polyterpenes appear to be produced in the cytosolic and endoplasmic reticulum (ER) compartments, whereas isoprene, the monoterpenes (C_{10}), diterpenes (C_{20}), tetraterpenes (C_{40}) and certain prenylated quinones originate largely, if not exclusively, in the plastids. The biosynthetic pathways for the formation of the fundamental precursor IPP differ markedly in these compartments, with the classical acetate/mevalonate pathway being active in the cytosol and ER and the glyceraldehyde phosphate/pyruvate pathway operating in the plastids (Figure 7.20) (Croteau *et al*., 2000).

The cytosolic pathway that leads to IPP formation involves the condensation of three molecules of acetyl-CoA. The resulting product, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), is subsequently reduced to form mevalonic acid, the six-carbon compound that gives its name to the pathway. Two sequential ATP-dependent phosphorylations of mevalonic acid and a subsequent phosphorylation/elimination-assisted decarboxylation yield IPP (Figure 7.21).

In the deoxyxylulose phosphate pathway, D-glyceraldehyde 3-phosphate and pyruvate are converted into 1-deoxy-D-xylulose 5-phosphate in a decarboxylative reaction. Subsequent rearrangement and reduction leads to 2C-methyl-D-erythritol

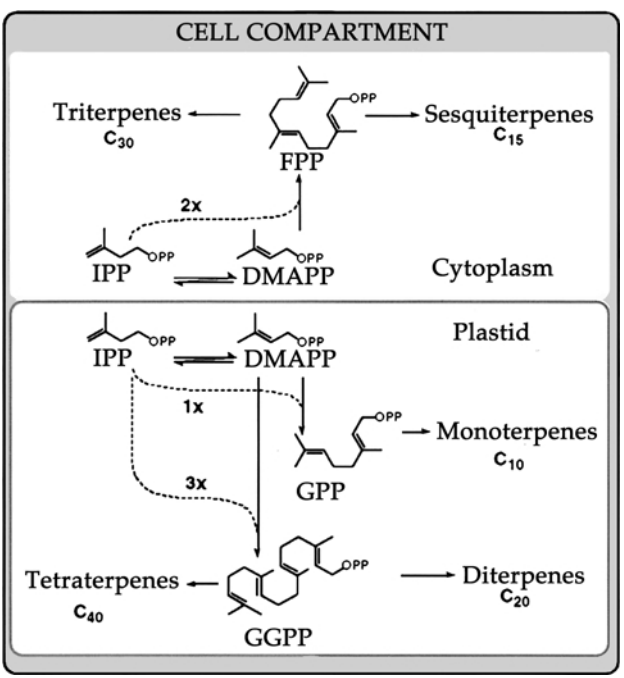

Figure 7.20 Compartmentalization of terpenoid biosynthesis. Sesquiterpenes and triterpenes are synthesized in the cytoplasm, whereas monoterpenes, diterpenes and tetraterpenes are made inside the plastids. (Croteau *et al*., 2000, copyright 1998 National Academy of Sciences, USA.)

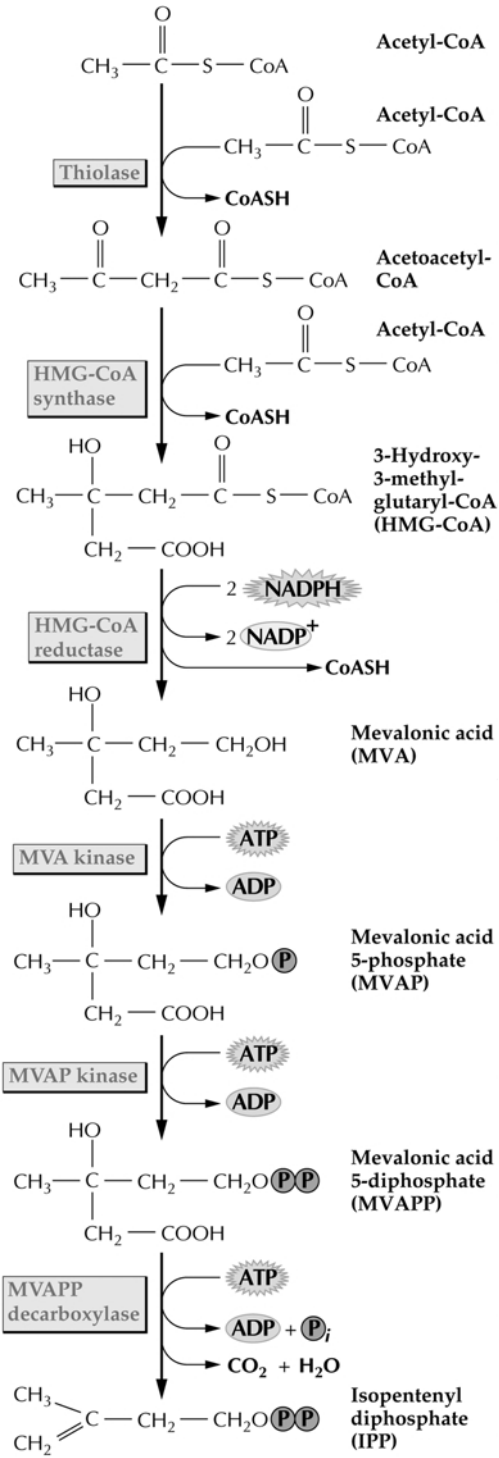

Figure 7.21 Mevalonic acid pathway. (See text for details. From Croteau *et al*., 2000, copyright of the American Society of Plant Biologists, reproduced with permission.)

4-phosphate, which is converted into 2C-methyl-D-erythritol 2,4-cyclodiphosphate via 4-diphosphocytidyl-2C-methyl-D-erythritol and 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate. The further reactions leading to IPP and DMAPP are still unknown (Figure 7.22) (Eisenreich *et al*., 2001).

By using the building blocks IPP and DMAPP, plants are able to synthesize a wide array of bioactive compounds, ranging from essential oil constituents to high-molecular-weight rubber. The vast number of bioactive isoprenoids would need an encyclopaedia to cover all biosynthetic aspects. I will focus on a few examples taken from the most popular bioactive compounds.

Monoterpenes

Peppermint oil is the major constituent of several over-the-counter remedies for symptoms of irritable bowel syndrome (IBS). As the aetiology of IBS is not known and treatment is symptomatic there is a ready market for such products. Peppermint oil relaxes gastrointestinal smooth muscle by reducing calcium influx (Hills and Aaronson, 1991). The main constituents of peppermint oil are menthol, menthone and the undesired compounds pulegone and menthofuran. Several pharmacological and biological effects have been associated with these oil components. In particular: menthol is a known antifungal and antimicrobial agent (Morris et al., 1979) and also a larva repellent (Kelsey et al., 1984). For many years menthol has been used in the treatment of respiratory disorders. It has been found to interact at the cell level with cytosolic Ca^{2+}, probably through an intracellular Ca^{21} store release (Takeuchi *et al*., 1994) and Ca^{2+} channel blocking (Taylor *et al*., 1985; Hills and Aaronson, 1991). Wright and

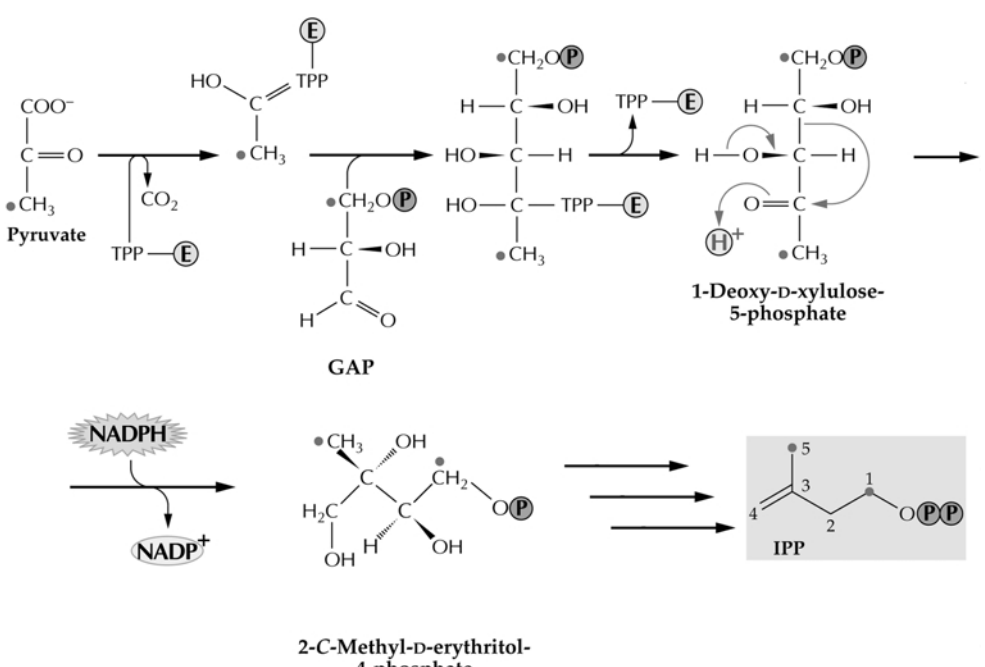

Figure 7.22 The deoxyxylulose pathway. (See text for details. From Croteau *et al*., 2000, modified.)

co-workers (1997) have demonstrated that menthol attenuates both capsaicin- and NKA-induced (neurokinin-A) bronchoconstriction *in vivo* and relaxes KCl and ACh (acetylcholine) preconstricted bronchi *in vitro*. Menthol inhibition of NKA- and capsaicin-induced bronchoconstriction could be, in part, explained by a direct action of menthol on bronchial smooth muscle. Menthone is a growth inhibitor (Slama, 1978), whereas pulegone, a potent abortifacient, and menthofuran are metabolized by hepatic microsomal mono-oxygenases to a series of hepatotoxins that cause liver cancer (Thomassen *et al*., 1990; Nelson, 1995) and are involved in many reported cases of intoxication in humans and animals (Bakerink *et al*., 1996). Furthermore, pulegone is considered one of the most powerful allelochemicals and has been demonstrated to be about twice as toxic as HCN (Asplund, 1969; Fischer, 1991).

Figure 7.23 depicts the pathway for monoterpene synthesis in the genus Mentha. Menthol biosynthesis starts from cyclization of the building block geranyl

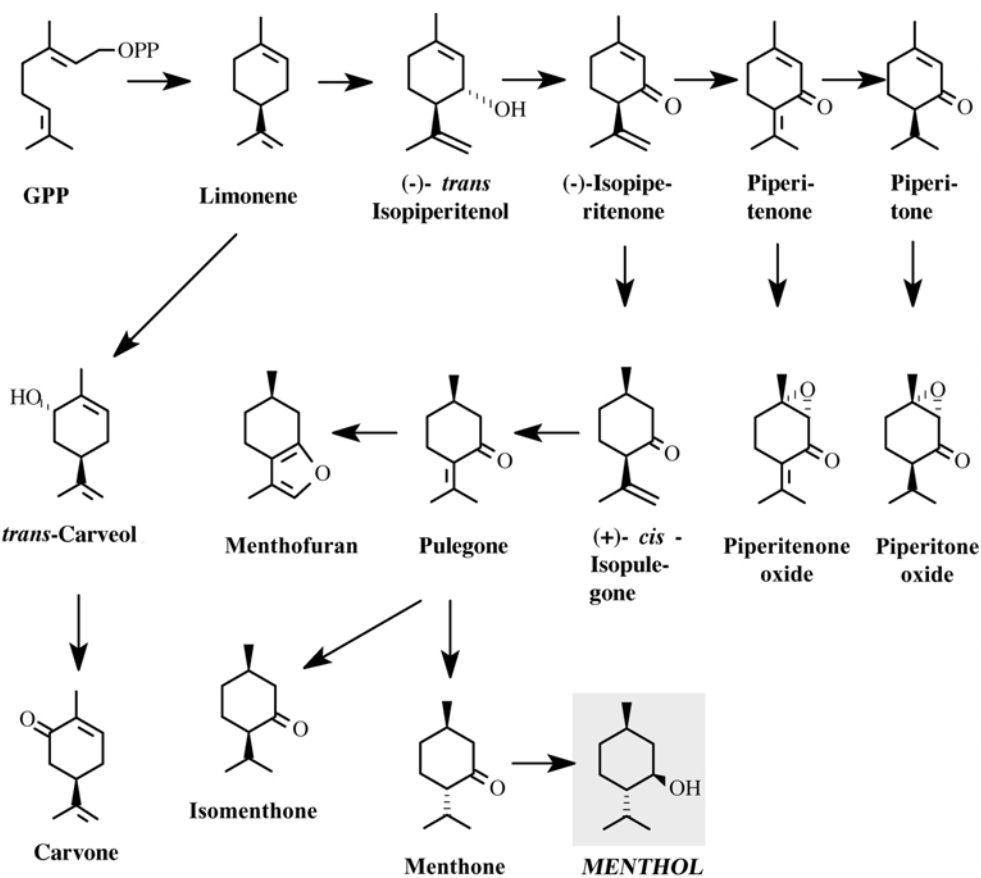

Figure 7.23 Peppermint and spearmint are the two major essential-oil-producing crops in terms of global production. In peppermint and in spearmint the cyclization of geranyl pyrophosphate (GPP) gives limonene. In peppermint limonene is hydroxylated and reduced to form the main compounds menthol and menthone, whereas in spearmint hydroxylation leads to the formation of carvone. Pulegone and menthofuran, which are intermediate and end products of peppermint, are toxic compounds. (See text for details.)

pyrophosphate to yield limonene. This monoterpene olefin is then hydroxylated to *trans*-isopiperitenol. Oxidation of the latter compound produces isopiperitenone, which is reduced to *cis*-isopulegone. Isomerization of *cis*-isopulegone yields pulegone, the precursor of menthone, menthofurnan and isomenthone. Reduction of menthone gives rise to menthol. Alternatively, isopiperitenone can be isomerized to piperitenone which can be reduced to piperitone or oxidized to piperitenone oxide. Another monoterpene hydroxylase acting on limonene gives rise to *trans*-carveol the precursor of carvone.

Sesquiterpenes

Malaria is responsible for the deaths of 1 to 2 million people each year, most of them children. There is an increasing amount of experimental evidence that shows that the sesquiterpene endoperoxide, artemisinin, and related compounds, have a potential antimalarial activity. The mechanism of action of this compound is a two-step process. First artemisinin interacts with the haem residue remaining after the digestion of haemoglobin by the malaria parasite; this process produces highly reactive free radicals. In the second step, free radicals deriving from artemisinin react with several molecular components of the parasite, eventually leading to death of the parasite (Wright and Warhurst, 2002). The biosynthesis of artemisinin starts from the condensation of the building block farnesyl pyrophosphate to yield the cadinyl cation. Via modest oxidative processes artemisinic acid and arteannuin B are produced. Further modifications lead to several intermediate compounds via the cleavage of the C–C bond of the epoxide function and the generation of the endoperoxide linkage. The reduction of artemisitene leads to the formation of artemisinin (Figure 7.24) (Dewick, 1998).

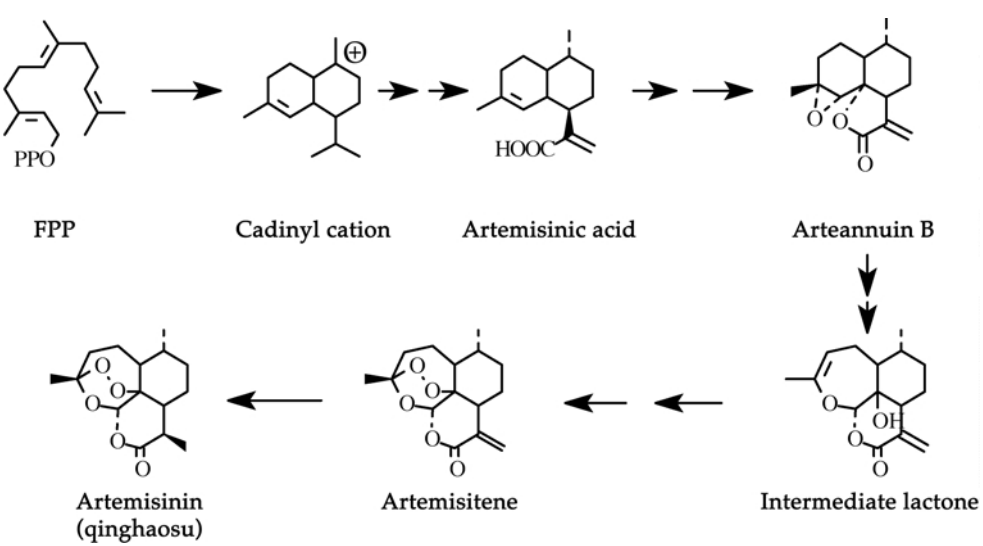

Figure 7.24 Biosynthesis of the endoperoxide antimalarial sesquiterpene artemisinin.

Diterpenes

Taxoid is the generic name of a large class of diterpenes like taxin, which was first isolated from the European yew tree *Taxus baccata*. Two members of this class of compounds, taxol and its semi-synthetic analogue, Taxotere, are used successfully for chemotherapy of solid tumours, especially in cases of resistance to classical cytostatic agents (Eisenreich *et al*., 1996). Impressive progress has been made by the demonstration of taxadiene as the first committed intermediate in taxol biosynthesis and by the purification and characterization of taxadiene synthase catalysing the cyclization reaction conducive to the taxane system from the universal diterpene precursor, geranylgeranyl pyrophosphate, in a single enzymatic step (Eisenreich *et al*., 1996). Although taxol is sometimes classified as a diterpenoid alkaloid, the nitrogen atom is not incorporated into the diterpene skeleton, but derives from shikimate via phenylalanine (Figure 7.25).

The use of complementary medicines such as plant extracts in dementia therapy varies according to the different cultural traditions. In orthodox Western medicine, contrasting with that in the Far East for example, pharmacological properties of traditional cognitive or memory-enhancing plants have not been widely investigated in the context of current models of Alzheimer's disease. An exception is *Ginkgo biloba* in which the ginkgolides have antioxidant, neuroprotective and cholinergic activities relevant to Alzheimer's disease mechanisms (Perry *et al*., 1998). Although detailed evidence is still lacking, extensive modification of the diterpene skeleton of copalyl pyrophosphate is probably responsible for generations of ginkgolides (Figure 7.26) (Dewick, 1998).

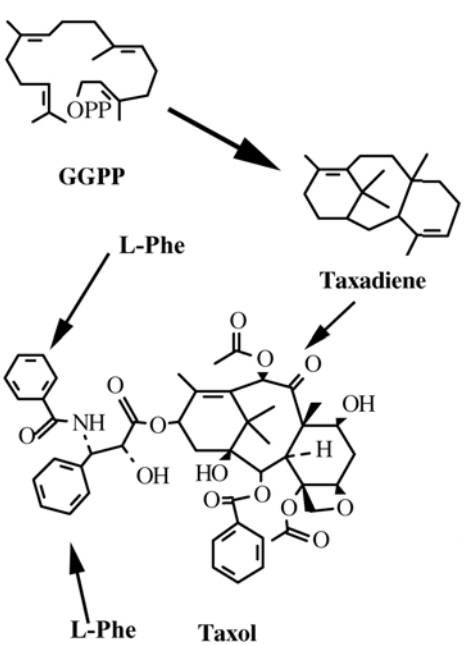

Figure 7.25 Cyclization of the diterpene precursor geranylgeranyl pyrophosphate (GGPP) gives rise to taxadiene, which after several transformations yields taxol. The nitrogen atom of taxol derives from phenylalanine.

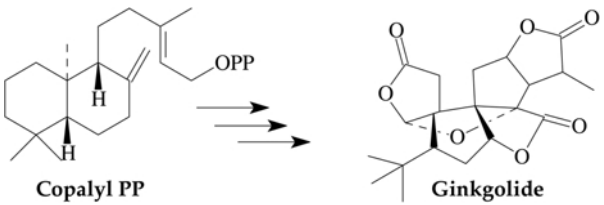

Figure 7.26 Ginkgolide is a bioactive compound derived from successive modifications of the diterpene copalyl pyrophosphate.

Triterpenes

The triterpenes comprise a variety of structurally diverse compounds, including steroids. Found especially in plant cell membranes, sterols decrease the permeability of the membrane to small molecules by decreasing the motion of the fatty acid chains. Other steroids are defensive secondary products. Triterpenes that are bioactive compounds include cardenolides and saponins.

Cardenolides are glycosides (compounds containing an attached sugar or sugars) that taste bitter and are extremely toxic to higher animals. In humans, they have dramatic effects on the heart muscle through their influence on Na^+/K^+-activated ATPases. In carefully regulated doses, they slow and strengthen the heartbeat. Cardenolides extracted from species of foxglove (*Digitalis*) are prescribed to millions of patients for the treatment of heart disease. Digitoxigenin is the aglycone triterpene portion of the naturally occurring digitanides (Taiz and Zeiger, 1998). The biosynthesis of the cardenolide digitoxigenin and of the bufadienolide hellebrigenin starts from the shortening of the side chain of cholesterol, with the synthesis of the intermediate progesterone. The latter undergoes subsequent transformations that give rise to bufalin, and then to hellebrigenin, and to digitoxigenin, which is the precursor of other cardenolides such as digoxigenin and gitoxigenin (Dewick, 1998) (Figure 7.27).

Saponins are steroid and triterpene glycosides, so named because of their soaplike properties, and are common constituents of plants. The presence of both lipid-soluble (the triterpene) and water-soluble (the sugar) elements in one molecule gives saponins detergent properties, and they form a soapy lather when shaken with water. The toxicity of saponins is thought to be a result of their ability to form complexes with sterols. Saponins may interfere with sterol uptake from the digestive system or disrupt cell membranes after absorption into the bloodstream. The yam *Dioscorea* contains saponins, e.g. yamogenin, that are widely used as starting materials in the synthesis of progesterone-like compounds for birth control pills (Taiz and Zeiger, 1998). Another source of triterpenoid saponins is obtained from the bark of *Quillaja saponaria* Molina (the soapbark tree) and have been known since the 1920s to cause substantial enhancement of immune responses (Barr *et al*., 1998). Saponins have long been known to possess properties useful to man and were used by Australian Aborigines to harvest fish and snails (Oakenfull and Sidhu, 1989). There is a need with some vaccines to induce an immune response capable of eliminating virus-infected or malignant cells. This is an activity which saponin-based adjuvants, but few others, appear to possess. This feature is due to the ability of saponins to stimulate the cell-mediated arm of the immune system as well as enhancing antibody production. According to Barr and

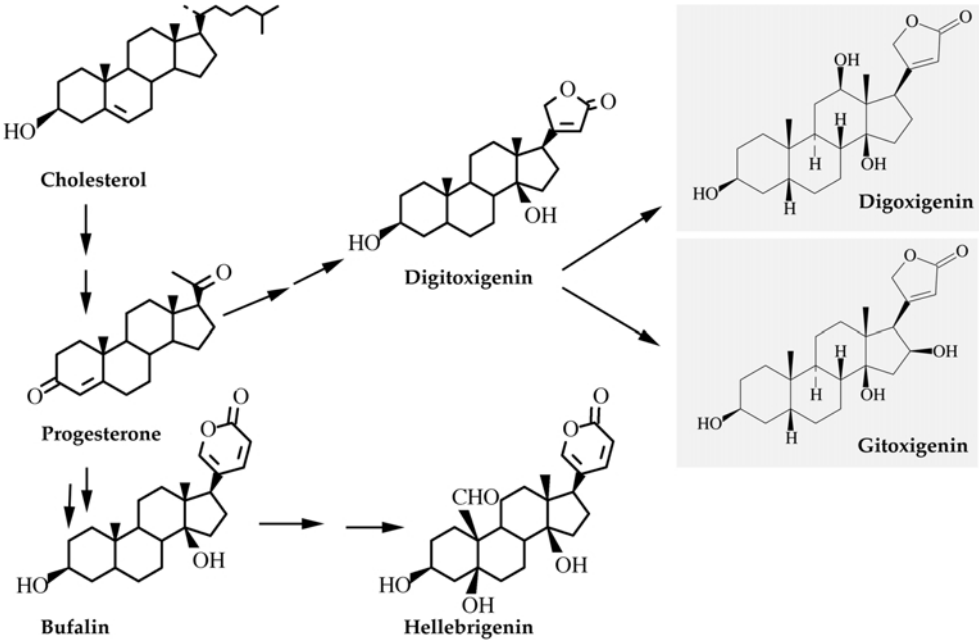

Figure 7.27 Biosynthesis of the triterpene cardenolides digoxigenin and gitoxigenin from cholesterol. Transformations of progesterone to bufalin lead to the biosynthesis of hellebrigenin, a bufadienolide.

co-workers (1998), the promising area of saponin-based human vaccines has entered a significant stage in their development with ongoing human clinical trials, and the results over the next few years will determine their future applications in human medicine. Figure 7.28 shows a typical structure of a saponin purified from *Quillaja saponaria* barks.

Tetraterpenes

Plant carotenoids are tetraterpenes, 40-carbon isoprenoids, with polyene chains that may contain up to 15 conjugated double bonds. Because of their chemical properties carotenoids are essential components of all photosynthetic organisms. Carotenoids, which occur in many fruits and vegetables, are potentially antioxidants; in addition, some of them (including the carotenes) are precursors of the essential micronutrient, the 20-carbon compound retinol, or vitamin A. In humans, the levels of carotenoids in the blood are determined by dietary intake, but the concentration of retinol is not (instead being regulated via the liver, where it is stored). The antioxidant potential of carotenoids has been demonstrated in various *in vitro* systems, and recently the possibility that they are effective antioxidants *in vivo* (with DNA damage as the endpoint) has been tested; however, the evidence for a cancer-protective action of carotenoids in humans is, to say the least, equivocal (Collins, 2001). Effects of dietary carotenoids in animals (Duthie *et al*., 1998) and the uptake, metabolism and transport of carotenoids in humans (Parker, 1996) have been reviewed. Carotenoids are lipid-soluble, and

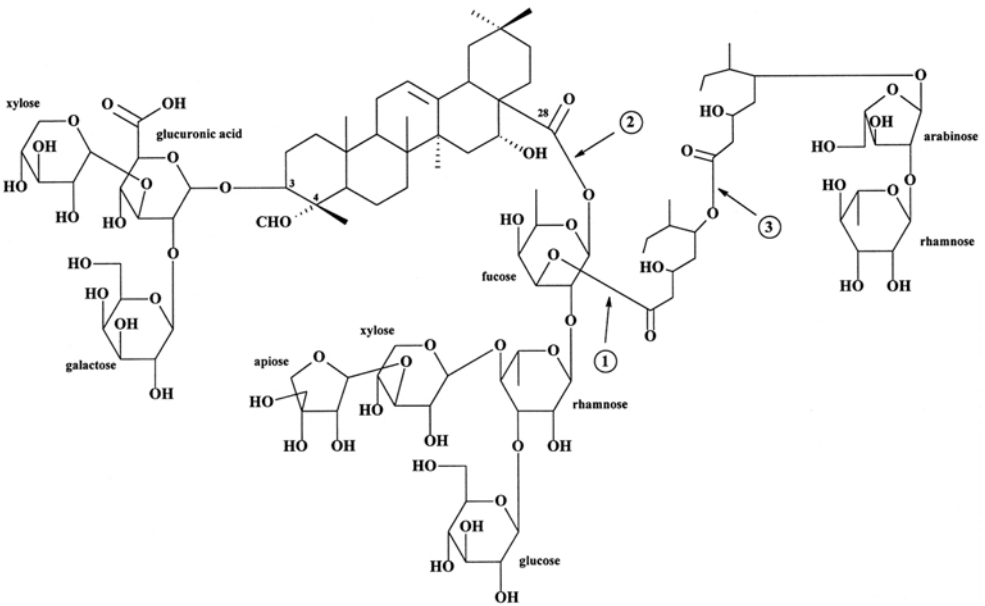

Figure 7.28 Structural formula of a saponin purified from *Quillaia saponaria* barks. (From Barr *et al*., 1998.)

appear to be absorbed by passive diffusion (within lipid micelles) into the cells of the intestinal mucosa. Molecules such as lutein, zeaxanthin, lycopene, β-cryptoxanthin and α- and β-carotene are commonly measured in serum or plasma, with individual variations in concentration depending on dietary intake (Collins *et al*., 1998). Some tissues are known to accumulate particular carotenoids; for instance, the macula of the retina has high concentrations of zeaxanthin and lutein (Handelman *et al*., 1988).

The carotenoid biosynthesis pathway has recently been reviewed (Hirschberg, 2001). In plants, carotenoids are synthesized within the plastids by enzymes that are nuclear-encoded. Like all other isoprenoids, carotenoids are built from the building block IPP produced via the pyruvate/triose phosphate pathway (Figure 7.29). The sequential addition of three IPP molecules to DMAPP, which is catalysed by a single enzyme, geranylgeranyl diphosphate (GGPP) synthase (GGPS), gives the 20-carbon molecule geranylgeranyl pyrophosphate (GGPP). The first committed step in the carotenoid pathway is the condensation of two GGPP molecules to produce 15-*cis* phytoene, which is catalysed by a membrane-associated enzyme, phytoene synthase (PSY) (Camara, 1993). Two structurally and functionally similar enzymes, PDS and ζ-carotene desaturase (ZDS), convert phytoene to lycopene. Cyclization of lycopene marks a branching point in the pathway: one branch leads to β-carotene and its derivative xanthophylls, whereas the other leads to δ-carotene, α-carotene and lutein. In leaves, violaxanthin can be converted back to zeaxanthin by violaxanthin de-epoxidase. Zeaxanthin is effective in the thermal dissipation of excess excitation energy in the light-harvesting antennae and, thus, plays a key role in protecting the photosynthetic system from damage by strong light. The interconversion of zeaxanthin and violaxanthin is known as the 'xanthophyll cycle' (Hirschberg, 2001).

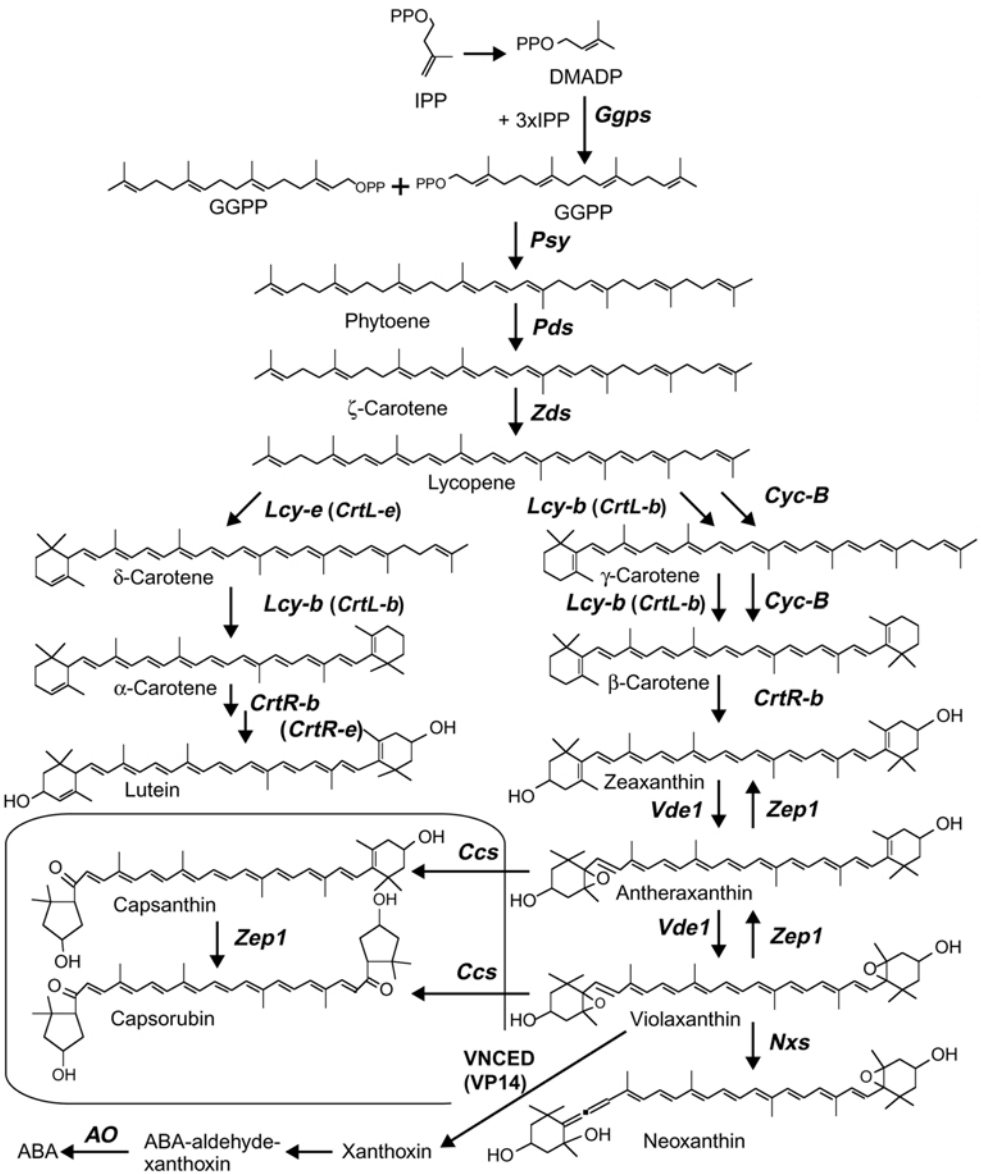

Figure 7.29 The carotenoid biosynthesis pathway in plants. Enzymes are named according to the designation of their genes. The pathway in the box takes place in chromoplasts of pepper fruit. A, DPME; B, 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate; C, 2C-methyl-D-erythritol 2,4-cyclodiphosphate. AO, aldehyde oxidase; *CrtR-b*, β-ring hydroxylase, *CrtR-e*, ϵ-ring hydroxylase; DMADP, dimethylallyl pyrophosphate; DOXP, 1-deoxy-D-xylulose 5-phosphate; ispF, 2C-methyl-D-erythritol 2,4-cyclodiphosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; VNCED (VP14), 9-*cis*-epoxycarotenoid dioxygenase. (Reprinted from *Current Opinion in Plant Biology*, Hirschberg, 2001, with permission from Elsevier Science.)

Nitrogen-containing plant products

Nitrogen-containing compounds are a vast category of molecules produced by a consistent percentage of plant species. Alkaloids, which are surely the most important class of bioactive nitrogen-containing compounds, accumulate in about 20 per cent of plant species; other compounds such as glucosinolates (mustard oil glycosides) are restricted to few plant families (e.g. the Brassicaceae), whereas cyanogenic glycosides (the HCN-releasing compounds) are common in some food plants (e.g. cassava, apricot stones). In this section I will briefly skim over the ocean of metabolic pathways leading to the various nitrogen-containing bioactive molecules, trying to focus our attention on molecules mainly present in foods.

Alkaloids

Alkaloids are low-molecular-weight nitrogen-containing substances with characteristic toxicity and pharmacological activity. These properties, which have traditionally been exploited by humans for hunting, execution and warfare, have also been used for the treatment of disease (Mann, 1992). Many alkaloids have complex chemical structures and contain multiple asymmetric centres, complicating the elucidation of their structure and making study of the biosynthesis of alkaloids quite difficult (Croteau *et al*., 2000). Alkaloids are mostly derived from the amino acids, Phe, Tyr, Trp, Lys and Orn. In addition, the monoterpenoid indole alkaloids, which form a large class of complex compounds, are derived from Trp and terpenoid precursors (De Luca and St Pierre, 2000). Over 12,000 different alkaloids have been described, indicating their structural and biosynthetic diversity compared to that of other secondary metabolites (Wink, 1999). The ability of plants to couple amines to different chemical partners produces a restricted number of versatile chemical backbones (i.e. central intermediates) from which the diversity of alkaloids is produced (De Luca and Laflamme, 2001). Building blocks from the shikimate, mevalonate, pyruvate/triose phosphate and acetate pathways are often incorporated into the alkaloid structures. Below are described some biosynthetic pathways of bioactive molecules present in selected food plants.

CAPSAICIN: CHILLI PEPPER (*CAPSICUM ANNUUM*)

Chilli pepper contains a pungent principle, capsaicin. Besides having culinary importance, the plant is used medicinally (see Chapter five). The burn effect of capsaicin affects the pain receptors, making them less sensitive. Biosynthetically, the aromatic portion of capsaicin derives from Phe, via a series of metabolic transformations leading to ferulic acid, vanillin and then, through transamination, to vanillylamine. Starting from valine a branched-chain fatty acyl-CoA is produced and condensed with vanillylamine to give capsaicin (Dewick, 1998) (Figure 7.30).

CAFFEINE, THEOBROMINE AND THEOPHYLLINE: COFFEE, TEA, COLA

Caffeine is found in seeds and leaves of cocoa, coffee, cola, maté and tea, and is one of the most consumed alkaloids. Even though caffeine is also used medicinally, much more effective is the alkaloid theophylline, because of its muscle-relaxant properties,

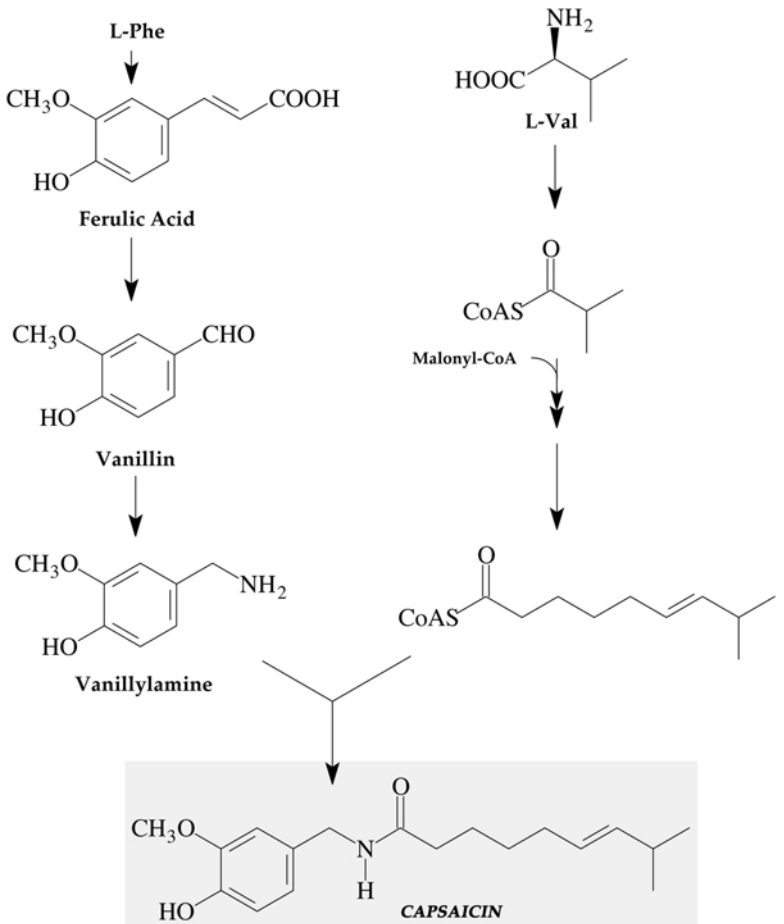

Figure 7.30 Biosynthesis of the alkaloid capsaicin. (From *Medicinal Natural Products* by P.M. Dewick, 1998, reproduced with permission from John Wiley & Sons.)

which is used to cure bronchial asthma. Theobromine is found in cocoa and related chocolate products (Dewick, 1998). Purine bases like adenine and guanine are transformed in these species to purine alkaloids. The biosynthesis of these compounds starts from xanthosine monophosphate methylation to give 7-methylxanthosine monophosphate. Alternatively the dephosphorylation of xanthosine monophosphate gives xanthosine. The latter is the precursor of theophylline, whereas hydrolysis of the phosphate group of 7-methylxanthosine monophosphate gives 7-methylxanthosine. The latter compound undergoes cleavage of the ribose to yield 7-methylxanthine, which after successive methylations is transformed to theobromine and finally to caffeine. The hydrolysis of the phosphate ester of xanthosine monophosphate gives xanthosine. The latter compound, after cleavage of ribose and two methylations, is finally transformed to theophylline (Figure 7.31).

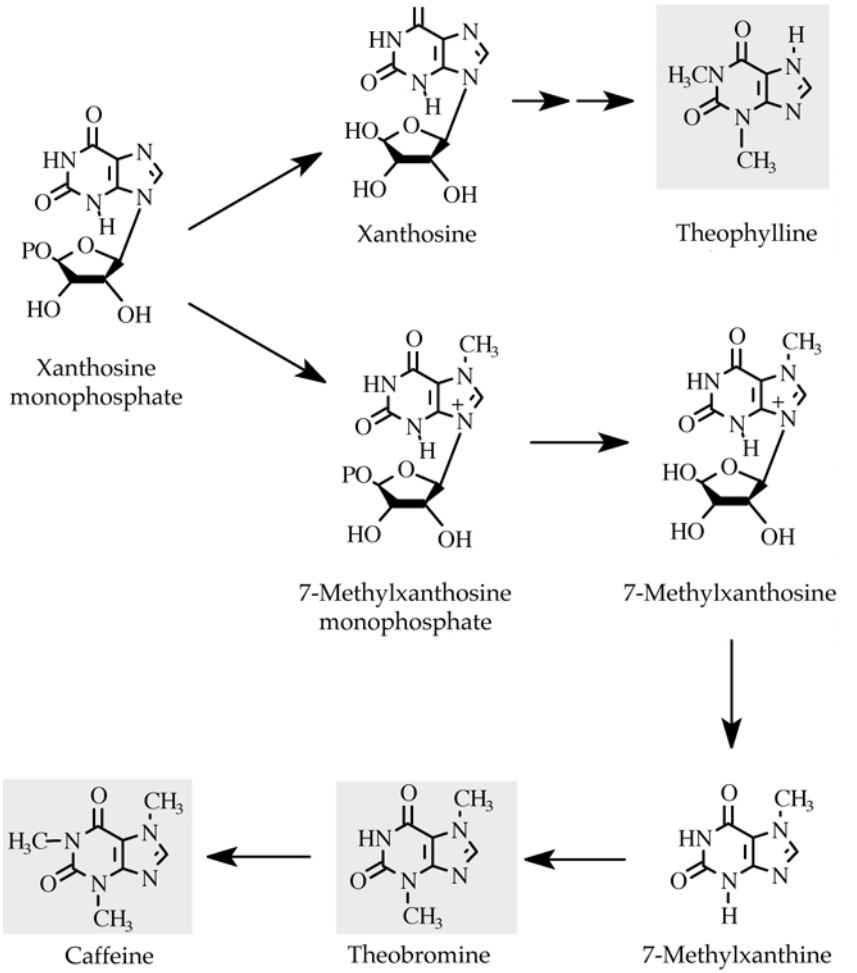

Figure 7.31 Biosynthesis of the purine alkaloids commonly occurring in beverages such as tea and coffee, as well as in chocolate. Caffeine and theobromine derive from 7-methylxanthosine monophosphate, whereas theophylline derives from xanthosine.

EPHEDRINE: MA HUANG

Dietary supplements of ephedrine plus caffeine for weight loss (weight loss being the current first-line recommendation of physicians for osteoporosis) show some promise, but are not sufficient in number of study subjects. Ephedrine is a sympathomimetic amine with effects similar to those of adrenaline; it is produced by *Ephedra* (or ma huang), which has been used by the Chinese for at least 5,000 years. Ephedrine has resulted in deaths and hence is worrisome as an over-the-counter dietary supplement (Fillmore *et al*., 1999). Ephedrine has been described as a causative factor of vasculitis but myocarditis has not yet been associated with either ephedrine or its plant derivative ephedra (Zaacks *et al*., 1999). The content of ephedra alkaloids in herbal dietary supplements containing *Ephedra* (ma huang) has been studied by Gurley and co-

workers (2000). The *Ephedra* alkaloid content of 20 *Ephedra*-containing supplements was determined and found to contain: (−)-ephedrine, (+)-pseudoephedrine, (−)-methylephedrine, (−)-norephedrine and (+)-norpseudoephedrine. Total alkaloid content ranged from 0.0 to 18.5 mg per dosage unit. Ranges for (−)-ephedrine and (+)-pseudoephedrine were 1.1–15.3 mg and 0.2–9.5 mg, respectively. (+)-Norpseudoephedrine, a Schedule IV controlled substance, was often present. Finally, half of the products exhibited discrepancies between the label claim for *Ephedra* alkaloid content and actual alkaloid content in excess of 20 per cent (Gurley *et al.*, 2000). Norpseudoephedrine and catinone are also contained in khat (*Catha edulis*), a small tree cultivated in Ethiopia. The leaves of khat are chewed for a stimulant effect (Dewick, 1998).

Biosynthesis of *Ephedra* alkaloids starts from condensation of benzoic acid with pyruvic acid to give dichetopropyl phenyl, which after transamination yields cathinone. The latter is reduced to the diastereoisomers norephedrine and norpseudoephedrine (cathine). Methylation of these two compounds gives ephedrine and pseudoephedrine, respectively (Figure 7.32).

Cyanogenic glycosides

The cyanogenic glycosides are glycosides of α-hydroxinitriles and they are amino-acid-derived plant constituents, present in more than 2,500 plant species. The generation of cyanide (HCN) from cyanogenic glycosides is a two-step process involving a deglycosilation and a cleavage of the molecule (regulated by β-glucosidase and α-hydroxynitrilase). Furthermore, on enzymatic hydrolysis, cyanogenic glycosides yield the

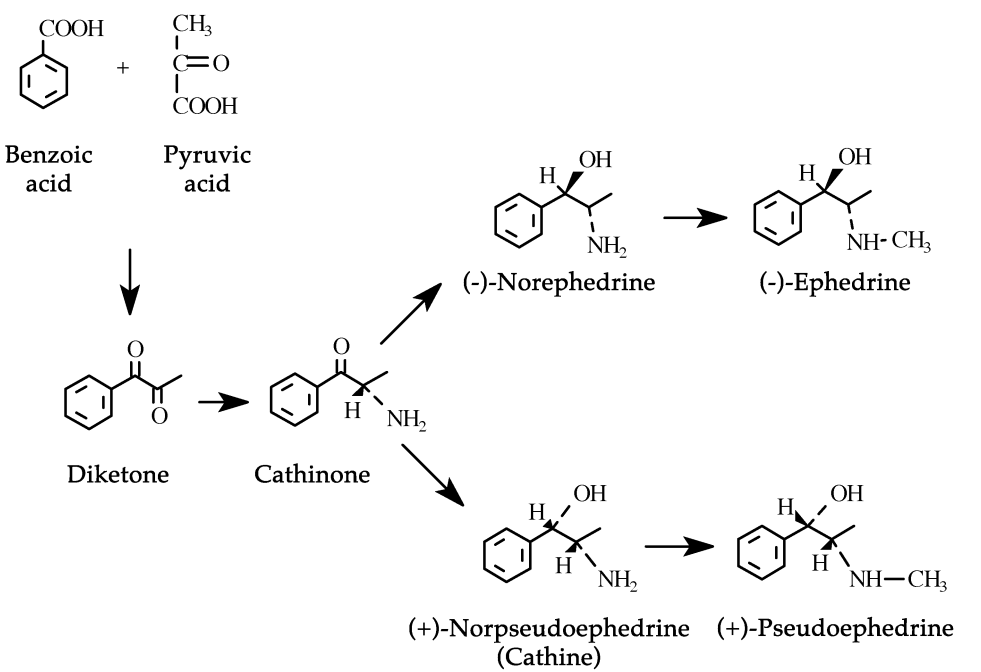

Figure 7.32 Biosynthesis of the bioactive ephedra (ma huang) alkaloids ephedrine and pseudoephedrine.

aglycone (which is an α-hydroxynitrile) and the sugar moiety. The aglycones can be grouped into aliphatic and aromatic compounds; the sugar is mostly D-glucose, but can be other sugars too, for example, gentiobiose or primeverose (Vetter, 2000).

HCN is toxic to most living organisms due to its ability to bind to the metal (Fe, Zn and Cu) functional groups or ligands of many enzymes. Inhibition of oxygen reduction in the respiratory electron chain is one example, but inhibition is also exerted towards plastocyanin reduction (during photosynthesis) and catalase activity. All plants produce cyanide as a by-product of ethylene synthesis (McMahon *et al*., 1995) and between 3,000 and 12,000 plant species produce cyanogenic compounds (Kakes, 1990).

Several food plants are cyanogenic, including white clover, flax, almonds, wild lima bean and, particularly, cassava. This last plant is the major source of calories for more than 500 million people, with over 150 million tons of cassava root being harvested annually (Boccas, 1987). Cassava is thus one of the major food crops in which cyanogenesis is a real problem. This tropical root crop is grown extensively in Africa, Asia and Latin America. Its use provides up to 60 per cent of dietary calories in some areas. Furthermore, cassava roots are an imporant insurance crop for subsistence farmers throughout the tropics. All tissues of cassava, particularly leaves and root peel, contain high amounts of the cyanogenic glycoside linamarin and lesser amounts of lotaustralin (Bradbury and Egan, 1992). Some culivars of cassava may contain up to 500 mg/kg^{-1} cyanogenic glycosides (McMahon *et al*., 1995).

In general, the dangers of long-term exposure to HCN are not completely understood, but toxic effects involving the central nervous system, the gastrointestinal tract and the thyroid have been observed. According to the hypothesis of Kamalu (1993), linamarin absorbed from cassava diets causes inhibition of Na-K-ATPase, giving rise to electrolyte imbalance with potassium depletion. This depletion causes cellular swelling, vacuolation and rupture of the epithelial cells of the proximal tubules, which results in proteinuria and causes low serum albumin concentration. Such conditions are endemic in areas where people use high amounts of food plants containing cyanogenic glycosides. The problem is even higher in areas where a low-protein diet is accompanied with the consumption of such plants. In fact, amino acids help detoxify cyanide once it has been ingested or released inside the body.

Determination of cyanogenic glycoside content in dietary supplements is done by means of qualitative, semi-quantitative or quantitative methods. The first group of determinations includes direct methods; the second group is based on preliminary hydrolysis and quantification of HCN. Because of the medical significance of cyanide, it is not surprising that most research data are reported in terms of potential cyanide yields rather than the glycoside content itself. A direct estimation of linamarin in beans, bean paste products and cassava flour has been preformed (Kawamura *et al*., 1993). A modern variant of the old picric acid method for the estimation of HCN has been developed by Hin *et al*., (1996). This system is based on the hydrolysis of linamarin by stabilized leaf linamarase with detection of the cyanide by an alkaline picrate reagent.

In the general pathway of biosynthesis of cyanogenic glycosides, the α-amino acids are hydroxylated to form an *N*-hydroxylamino acid, which is then converted to an aldoxime and this in turn to a nitrile. The nitrile is hydroxylated to form an α-hydroxynitrile, which is glucosylated to form the corresponding cyanogenic glycoside (McFarlane *et al*., 1975). The precursor of the linamarin synthesis is the valine and the

conversion of valine to acetone cyanohydrin (non-glycosylated form of linamarin) is catalysed by NADPH-dependent cytochrome P450. The initial step is the *N*-hydroxylation of valine followed by the formation of 2-methyl-propanal oxime and its dehydration to yield 2-methylpropionitrile. The addition of oxygen forms acetone cyanohydrin, which is then glycosylated to form linamarin (Koch *et al*., 1992). The term 'cyanogenesis' means not only the synthesis or presence of a cyanogenic glycoside, but the enzymatic hydrolysis producing free HCN and other compounds. Since no HCN is released from intact cyanogenic plants, the substrates (the cyanogenic glycoside) and the enzymes must be located in different cell compartments (Vetter, 2000).

The generation of cyanide from linamarin is a two-step process involving the initial deglycosilation of linamarin and the cleavage of linamarin to acetone cyanohydrin to form acetone and cyanide. These reactions are catalysed by a β-glucosidase (linamarase) and by α-hydroxynitrile lyase (HNL). Since acetone cyanohydrin may enzymatically as well as spontaneously decompose, it has been generally assumed that the linamarase activity is the rate-limiting step. The second, i.e. the final, step of the cyanogenesis is the breakdown of acetone to cyanide and acetone. This can occur both spontaneously (at temperatures greater than 35 °C or at pH greater than 4.0) and enzymatically catalysed by α-hydroxynitrile lyase (Vetter, 2000).

Figure 7.33 summarizes the biosynthesis and the generation of HCN from cyanogenic glycosides.

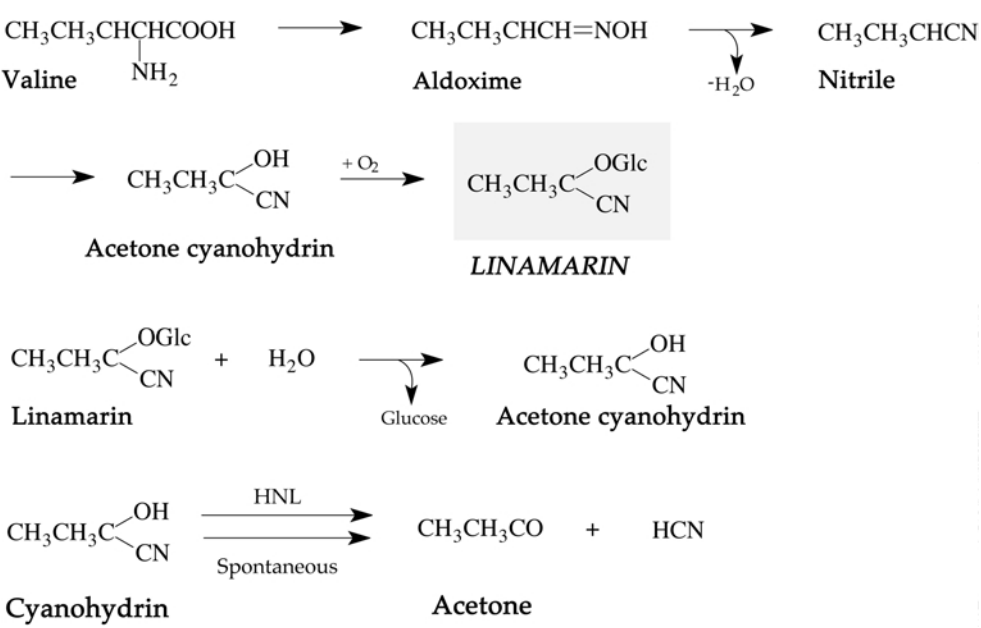

Figure 7.33 Biosynthesis of the cyanogenic glycoside linamarin and release of cyanide from cyanohydrin both spontaneously and by the action of the enzyme hydroxynitrile liase (HNL).

Glucosinolates and isothiocyanates

Glucosinolates (or β-thioglucose-*N*-hydroxysulphates), the precursors of isothiocyanates, are present in sixteen plant families, including a large number of edible species. These compounds have recently attracted intense research interest because of their cancer chemoprotective attributes. Moreover glucosinolate/isothiocyanates possess antibacterial, fungicidal, nematocidal and allelopathic properties (Fahey *et al*., 2001).

The consumption of food plants, such as cruciferous vegetables, has been found to be linked to reduced incidence of many types of cancer (Michaud *et al*., 1999; Talalay, 1999). At least some of the cancer chemoprotective activity of these vegetables is widely believed to be due to their content of minor dietary components such as glucosinolates (Fahey *et al*., 2001). Some glucosinolates have been reported to induce mammalian Phase 2 enzymes of detoxication (Fahey *et al*., 1997). Conversion of glucosinolates to thiocyanates, nitriles and isothiocyanates by the enzyme myrosinase (which is present in the microflora of the human digestive tract) is the important step in the process of cancer prevention. In fact these molecules possess potential antiproliferative, apoptosis-promoting, redox regulatory and Phase 1 enzyme-inhibiting roles (Nakamura *et al*., 2000; Fahey *et al*., 2001). For an extended revision of cancer-preventive effects of glucosinolate/isothiocyanate see Fahey *et al*. (2001).

Glucosinolates are tasteless and odourless compounds, whereas isothiocyanates are liquids with a sharp smell and taste (mustard oils). Black mustard (*Brassica nigra* L.) contains sinigrin. The drug is used as a condiment because of the sharp taste of the allyl isothiocyanate, whereas white mustard (*Sinapis alba*) contains sinalbin and is used as a spice. Powdered black and white mustard can be stirred and taken as an emetic (Samuelsson, 1992; Mithen *et al*., 2000).

In the same way as for cyanogenic glycosides, the biosynthesis of glucosinolates starts from an α-amino acid. Elongation of the amino acid side-chains occurs before *S*-glycosylation, whereas side-chain modification probably occurs after addition of the aglycone moiety. In the same way as for cyanogenic glycosides, the initial step in the biosynthesis proceeds by *N*-hydroxylation of a precursor amino acid, followed by decarboxylation to form an aldoxime (Bennett *et al*., 1995; Mithen *et al*., 2000). In the biosynthesis of sinalbin, tyrosine is the precursor. Biosynthetic steps after aldoxime formation are believed to involve conversion to a thiohydroximic acid, introduction of the thioglucoside sulphur from cysteine, *S*-glycosyl transfer from UDP-glucose, and sulphation by the donor 3′-phosphoadenosine-5′-phosphosulphate (PAPS) (reviewed by Fahey *et al*., 2001).

Glucosinolates are very stable water-soluble precursors of isothiocyanates. Conversion of glucosinolates to isothiocyanates occurs upon wounding of the plant, mastication of fresh plants (i.e. vegetables), or by damage caused by shipping, handling or bruising. Tissue damage releases myrosinase, an enzyme sequestered within aqueous vacuoles that hydrolyses glucosinolates. After hydrolytic cleavage of glucose, the sulphate moiety is released non-enzymatically to form thiohydroxamate-*O*-sulphonate. This unstable intermediate then rearranges to form isothiocyanates, or other breakdown products (such as thiocyanates, nitriles, epithionitriles and oxazolidine-2-thiones) in a manner that depends upon glucosinolate substrate as well as the reaction conditions (Fahey *et al*., 2001). Figure 7.34 depicts the pathway of the glucosinolate sinalbin formation and the breakdown products of myrosinase activity.

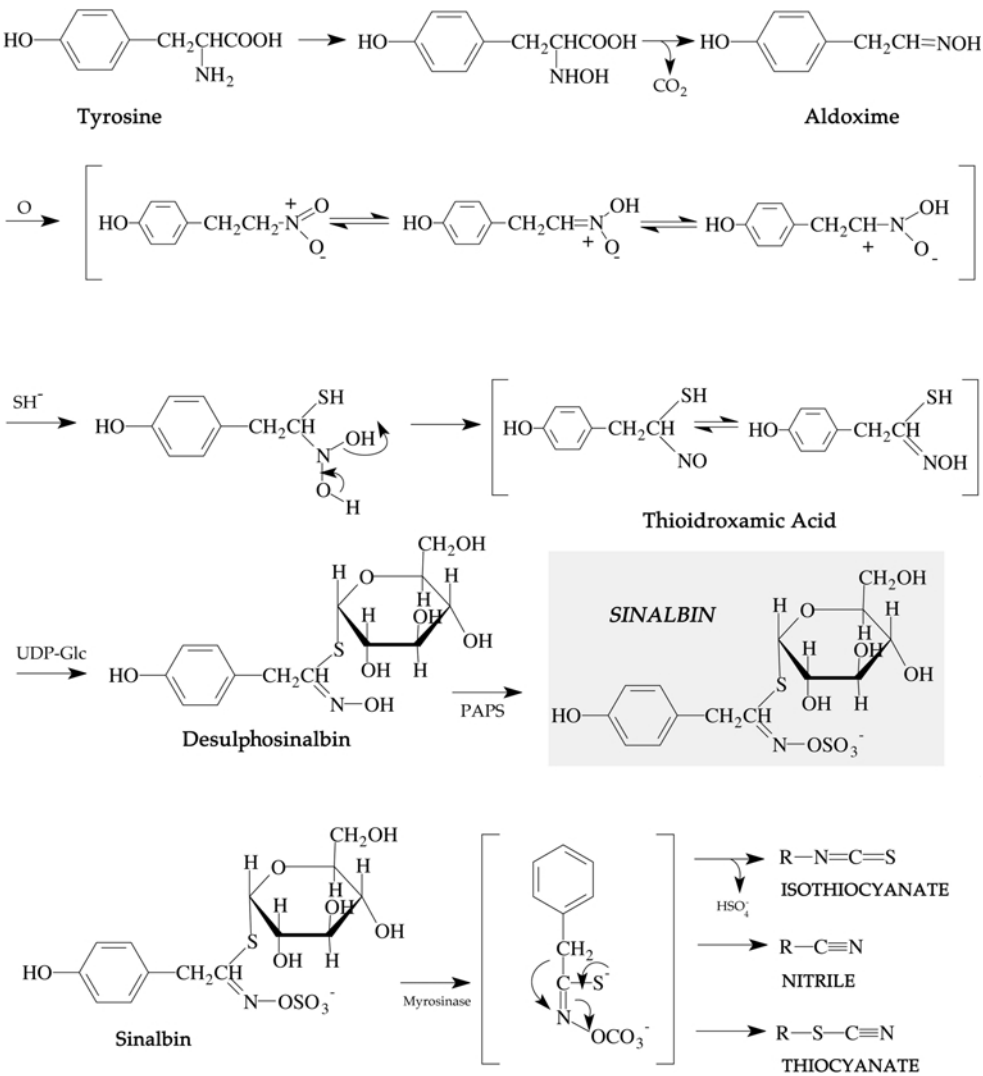

Figure 7.34 Biosynthesis of the glucosinolate sinalbin and breakdown products isothiocyanates, nitriles and thiocyanates by the action of the enzyme mirosinase.

Other bioactive compounds

Several other compounds, deriving from different pathways, have bioactive properties. Most of these molecules belong to the so-called acetate pathway, which leads to fatty acids and polyketides. Polyketides represent a large class of natural products that are grouped together on purely biosynthetic grounds. Included in such compounds are fatty acids, polyacetylenes, prostaglandins, macrolide antibiotics and many aromatic compounds (Dewick, 1998). Below are described biosynthetic patwhays to some of the most interesting compounds found in dietary supplements: from St John's wort hypericin to the widely studied omega-3 fatty acids.

Hypericin and hyperforin: St John's wort

There is a growing increase in the sale of herbal medicines. This is particularly the case for St John's wort (*Hypericum perforatum*), a herbal antidepressant whose sales in the USA in 1998 were estimated at $200 million, while in Europe in 1999 sales amounted to $6 billion (McIntyre, 2000). Indeed, in Germany St John's wort is the leading treatment for depression, outselling fluoxetine (Prozac®) by a factor of four (Ernst, 1999). St John's wort contains numerous biologically active constituents, including naphthodianthrones (e.g. hypericin and its derivatives), phloroglucinol derivatives (e.g. hyperforin) and flavonoids (e.g. rutin, quercetin, quercitrin and biapigenin). For the treatment of depression, standardized alcoholic (60 per cent ethanol or 80 per cent methanol) extracts are commonly used. These are prepared from the dried plant and formulated into tablets, capsules and syrups for oral administration. Alcoholic extracts can contain 0.1–0.3 per cent hypericin, 2–4 per cent flavonoids and up to 6 per cent hyperforin. Commercial extracts are standardized to 0.3 per cent hypericin (Di Carlo *et al*., 2001). Inhibition of monoamine oxidase (MAO) by hypericin was believed to be the primary mode of action of the antidepressant effect of St John's wort. However, this initial assumption has not been confirmed in several subsequent studies (Muller *et al*., 1998; Nathan, 1999). In fact, the current standardization of *H. perforatum* extracts based on hypericin content correlates poorly with clinical potency because the antidepressant effect of *H. perforatum* extracts depends on their hyperforin content (Laakmann *et al*., 1998). St John's wort activates the pregnane X receptor (PXR, a member of the steroid thyroid hormone receptor family that serves as a key regulator of cytochrome P450 enzyme system transcription) and consequently induces the expression of cytochrome P450 in human hepatocytes. Hyperforin, but not hypericin, is the chemical component of St John's wort responsible for PXR activation (Moore *et al*., 2000). *Hypericum* extracts have only weak activity in assays related to mechanisms of the synthetic antidepressants, that is, inhibition of MAO, catechol O-methyltransferase or serotonin re-uptake. It has been postulated that the clinical efficacy of St John's wort could be attributable to the combined contribution of several mechanisms, each one too weak by itself to account for the overall effect. The recent demonstration of a significant affinity of hypericin for sigma receptors presents new possibilities for consideration (Bennett *et al*., 1998). However, recent findings indicate that acute or chronic treatment with *Hypericum* extract does not alter mouse brain MAO activity and extracts devoid of hypericin still retain antidepressant activity (Di Carlo *et al*., 2001).

Another matter of concern is the phototoxic effects of hypericin and its derivatives – a disease called hypericism. Pseudohypericin and hypericin, the major photosensitizing constituents of *H. perforatum*, are believed to cause hypericism. Since hypericin has been proposed as a photosensitizer for photodynamic cancer therapy, the photocytotoxicity of its congener pseudohypericin has been investigated. Pseudohypericin, in contrast to hypericin, interacts strongly with constituents of fetal calf serum (FCS), lowering its interaction with cells. Since pseudohypericin is two to three times more abundant in *Hypericum* than hypericin and the bioavailabilities of pseudohypericin and hypericin after oral administration are similar, results from the work of Vandenbogaerde and collaborators (1998) suggest that hypericin, and not pseudohypericin, is likely to be the constituent responsible for hypericism. Moreover, the dramatic decrease of photosensitizing activity of pseudohypericin in the presence of serum may restrict its applicability in clinical situations. Recently, studies done by Bernd

and co-workers (1999) have confirmed the phototoxic activity of *Hypericum* extract on human keratinocytes. However, the blood levels that are to be expected during antidepressive therapy are presumably too low to induce phototoxic skin reactions.

The biosynthesis of the naphthodianthrone hypericin starts from the cyclization of a polyketide containing eight C_2 units. After several modifications and the aromatization of the molecule, several tetrahydronaphthalene intermediates are formed, to end up with the anthrone, emodin anthrone. A further oxidative step can create a dehydrodianthrone, and then coupling of the aromatic rings through protohypericin gives the naphthodianthrone hypericin (Dewick, 1998). Hydroxylation of the latter gives rise to pseudohypericin (Wink, 1999).

The phloroglucinol derivative hyperforin is probably synthesized from chalcone derivatives (Torssell, 1997).

Figure 7.35 depicts the biosynthetic pathway to hypericin and the structural formula of hyperforin.

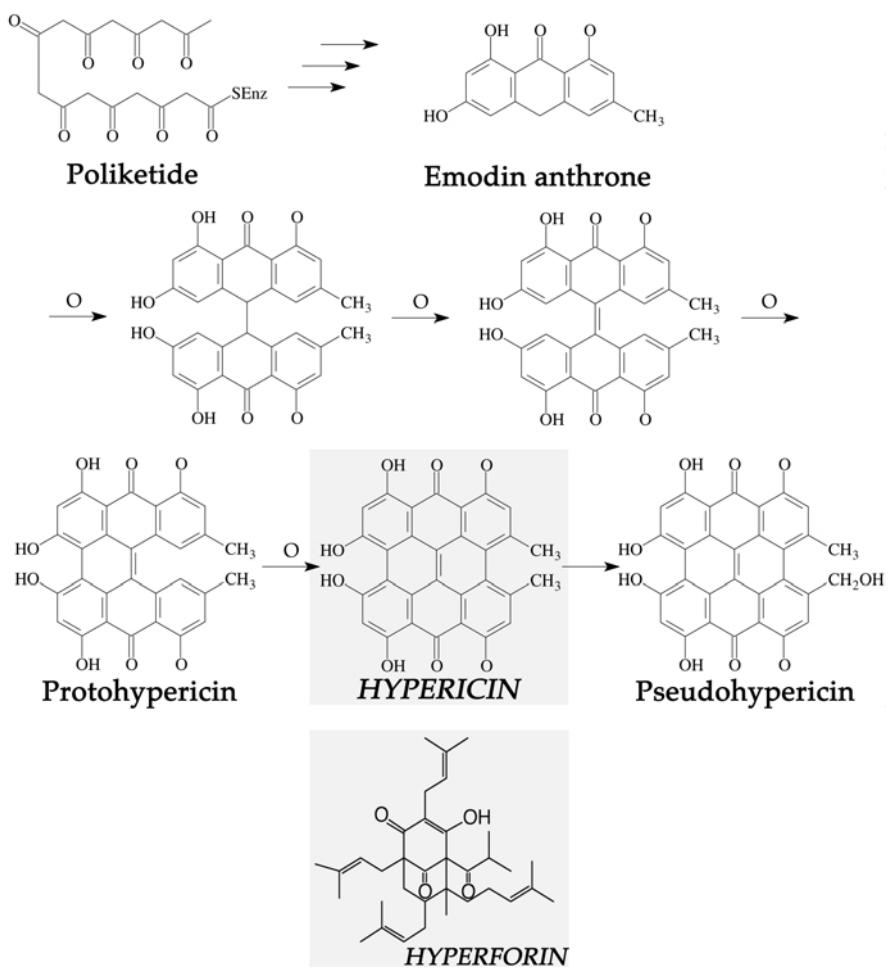

Figure 7.35 Biosynthesis of the naphthodianthrones hypericin and pseudohypericin. The structural formula of the phloroglucinol derivative hyperforin is also shown.

Resveratrol: grape and wines

Resveratrol (3,4,5-trihydroxy-*trans*-stilbene) is a phenolic compound of the stilbene family present in wines and various parts of the grape, including the skin, and shows antioxidant and antiproliferative activities. Given that it is present in grape skins but not in the flesh, white wine contains very small amounts of resveratrol, compared to red wine. In red wines the concentrations of the *trans*-isomer, which is the major form, generally ranges from 0.1 to 15 mg/L (Frémont, 2000). Several researchers have investigated the antioxidant and pro-oxidant activities of resveratrol and compared them with other antioxidants widely used in foods (Murcia and Martinez-Tomé, 2001; Stivala *et al*., 2001). The results showed that the hydroxyl group in the 4 position is not the sole determinant for antioxidant activity. In contrast, the presence of 4-OH together with stereoisomery in the *trans*-conformation (4-hydroxystyryl moiety), was absolutely required for inhibition of cell proliferation. Enzymatic assays *in vitro* demonstrated that inhibition of DNA synthesis was induced by a direct interaction of resveratrol with α- and δ-DNA polymerases (Stivala *et al*., 2001). Moreover, a direct comparison of resveratrol with other antioxidants showed that the order of HOCl scavenging activity was prolyl gallate > resveratrol > α-tocopherol > phenol; however, resveratrol was found to be inefficient in scavenging OH* and H_2O_2 (Murcia and Martinez-Tomé, 2001).

Recent studies indicate that resveratrol can block the process of multistep carcinogenesis through mitotic signal transduction blockade and can also reduce risk of cardiovascular disease owing to its phyto-oestrogenic activity. Furthermore, it has been suggested that the antitumour and antimetastatic activities of this molecule might be due to the inhibition of DNA synthesis in Lewis lung carcinoma cells (Kimura and Okuda, 2001). Resveratrol was also found to strongly inhibit nitric oxide (NO) generation and reduce the amount of cytosolic inducible nitric oxide synthase (Lin and Tsai, 1999).

Resveratrol is glucuronated in the human liver and glucuronidation may reduce the bioavailability of this compound. However, recent findings that the flavonoids quercetin, myricetin, catechin, kaempferol, fisetin and apigenin may inhibit resveratrol glucuronidation suggest that such an inhibition might improve the bioavailability of resveratrol (De Santi et al., 2000).

Resveratrol biosynthesis starts from the phenolic compound building block *trans*-4-hydroxycinnamoyl CoA and proceeds through addition of 3-malonyl-CoA units to form a polyketide intermediate. The latter undergoes decarboxylation and cyclization to form the stilbene skeleton of resveratrol (Dewick, 1998) (Figure 7.36).

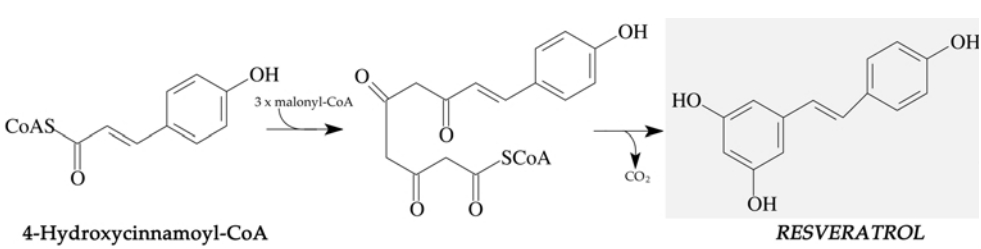

Figure 7.36 Biosynthetic pathway to the stilbene resveratrol.

Omega-3 fatty acids

Future advances in clinical cancer research will come from an emphasis on prevention rather than the treatment of metastatic disease. This research effort encompasses epidemiological studies, such as those responsible for the recognition that high consumption of vegetables and fruits is associated with a reduced risk of some cancers, laboratory experiments to evaluate potential natural and synthetic products as chemopreventive agents, and the execution of clinical preventive trials (Greenwald *et al*., 1993). Experimental studies performed in several laboratories suggest that the omega-3 fatty acids (FAs) can have a chemosuppressive effect on the progression of microscopic metastatic foci (Rose *et al*., 1996). These remarkable nutrients have attracted interest because of their importance in normal brain development, as dietary supplements for the prevention and treatment of chronic cardiovascular disease, and in the treatment of arthritic disorders and diabetes mellitus (Rose and Connolly, 1999).

The unsaturated FAs comprise monounsaturates and polyunsaturates. The conventional chemical nomenclature is to begin the systematic numbering of carbon atoms from the carboxyl terminal group. The carbon atom numbers 2 and 3 from the carboxyl group are referred to as the α and β carbons, respectively, the last carbon is the ω- or *n*-carbon, and the position of a double bond is indicated by the symbol Δ, followed by a number: for example, Δ9 refers to a double bond between carbon atoms 9 and 10 from the carboxyl group. However, an accepted practice in describing the chemical structure of FA molecules is to start numbering the carbons at the methyl group (ω- or *n*-). The omega-3 (*n*-3) and omega-6 (*n*-6) polyunsaturated FAs cannot be synthesized by mammals, and because they must be obtained from the diet, they are referred to as 'essential fatty acids'. The *n*-3 FAs are represented by α-linolenic acid (LNA) and the *n*-6 FAs by linoleic acid (LA). Both LNA and LA are metabolized to longer-chain FAs, largely in the liver; LNA is converted to eicosapentaenoic acid (EPA), and thence to docosahexaenoic acid (DHA), while LA is the metabolic precursor of arachidonic acid (AA) (Rose and Connolly, 1999). It has been demonstrated that while dietary LA may influence eicosanoid formation by increasing the tissue AA pool, this contribution diminishes as dietary AA intake increases (Whelan *et al*., 1993).

FAs are found naturally in high concentration in fish. In addition to fish and fish oils, soybean and canola (low erucic acid rapeseed) oils may provide a significant source of dietary *n*-3 FA in the form of LNA (Hunter, 1990), the major FA in chloroplast lipids. In North American diets, the principal food sources of LNA are salad and cooking oils and salad dressing products. The per capita intake in the United States has been estimated to be *c.*16–20 g/day for men and 12 g/day for women (Kim *et al*., 1984). Rose and Connolly (1999) have recently reviewed the potential that long-chain *n*-3 FAs exhibit as breast and colon cancer chemopreventive agents.

In higher plants there are two sets of reactions leading to production and accumulation of polyunsaturated fatty acids. The set of reactions occurring solely within the chloroplast are termed the 'prokaryotic pathway'; those involving glycerolipid synthesis in the endoplasmic reticulum and subsequent transfer to the chloroplast constitute the 'eukaryotic pathway'. Figure 7.37 shows the early steps in the biosynthesis of fatty acids. Fatty acids grow by the addition of two-carbon units; acetyl-CoA is the building block for the synthesis of both malonyl-CoA and the condensation reactions that lead to chain elongation. The first product of condensation is 3-ketobutyryl-ACP, which is reduced to 3-hydroxybutyryl-ACP, a reaction catalysed by the enzyme

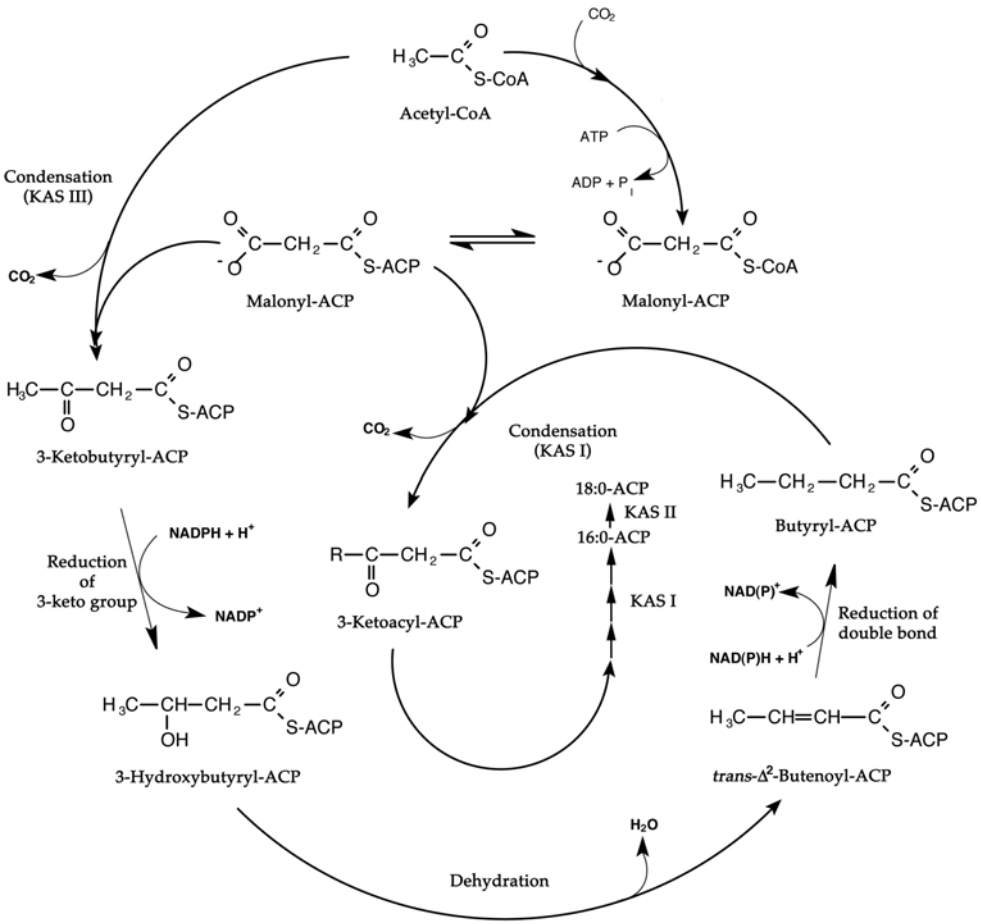

Figure 7.37 Biosynthesis of fatty acids starting from the building block metabolite acetyl-CoA. The synthesis goes through several condensations of malonyl-CoA to yield 18:0-ACP. (See text for details.)

ketoacyl-ACP synthase (KAS) isoform III. The latter compound is dehydrated and then reduced to butyryl-ACP which undergoes condensation with malonyl-ACP to form 3-ketoacyl-ACP, a reaction catalysed by KAS I. For the next six turns of the cycle, the condensation reaction is catalysed by KAS I and, finally, the conversion of 16:0 to 18:0 is catalysed by isoform II of KAS. Each condensation is accompanied by a decarboxylation, and the reaction goes on by addition of C_2 units (Somerville *et al.*, 2000). Figure 7.38 depicts the several steps involved in FA synthesis in leaves of *Arabidopsis thaliana.*

Bioengineering of bioactive plant compounds

The first genetically modified (GM) crops were put on the market in the mid-1990s. Since then, uneven developments have occurred in various parts of the world. In the

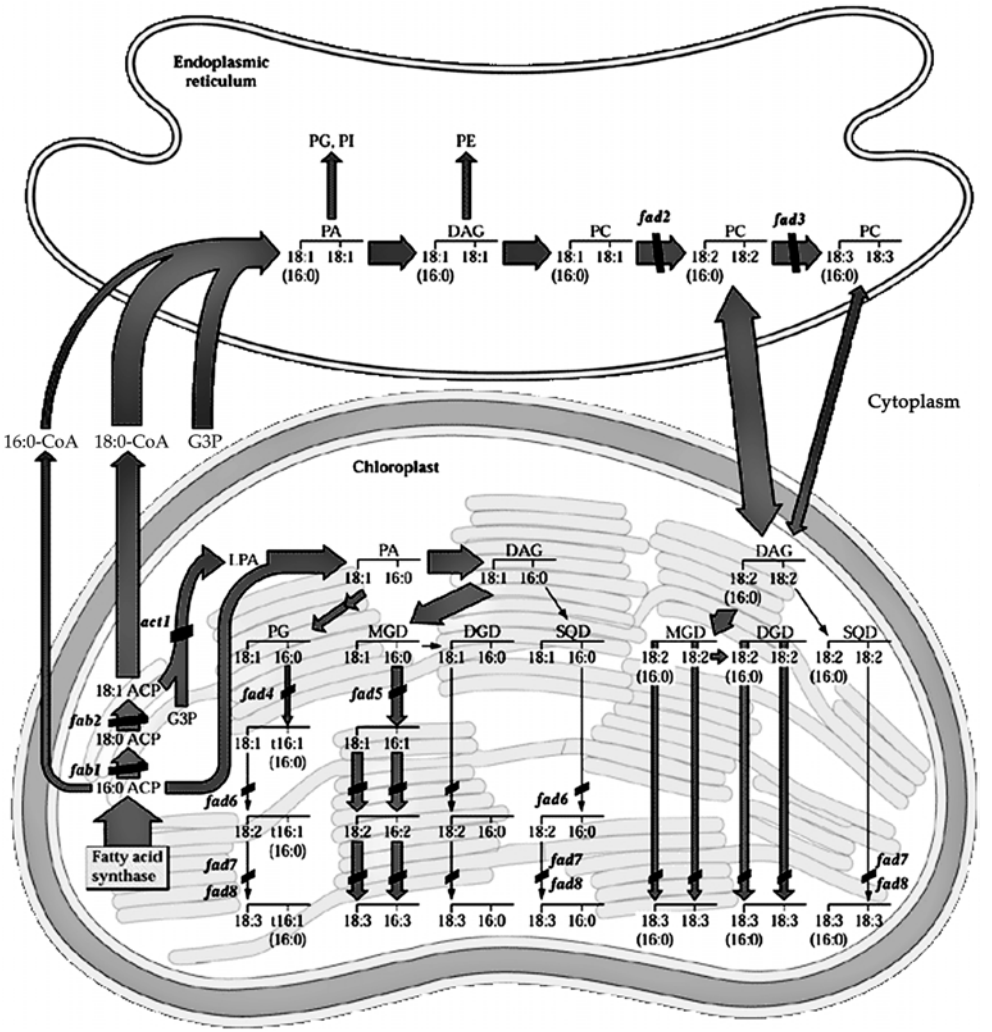

Figure 7.38 Further transformation of fatty acids in the chloroplast and endoplasmic reticulum as demonstrated in leaves of *Arabidopsis thaliana*. (From Somerville *et al*., 2000, copyright of the American Society of Plant Biologists, reproduced with permission.)

future, the proportion of acreage planted with transgenic crops and the range of transgenic crops are sure to increase. As with other innovations, the rapid uptake of GM crops is driven by profitability expectations (EC Working Document, 2000).

Before entering the world of bioengineering let us recall some basic definitions; then we will move to the discussion of food biotechnology, international policy, regulation and public concern. We will end the chapter with the last frontier in agro-biotechnology: molecular farming.

GENETIC ENGINEERING

This is the manipulation of an organism's genetic endowment by introducing or eliminating specific genes through modern molecular biology techniques. A broad definition of genetic engineering also includes selective breeding and other means of artificial selection.

GENETICALLY MODIFIED ORGANISMS (GMO)

These are organisms produced from genetic engineering techniques that allow the transfer of functional genes from one organism to another, including from one species to another. Bacteria, fungi, viruses, plants, insects, fish and mammals are examples of organisms whose genetic material has been artificially modified in order to change some physical property or capability. 'Living modified organism' (LMO), and 'transgenic organism' are other terms that are often used in place of GMO.

GENETICALLY MODIFIED FOOD

This is food or food ingredients consisting of or containing genetically modified organisms, or produced from such organisms.

NOVEL FOOD

Novel food is GM food or other foods or food ingredients consisting of or isolated from microrganisms, fungi, algae, plants or animals, or which has been obtained through new processes.

TRANSGENIC PLANTS

Transgenic plants result from the insertion of genetic material from another organism so that the plant will exhibit a desired trait.

Food biotechnology

Public concern and regulation

Food biotechnology is a process that uses the tools of modern genetics to enhance the beneficial traits of plants and microorganisms for food production. It offers controlled, precise and time-effective ways to modify fruits, vegetables, grains and other food crops to produce desirable attributes for foods. These innovations include the way plants and foods are grown, processed and tested for purity and safety.

The major concern about GM foods is the 'fear of the unknown'. I will briefly try to point out the profits and losses, the danger and the advantages, the fear of the unknown and the faith of science that surrounds GM foods. In a recent brochure (2000) on GM foods by Maarten J. Chrispeels, Director of the San Diego Center for Molecular Agriculture, there is an interesting list of 'if yous' that is worth quoting:

If you worry about food safety you should know that GM foods are as safe as other foods and that GM crops are grown with fewer pesticide applications than traditional crops.

If you treasure butterflies you need to know that pesticides used in conventional farming are far worse than GM crops for butterflies.

If you have allergies you need to know that GM technology can eliminate food allergens and that all GM crops are extensively tested to make sure that no new allergens are introduced. In addition, GM crops are being created in which the major allergens have been eliminated.

If you are worried about cancer you should take note of the fact that 99.99 percent of the carcinogens in your food supply are natural chemicals that humans have been eating for thousands of years. However, GM technology provides the means of increasing levels of phytoestrogens, isoflavones, carotenoids, and other antioxidants known to prevent cancer.

If you are a woman and worried about getting sufficient iron you should know that genetic modification can increase the iron content of cereals and has eliminated chemicals (phytic acid) that prevent iron absorption.

If you have doubts about the government's approval of GM crops you need to know that extensive testing and a long approval process accompany every GM crop introduction.

If you care about the environment you may want to know that GM foods can make a significant contribution to alleviating the negative impact that agriculture has on our environment.

If you care about developing countries you should take note of the fact that the most eminent plant breeders in those countries want to have access to GM technology to breed more productive and more nutritious crops.

If you don't trust industry spokespersons then listen to independent university scientists.

If you are worried about eating genes you should know that a GM-free meal that has ten ingredients (wheat, potato, broccoli, meat, etc.) has billions of copies of 250,000 different genes. If five of those ingredients are GM crops you will eat an additional ten to fifteen genes. All those genes are quite readily digested by your stomach juices.

So there seems to be no reason to worry about GM foods, but what are consumers' attitudes towards food biotechnology? For more than a decade, American consumers have consistently expressed positive attitudes about biotechnology. Historically, between two-thirds and three-quarters of US consumers have supported biotechnology and are willing to accept food enhanced by biotechnology techniques. A survey conducted of 1,000 US adults in February 1999 by the Wirthlin Group found high awareness of food biotechnology, strong support for its benefits, and endorsement for current labelling requirements. The survey supports the fact that eight out of ten Americans expect to derive benefits from biotechnology within the next five years (NFPA, 2001). It has become customary to contrast North American consumers' perceptions of GMOs with those of European consumers. While Americans and Canadians would hold benevolent views or simply be indifferent, European consumers would display more scepticism for reasons which are said to be: cultural (degree of faith in science, relationship to food), historical (recent food scares in Europe), and political (degree of

trust in public and private actors). This dichotomy reflects clear regional cleavages, yet needs qualifying for at least three reasons. First, civil society groups have early on organized global, transregional mobilization campaigns against GMOs. Second, some differences that were once apparent between European and North American public opinions have eroded with time. Finally, the two blocs overlap only loosely with geographic boundaries. Not all European countries share the same concerns over GMOs; conversely, some countries outside Europe – Australia, New Zealand – have joined in the mobilization against transgenic food (EC Working Document, 2000).

It is clear that acceptance of GM foods varies round the globe. But what is the situation with regard to regulation? The development of output-enhancing and/or cost-reducing GMOs on crops expected to exhibit strong export demand growth in the US provided a strong push for their rapid development and commercial use. In the EU, consumer resistance, linked not only to concerns for food safety and environmental stewardship but also to the absence of identifiable benefits from GMOs, resulted in a slower approval process (Haniotis, 2000).

As we noted above, there are distinct differences in the perception of risk between US and EU consumers and citizens. In general, the US consumer holds a great deal of confidence in the established governmental approval agencies such as the Food and Drug Administration (FDA) and the United States Department of Agriculture (USDA). On the other hand, the typical EU consumer is more risk-averse than the US consumer on food safety, and exhibits less trust in governments (Haniotis, 2000).

Based on consumer resistance, many food suppliers have taken a restrictive stance to GM food. In the EU, food processors and retailers are trying to avoid or to restrict GM food. In the US and in Canada, some grain traders and processors are considering segregating GM and non-GM crops to meet differentiated export, and even domestic, demand. Segregating implies setting up, organizing and monitoring separate market channels for GM and non-GM products, throughout the food chain. One step further is identity preservation, a production and marketing process which preserves the source and the nature of a specified crop. In the case of GM crops with quality traits (second-generation), identity preservation is necessary for preserving their value. Identity preservation of GM products would be a move away from the mainstream of commodity-based trading. However, identity preservation is already implemented for some speciality products in the EU (EC Working Document, 2000).

Why is the European public neutral to genetically modified crops but strongly opposed to GM foods? The differential support is associated with different perceptions of use, risk and moral acceptability. The negative perceptions of GM foods may be the result of the BSE crisis and other food scares, which have heightened people's sensitivities to what they eat. People simply do not want to take the risk of eating GM foods, and the absence of labelling and consequent denial of choice in the matter is the crucial concern (Gaskell, 2000).

It is within this context that labelling issues grew in importance in the EU in the past decade. The main idea behind labelling food products according to ingredients and processes responds to the Amsterdam treaty idea of the consumers' 'right to know'. This philosophy, coupled with the use of the 'precautionary principle' in food safety regulation, leads to a long-term view of potential costs and benefits for each product before it is approved, thus covering all potential consumer, social and environmental risks (Haniotis, 2000). Labelling has been recommended as a tool to provide consumer choice between products and to avoid further market and trade disruptions.

However, labelling systems in which consumers have confidence would require at least segregation of product lines throughout the processing system. Moreover, identity preservation would be required to distinguish the different types of products according to their contents of GM material or the way they have been produced whether using GM technologies or not. Segregating and identity preservation are attempts to create and establish a separate market for a 'new' product, a specific crop. The success of such attempts will depend on supply and demand concerning the new product (EC Working Document, 2000).

Because of the public's response, the European approval procedures for GMOs are stringent and thorough. European resistance to the introduction of GMOs is so strong that approval has practically come to a stop and Europe is in danger of rejecting this new science of biotechnology despite its enormous potential for good (Richardson, 2000).

Who regulates GM crops in the US? The US Department of Agriculture (USDA) regulates the transport, growth and propagation of plants. Special regulations deal with GM crops. Companies that develop such crops need to apply for a permit to conduct field tests of new GM crops or new varieties of already approved crops (e.g. introduction of a different gene). Regulators try to predict the environmental impact of the new GM crop. Especially important is the presence or absence of wild relatives of the crop and the possibility that genes could spread to those wild relatives. The USDA also oversees the nutritional content labelling of foods. The Environmental Protection Agency (EPA) regulates new chemical substances, especially hazardous ones. The EPA decided some years ago that genetically engineered pest control 'agents' such as Bt genes should be considered as chemical substances and that the EPA should regulate GM crops engineered to be pest-resistant. This does not make much sense to most scientists because these agents are usually proteins or other natural substances that are already present in plants. Alternatively, they may be proteins that are toxic to insects but easily digested by people. There is no evidence that these agents pose an environmental threat. The involvement of the EPA does not rest on sound scientific principles because both conventional and GM crops contain natural pesticides. Despite this, the EPA regulates only the GM crops, not traditional crops (Chrispeels, 2000).

What is the role of the FDA? The Food and Drug Administration (FDA) regulates both new foods (and drugs) that are introduced and foods derived from conventional or GM crops. Its primary concern is with food safety. The FDA is not particularly interested in how the food is produced (GM or non-GM) and treats all foods equally. Because some people maintain that GM foods are unsafe, the FDA has become involved in the issue of labelling them. However, so far there are no indications that GM foods are either more or less safe than other foods. Scientists generally support the idea that regulation is important and they agree that it should be based on sound scientific principles and be free of political considerations. Having GM crops regulated by three different federal agencies is cumbersome and the United States government is moving towards creating a single food safety agency to ensure the safety of all foods, including GM foods (Chrispeels, 2000).

GMO testing methods

A genetically modified organism can be distinguished from a non-GMO by the fact that it contains unique novel deoxyribonucleic acid (DNA) sequences and/or unique novel proteins not present in its conventional counterpart. Two methods are actually applied: a PCR (polymerase chain reaction) test based on DNA detection and the ELISA (enzyme-linked immunosorbent assay) which is based on protein detection. Validation programmes for both methods are currently exercised by the EU Joint Research Centre (Lipp *et al*., 2000).

PCR

The polymerase chain reaction (PCR) is based on the detection of DNA fragments that are inserted in the plant genome. This method allows amplification in a few hours of specific DNA fragments to a degree that they can be analysed qualitatively and quantitatively by common laboratory techniques (e.g. electrophoresis). However, it requires specialized equipment and training. PCR testing is applicable and extremely sensitive in the case of unprocessed food where the DNA is still intact. This is not the case for processed food where it is more difficult to isolate high-quality DNA and where GM material from more than one GM species can be present. In the latter case, the method is laborious and costly. PCR requires little reagent development time compared to immunological assays, but it can still take 1 to 3 days to receive results from a testing laboratory. The test is estimated to be about 99.9 per cent accurate (EC Working Document, 2000).

ELISA

This method is able to detect and quantify the amount of a certain protein which is of interest in a sample that may contain numerous other dissimilar proteins. ELISA uses antibodies to bind specific proteins. Antibodies are soluble proteins produced by the immune system of animals in response to exposure to a foreign substance (called an antigen). For GMOs, the antigen can be the newly synthesized protein. A colorimetric or fluorometric reaction can visualize and measure when the antigen and specific antibody bind together. One restriction for using ELISA is the denaturation of proteins in some food processes. Similar to PCR, ELISA requires trained personnel and specialized equipment. This method also requires high investments to develop the assay and to generate antibodies and protein standards. However, once reagents are developed, the cost per sample is low. The test is reported to be 95 per cent reliable (EC Working Document, 2000).

The DNA-based PCR test takes 1–3 days, at a cost of €104–310 per test. ELISA takes only 2–8 hours and may cost up to €10 per test. A faster and simpler ELISA dipstick test to provide a 'yes or no' result takes 5–10 minutes and costs only €3.6 per test (ACPA, 1999; EC Working Document, 2000).

Biosafety Protocol

The Biosafety Protocol provides a framework for addressing environmental impacts of bioengeneered products that cross international borders. It was concluded in Montreal in January 2000 by delegates from 138 countries.

> In accordance with the precautionary approach (. . .), the objective of this [Biosafety] Protocol is to contribute to ensuring an adequate level of protection in the field of safe transfer, handling and use of Living Modified Organisms resulting from modern biotechnology that may have an adverse effect on the conservation and sustainable use of biological diversity, taking also into account risks to human health, and specifically focussing on trans-boundary movements.
>
> (Article 1).

The procedures foreseen under the Protocol are different for living modified (LM) seeds and commodities.

- For LM seeds: Advance Informed Agreement procedures shall apply before the first *trans*-boundary movement of seeds. Notification of exporter before movement. Accompanying documentation with precise identification and requirements.
- For LM commodities used as food, feed or for processing:
  - Information sharing on approved LMOs through Biosafety Clearing House. Possibility for developing countries without domestic regulation on LMOs to take decisions on imports under the Protocol, to benefit from assistance (financial, technical, capacity-building).
  - Documents accompanying *trans*-boundary movements of LMO commodities stating that they 'may contain LMOs'. Detailed requirements on the identification of LMOs should be adopted within two years after the entry into force of the Protocol (entry into force itself might require 2 years).

But how safe is conventional plant breeding? It has been reported that potato breeding inadvertently produced a cultivar that caused gastrointestinal, circulatory, neurological and dermatological problems associated with alkaloid poisoning. It reached the marketplace before withdrawal (Valkonen *et al*., 1996). Any toxicity or allogenicity associated with a food is universal to all human populations. Therefore, those traits in GM food that have gained approval for use in the developed world are likely to be of value to the developing world, provided that usage is similar (Atkinson *et al*., 2001).

The last frontier: molecular farming

'Molecular farming' is the term used to indicate use of GM plants to produce high-value protein and other important molecules. The FAO estimates that 124 million children suffer from vitamin A deficiency and that 250,000 go blind every year because they lack this essential vitamin in their diet. This deficiency depends on poor foods in terms of vitamins and other mineral content. The recent production of 'golden rice' is a brilliant application of GM technology. This rice is rich in the precursor of vitamin A, which the body readily converts into the vitamin itself. Genes that cause the yellow colour of daffodils were re-engineered so that they would be expressed in rice seeds and the resulting GM rice looks faintly yellow. Vitamin A deficiency is extremely common in Southeast Asia, Africa and Latin America among poor people for whom rice is the staple food and often just about the only food available (Chrispeels, 2000).

Proteins of microbial and viral pathogens were some of the earliest examples chosen to show the feasibility of transgenic plant expression systems (Mason *et al*., 1996). The practical aspects of choosing particular foodstuffs in which to deliver defined doses of a vaccine are being explored, and efforts are under way to establish clear regulatory paths for the development of edible vaccines (Daniell *et al*., 2001). The potato is one of the food plants in which introduction of genes for the production of vaccine has been achieved in order to fight one of the most common causes of children's death – diarrhoea. Similar applications have been done in banana, tobacco and tomato (Haq *et al*., 1995; Mason *et al*., 1996). Potato has also been the subject of other studies aimed at obtaining oral immunization. However, even though Norwalk virus capsid protein expressed in potatoes caused oral immunization when consumed as food, expression levels were too low for large-scale oral administration (0.37 per cent of total soluble protein) (Tacket *et al*., 2000). The expression of vaccine components in plants has been increased by using a range of leader and polyadenylation signals 31 (Richter *et al*., 2001) and by optimizing codon usage for plants (Streatfield *et al*., 2000). The most attractive species for expressing subunit vaccine components should have high levels of soluble protein that is stable during storage; seed crops such as cereals are particularly suitable. The choice of crop defines the type of material to be fed. Many plant tissues can be consumed raw but others must be processed. Processing facilitates the creation of a homogeneous sample, enabling a defined dose size, but it is important that any heat or pressure treatments involved do not destroy the antigen (Daniell *et al*., 2001). Furthermore, plant-expressed antigens have been shown to be able to induce mucosal and serum immune responses when administered parenterally or orally to experimental animals and, in some test cases, they have offered protection against a subsequent pathogen challenge or challenge model (Wigdorovitz *et al*., 1999).

It is clear that molecular-farming-derived biopharmaceuticals should meet the same standards of safety and performance as conventional production systems. However, many herbal medicines are now exempt from such close scrutiny and are not required to meet the same standards because of their classification as nutritional or dietary supplements. Because several environmental concerns have been raised by interest groups to confuse public perception, it is of paramount importance that regulating agencies distinguish between real and perceived public concerns (scientific versus non-scientific) (Daniell *et al*., 2001).

A hotly debated environmental concern has been the outcrossing of transgenic pollen to weeds or related crops (Daniell, 1999). Another public concern is the presence of antibiotic resistance genes or their products (which are used as selective markers) in edible parts of genetically modified crops. However, several approaches are now available to generate plants with transgenes in their nuclear or chloroplast genomes without the use of antibiotic selection (Puchta, 2000).

Both those for and those against transgene technology must judge each case on its merits. Atkinson and co-workers (2001) in a recent review advocate establishing a standard that GM crops must achieve so that they provide benefits but avoid risks. The trait being considered should offer no risk to humans and pose no greater risks to the environment than other, current practices of farmers. A benefit for resource-poor farmers should be demonstrated and the trait should be offered at no additional cost to them. Furthermore, no or only minimal changes to normal agricultural practices should be required (Atkinson *et al*., 2001).

Concluding remarks

There is a growing body of evidence about public awareness of the role of diet in preventing and, in some cases, healing disease. Bioactive molecules present in foods and supplied to foods as additives are a large portion of the natural way we can follow to improve quality of life. In this chapter we learned where, when and why bioactive molecules are produced by plants and we scratched the surface of the biochemistry of bioactive molecule production in plants, thus answering the question on how they are constructed. In the last decade we have seen new frontiers emerge in the struggle against illness by the use of natural remedies.

Since humans and animals cannot avoid eating, because of their nature as heterotrophic organisms, they must obtain calories and proteins from plants or from animals that feed on plants. Through diet they can get much more than nutrition. Scientists are beginning to understand how some components of food could promote health and reduce risk of disease. Plant bioactive compounds, like bullets targeted at a bull's eye, can be directed to diseased organs and can improve health. Well-recognized diseases like cancer and cardiovascular diseases, and others that are less recognized but are as widespread, like depression, obesity and anorexia, have been demonstrated to be potentially treatable with phytochemicals, the bioactive non-nutritional molecules produced by plants.

Biotechnology can play an important role in improving the nutritional and phytochemical value of new functional foods, by allowing the functional components of foods to be modified, improved and produced in consistent quantities per serving.

It is clear that, with or without biotechnology, bioactive uptake through diet has to be regulated. Public policy regarding health claims regarding food has experienced dramatic changes during the past decade and the emerging world of bioengineering is complicating the whole picture. Labelling of GM and non-GM foods containing bioactive compounds is becoming a must, owing to the still unknown interaction of bioactive compounds with pharmaceuticals or possible allergenic effects on susceptible people. Certification and standardization of herbal extracts, herbs and bioactive principles is an indispensable prerequisite to better understanding the effects and actions of bioactive plant products on human health.

As people improve their quality of life through better nutrition and by means of phytochemical uptake through diet, become more affluent, and hence more aware of the power of some foods in preventing chronic disease, their demand for more information and quality improvement will grow. Through validation, selection and filtering of information, regulatory policy will provide consumers with the right information, avoiding the widespread self-curing attitude that derives from lack of information.

Consumers must get closer to the science of nutrition to fully get the power hidden in functional foods, and scientists must put all their effort into making simpler what indeed is still a complicated molecular puzzle.

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8 Interaction of herbs with other medicines

The example of St John's wort

Jerry Cott

Introduction

Increasing numbers of people are seeking symptomatic relief of psychiatric disorders by using dietary supplements. Since this is generally without medical supervision (Fugh-Berman and Cott, 1999; Wong *et al.*, 1998), it is essential that clinicians avail themselves of the extensive literature available on natural products so that potential problems can be avoided. Herb–drug interactions can be of two primary types: pharmacokinetic and pharmacodynamic. Dynamic interactions are those having to do with the mechanism of action, e.g. where the drug's pharmacologic actions may be in opposition to or in addition to one another. Pharmacokinetic interactions are the result of alterations in the absorption, distribution, metabolism or excretion of medications when given together with specific drugs, foods or supplements. Interactions between botanical products and prescribed medications could increase or decrease the action of the drug, though the majority of interactions are likely to go unnoticed due to their having only minor effects on drug plasma levels. For a few, this is not the case, and illustrations will be given to provide a basis by which many of the most serious interactions can be prevented. Since kinetic interactions are of much greater relative importance, only they will be considered here. While some pharmacokinetic information on herbal medicines is available (De Smet and Brouwers, 1997), much more is needed in order to put in proper perspective the vagaries of clinical anecdotes and *in vitro* experiments. Therefore, this chapter will not be a laundry list of putative interactions, but will offer an explanation of the primary types of kinetic interactions as well as a critical summary of reports concerning specific, illustrative herb–drug interactions, especially St John's wort (*Hypericum perforatum*; SJW).

Cytochrome P450 enzymes

Biotransformation of foreign exogenous compounds, such as drugs, takes place by various enzymatic reactions traditionally classified as phase I or phase II. Both phase I and phase II enzymes result in increased polarity of lipophilic compounds, thus aiding in their eventual elimination. The cytochrome P450 (CYP450) system is a family of enzymes, particularly concentrated in the liver and intestinal mucosa but also found in the kidneys, skin, lungs and other tissues. These enzymes are important for phase I drug metabolism. They normally attach small polar groups such as hydroxy or carboxyl groups to their substrates to produce a more water-soluble compound. Phase II metabolism involves conjugation, primarily as glucuronides and sulphates but also

with glycine, glutathione, acetylation and methylation. While less is known about these reactions, they can also be induced and inhibited, resulting in drug–drug or supplement–drug interactions (Liston *et al.*, 2001). The phase I cytochrome P450 enzymes catalyse predominantly oxidative reactions. These P450 enzymes may be thought of as a 'garbage disposal' that helps rid the body of various poisons and toxins before they harm us (Vogel, 2001a). Thus, foods or foreign substances that induce the enzymes may have served a beneficial role in our evolution. While twelve gene families have been identified, three categories of these enzymes are of the greatest significant in humans: the CYP2C, 2D6 and 3A4 (Hardman *et al.*, 1996; Caraco, 1998). CYP2C (particularly 2C9 and 2C19) is responsible for the metabolism of many anticonvulsants, proton pump inhibitors, antidepressants and NSAIDS (non-steroidal anti-inflammatory drugs). CYP2D6 is found in the liver, intestine, kidneys and brain where it mediates oxidative metabolism of many antidepressants, beta blockers, antipsychotics and other medications. This enzyme shows genetic polymorphism, and 'poor metabolizers' make up approximately 7 per cent of the caucasian population. While this enzyme is not itself induced, it has been suggested that persons deficient in CYP2D6 may be more susceptible to certain drug interactions after induction of other enzymes (Spina *et al.*, 2001). CYP3A4 is the most abundant hepatic enzyme and accounts for the oxidation of over half of all medications subject to oxidative metabolism. Due to the liberal presence of the enzyme in the enterocytes of the small intestine, orally administered substrates of CYP3A4 undergo a significant extrahepatic metabolism prior to absorption. While the activity of CYP3A4 is monomorphically distributed, there is significant interindividual variation. The benzodiazepine, alprazolam, is a rather pure substrate for CYP3A4 and is even used as a marker for the enzyme.

Many foods and drugs induce or inhibit (or both) the activity of CYP450 enzymes. For example, multiple oral dosing (twice daily for 10 days) of grapefruit juice to rats has been reported to inhibit the intestinal metabolism of nifedipine while simultaneously inducing liver microsomal metabolism (Mohri *et al.*, 2000). Induction is a slow process, since it depends on the rate of synthesis of new enzymes. It is usually noticeable after a few days, and may be maximal after two weeks. Inhibition is more rapid, and can become maximal within the first 24 hours of exposure to the inhibitor – but likewise may reverse more rapidly.

Herbal products usually contain numerous pharmacologically active constituents including essential oils, tannins, coumarins, anthraquinones, saponins, glycosides, anthocyanins, alkaloids and flavonoids, all of which may potentially participate in herb–drug interactions. *In vitro* studies have shown the ability of plant saponins to inhibit the CYP450 enzymes (Kim *et al.*, 1997). Some coumarins may also inhibit specific CYP isoenzymes (Tirillini, 2000; Ohnishi *et al.*, 2000). While a large amount of *in vitro* data are available regarding the ability of plant flavonoids to inhibit CYP isoenzymes (Obermeier *et al.*, 1995; Henderson *et al.*, 2000), the effects vary with the tissue being studied (Ueng *et al.*, 2000). They may also be additive. A synergy between the coumarins and the flavonoids may be important in regard to grapefruit inhibition (Tirillini, 2000). For example, the naringenin bioflavonoids and the coumarins of grapefruit juice are reported to inhibit intestinal CYP3A4 and may cause clinically significant drug interactions with felodipine, cyclosporin, terfinadine and diazepam (Lown *et al.*, 1997a; Fuhr, 1998; Özdemir *et al.*, 1998). The flavonoid quercetin is a constituent of many herbal products, including SJW. It has been shown

to inhibit CYP3A4 *in vitro* (Li *et al.*, 1994). Cruciferous vegetables such as Brussels sprouts and broccoli induce CYP1A2 (Kall *et al.*, 1996; Fontana *et al.*, 1999). This enzyme metabolizes many carcinogens, including tobacco-related compounds and char-grilled meat. Red wine has also been reported to inhibit CYP3A4 (Offman *et al.*, 2001). A high-protein diet may increase liver metabolism while a low-protein diet may reduce it (Walter-Sack and Klotz, 1996; Stowe *et al.*, 2000).

Although *in vitro* screening is a common and non-invasive means of screening for potential drug interactions mediated by the cytochromes, it has considerable limitations that may prevent generalization to clinical situations. For instance, *in vitro* drug and enzyme concentrations must approximate those attained *in vivo*, since enzyme specificity may be lost at elevated concentrations. False positives may be generated when crude extracts are incubated directly with hepatocytes (often at thousands of times the physiological plasma level). The incubates often contain constituents which would never be absorbed if orally ingested. Additional contributing factors that are difficult to simulate are genetic and environmental influences on enzyme expression, the extent of protein binding, hepatic blood flow and extra-hepatic elimination. Finally, the phase 2 enzymes are also subject to polymorphism, can be induced and inhibited, and are subject to rate limiting kinetics due to the availability of cofactors, and the overall redox status of the organism.

Thus, whole-animal or human clinical studies are by far the most useful measures of metabolic alterations since they incorporate the variables mentioned above and take into account the effects of stomach acids, digestive enzymes, transport systems, absorption, and so on.

St John's wort

In spite of recent reports concerning interactions of SJW with prescription medications, its safety record is excellent (Schulz, 2001) and it is still considered a first-line treatment for mild to moderate depression in Europe (Di Carlo *et al.*, 2001).

Although SJW shows monoamine oxidase (MAO) inhibition *in vitro*, this effect has not been displayed *in vivo*, nor have there been any reported cases of MAO inhibitor-associated hypertension in humans using SJW (Cott, 1997; Upton *et al.*, 1997). Although SJW has been reported to non-selectively inhibit uptake of serotonin, norepinephrine and dopamine (and other amines as well) into synaptosomes *in vitro* (Müller *et al.*, 1997) and *in vivo* (Neary *et al.*, 2001), the concentrations required are unrealistically high (approximately 1,000 times less potent than synthetic uptake inhibitors). In addition, the side-effects of SJW are not at all similar to those of serotonin (or other amine) uptake inhibitors (Woelk *et al.*, 1994; Ernst *et al.*, 1998). The phloroglucinol derivative hyperforin (up to 5 per cent of total extract) is believed to be the primary uptake inhibitor (Chatterjee *et al.*, 1998; Müller *et al.*, 1998). However, more recent studies suggest that neither SJW nor hyperforin are true uptake inhibitors since they do not bind to the uptake site like synthetic uptake inhibitors (Singer *et al.*, 1999; Jensen *et al.*, 2001). Rather, they appear to release monoamines from synaptosomes, yielding the same net effect in the *in vitro* assay (Gobbi *et al.*, 1999). This 'pseudo' non-selective uptake inhibition has been suggested to be a release of transmitter related to increasing intracellular sodium concentration (Singer *et al.*, 1999), calcium mobilization (Koch and Chatterjee, 2001), and ion channel modulation (Krishtal *et al.*, 2001). Finally, the whole notion of the relevance of hyperforin and

uptake inhibition is brought into question when one considers that the relatively hyperforin-free (<0.2 per cent) formulation, Ze 117 (Wurglics *et al.*, 2001), shows clinical antidepressant efficacy when compared with placebo (Schrader *et al.*, 1998) and equivalence to 20 mg/day fluoxetine (Schrader, 2000) and 150 mg/day imipramine (Woelk, 2000) in major depression. SJW treatment also fails to inhibit uptake in human depressed patients, unlike tricyclic and SSRI (selective serotonin re-uptake inhibitor) antidepressants (Uebelhack and Franke, 2000, 2001). Thus, the true mechanism(s) of antidepressant action for SJW is yet to be determined (Gobbi *et al.*, 2001).

Remaining putative antidepressant mechanisms include direct neurotransmitter release (Chatterjee *et al.*, 2001), alteration of neuronal membrane fluidity (Eckert and Müller, 2001), adenosine receptor antagonism (Müller *et al.*, 2000) and inhibition of free radical production (Hunt *et al.*, 2001). Surprisingly, rat atrial tissue preparations showed SJW extracts to have serotonin antagonist activity and negative chronotropic and inotropic actions (Straumann *et al.*, 2001).

In vitro data

Since the recent publication of data showing that SJW reduced plasma levels of indinavir (Piscitelli *et al.*, 2000) and cyclosporin (Ruschitzka *et al.*, 2000), there has been increasing interest in determining the extent of the interaction problem with this herb. Commercially available SJW extracts were examined for the potential to inhibit the human CYP enzymes, 1A2, 2C9, 2C19, 2D6 and 3A4 (Obach, 2000). Crude SJW methanolic extracts showed inhibition of all these enzymes at very high concentrations – IC_{50}s ranged from 10 to 1,000 μg/mL. The flavonoid compound I3,II8-biapigenin inhibited 3A4, 2C9 and 1A2 activities with IC_{50} values of 0.08, 4.0 and 3.7 μM, respectively. Hyperforin inhibited 2D6, 2D9 and 3A4 with IC_{50}s of 1.6, 4.4 and 2.3 μM, respectively. The significance of these data is uncertain because the concentrations were higher than those attained clinically, e.g. hyperforin maximum plasma level is reported to be 280 nM (150 ng/mL) (Biber *et al.*, 1998). In addition, the activities of isolated chemical constituents may not be relevant to whole or crude plant extracts. However, within physiologically relevant concentrations, the SJW constituent hyperforin induces CYP3A4 in hepatocyte cells via the pregnane X nuclear receptor (K_i = 27 nM) (Moore *et al.*, 2000) and the steroid X receptor (Wentworth *et al.*, 2000). Thus, the preponderance of data suggest that hyperforin may be the constituent responsible for enzyme and transport protein induction.

In contrast, rats treated with oral doses of 300 mg/kg SJW extract for 10 days showed no changes in CYP450 liver enzyme activity (Nöldner and Chatterjee, 2001). Rats treated orally with SJW did show reduced plasma levels of warfarin, however (Nöldner and Chatterjee, 2001). Together, these data suggest (in rats at least) that the metabolic induction by SJW takes place in the intestine, rather than in the liver.

In vivo data

Direct (*in vivo*) evidence of SJW interaction with CYP450 is more useful for predicting clinical interactions. One study to evaluate effects on CYP3A4 was conducted on 13 healthy volunteers given 300 mg standardized extract SJW t.i.d. for 14 days. Urinary excretion ratios (over 24 hours) of 6-beta-hydroxycortisol/cortisol were used as

an index of 3A4 activity both before and after 14 days of SJW treatment. A significant increase (from a baseline ratio of 7.1 to an endpoint of 13) was seen and the authors concluded that SJW is an inducer of CYP3A4 (Roby *et al.*, 2000).

In another study, the effects of SJW on the activity of CYP2D6 and 3A4 were assessed in 7 normal volunteers (Markowitz *et al.*, 2000). Probe substrates included dextromethorphan (for 2D6 activity) and alprazolam (for 3A4 activity). They were administered orally with and without the coadministration of a standardized 300-mg extract of SJW 3 t.i.d. for 3 days. Urinary concentrations of dextromethorphan and dextrorphan were quantified. Plasma samples were collected (0–60 h) for alprazolam pharmacokinetic analysis sufficient to estimate t_{max}, C_{max}, $t_{½}$, and AUC (area under the concentration–time curve). No statistically significant differences were found in any estimated pharmacokinetic parameters, suggesting that short-term treatment with SJW is unlikely to inhibit CYP2D6 or CYP3A4 activity. The dosing regimen, however, was too short to draw conclusions regarding induction.

Similar probe methodology was used to examine 3A4 and 2D6 in 16 healthy volunteers divided into extensive and poor metabolizers. SJW extract (300 mg t.i.d.) was administered for eight days. There was a tendency for induction of 3A4, but there was no effect on 2D6. No significant (inhibitory) effect on either enzyme was seen after an acute dose of SJW (Ereshefsky *et al.*, 1999). The same group of investigators also reported on the effect of acute and eight days' SJW treatment in 16 subjects on CYP1A2 and the phase 2 enzyme N-acetyltransferase (NAT2) using a caffeine probe methodology. The results showed no significant interactions with CYP1A2 or NAT2 metabolic pathways (Gewertz *et al.*, 1999).

In a recent study, 12 healthy subjects (5 female, 7 male) received SJW extract for 2 weeks (Wang *et al.*, 2001). Probe drugs were given to estimate the acute and chronic enzyme activity of CYP2C9, CYP1A2, CYP2D6 and CYP3A (oral midazolam for intestinal wall and hepatic enzyme, and intravenous midazolam for hepatic enzyme) before and after 2 weeks of SJW administration (300 mg 3 t.i.d.). Short-term administration of SJW had no effect on CYP activities. Long-term SJW administration resulted in a significant and selective induction of CYP3A activity in the intestinal wall. The activities of CYP2C9, CYP1A2 and CYP2D6 were unaltered (Wang *et al.*, 2001). These data are consistent with other human findings and indicate that CYP3A4 is the only P450 enzyme affected by SJW. Of interest here is a report that the low-hyperforin formulation Ze 117 does not interact with the 3A4 substrate oral contraceptives (Brattström, in press).

P-glycoprotein

P-glycoprotein (Pgp) is an ATP-dependent pump that moves substrates out of cells. It is an inducible membrane transport protein that was initially discovered by cancer researchers studying multidrug resistance to certain cytotoxic anticancer drugs (Johnstone *et al.*, 2000). This resistence was found to result in cross-tolerance or cross-resistance to structurally unrelated compounds due to an overexpression of a family of transporter proteins (Pgp) under the control of the multidrug-resistance (MDR-1) gene (Yu, 1999). Pgp is found in normal human renal, intestinal and biliary epithelia, adrenals, testis and pregnant uterus where it is a barrier to xenobiotic accumulation and a determinant of oral bioavailability of many drugs (Tanigawara, 2000). It is also found in both the choroid plexus and the cerebral endothelium where it contributes to

the blood–brain barrier and limits entry of drugs into the brain (Sugiyama *et al.*, 1999). Pgp is expressed in normal human T lymphocytes where it appears to participate in the transport of cytokines (IL-2, IL-4 and IFN-gamma) (Drach *et al.*, 1996).

Pgp can also be affected by a range of naturally occurring compounds. Some of these, like grapefruit juice, also modulate CYP450 (Tirillini, 2000), although this may be a random rather than an intrinsic linkage between the two systems (Kim *et al.*, 1999). Also, while grapefruit juice has been reported to enhance Pgp transport (Soldner *et al.*, 1999) the effects appear to be rather weak (Becquemont *et al.*, 2001). With drugs that are substrates of both Pgp and 3A4 (such as indinavir and cyclosporin), pre-systemic metabolism would take place in a synergistic fashion (Hochman *et al.*, 2000). This could result in large decreases in plasma levels by agents that induce expression of both proteins. Reactive oxygen species (ROS) downregulate the expression of Pgp (Wartenberg *et al.*, 2000). Since many medicinal plant constituents are antioxidants, this mechanism could play a significant role in the proposed interactions. Several naturally occurring flavonoids (many of which are antioxidants) bind the protein with high affinity (Maitrejean *et al.*, 2000). Rosemary (*Rosmarinus officinalis*) extracts appear to inhibit transport into cells expressing Pgp by prevention of binding of the substrate to the Pgp protein (Plouzek *et al.*, 1999). The antioxidants in rosemary are polyphenols, rather than flavonoids (Offord *et al.*, 1997). Methoxyflavones from orange juice are reported to inhibit Pgp-mediated transport of vinblastine into Caco-2 cells (Takanaga *et al.*, 2000) while the antioxidant flavones, quercitin and kaempferol, induced expression of UDP-glucuronosyltransferases and Pgp protein in Caco-2 cell monolayers (Bock *et al.*, 2000).

SJW has recently been reported to induce Pgp as well as CYP3A4. The administration of SJW extract to rats or to humans for 14 days resulted in a 3.8-fold or 1.4-fold increase, respectively, of intestinal Pgp expression (Dürr *et al.*, 2000). However, the low-hyperforin formulation, Ze 117, lacks interaction potential with the Pgp substrate, digoxin (Brattström, in press). On the other hand, inhibition of Pgp can greatly increase transfer of certain drugs into tissues where they normally do not go, such as the HIV-1 protease inhibitor, nelfinavir, into testes and brain (Choo *et al.*, 2000). Thus, the therapeutic efficacy of many drugs might be increased by enhancing their tissue perfusion. Several categories of drugs including antihistamines and diuretics have been reported to result in significant inhibition of Pgp at therapeutically relevant concentrations (Ibrahim *et al.*, 2001).

Cyclosporin

The acute rejection of cardiac grafts in two male patients in their early sixties was recently reported (Ruschitzka *et al.*, 2000). In both cases, immunosuppression was maintained with a standard triple therapy of azothiaprine, cyclosporin and corticosteroids. Both patients were hospitalized because of early signs of rejection three weeks after beginning standardized SJW at 300 mg three times per day. In both cases, cessation of SJW led to an increase in cyclosporin levels; both patients were eventually stabilized and they recovered (Ruschitzka *et al.*, 2000). In another report (Barone *et al.*, 2000), a 29-year-old woman who received a cadaveric kidney and pancreas transplant, with stable organ function and stable cyclosporin concentrations began self-medicating with SJW. After taking SJW supplements for four to eight weeks, her cyclosporin concentrations became subtherapeutic; this was associated with organ

rejection. Four weeks after stopping SJW, her cyclosporin concentrations again became therapeutic. Two other kidney transplant recipients developed marked reduction of cyclosporin therapeutic activity after self-initiation of SJW but they did not reject the organ (Mai *et al.*, 2000; Moschella and Jaber, 2001). Thirty patients at one institution in Germany were reported to have precipitous drops in cyclosporin plasma levels after starting SJW (Breidenbach *et al.*, 2000). Two additional patients were just reported to have lowered plasma concentrations of cyclosporin due to SJW (Turton-Weeks *et al.*, 2001).

Cyclosporin is known to be a substrate of 3A4, but 3A4 induction by SJW cannot explain the magnitude of the cyclosporin interaction. Much of the oral bioavailability variation in cyclosporin was previously ascribed to 3A4 variability. However, this variability is now known to be due to Pgp variably reducing the rate of intestinal absorption (Lown *et al.*, 1997b). Thus, SJW extracts may have reduced oral bioavailability of cyclosporin by inducing Pgp as well as 3A4 (Dürr *et al.*, 2000). In any event, since the potential SJW interaction with cyclosporin is marked, coadministration of the two agents should be avoided.

Digoxin

Since digoxin is a known substrate of Pgp transport, but is not metabolized by P450 enzymes, a recent clinical study on the interaction of digoxin and SJW suggests that Pgp modulation may be induced. Healthy volunteers were brought to steady state after five days' treatment with digoxin (Johne *et al.*, 1999). The subjects continued to receive digoxin (0.25 mg/day) either with placebo ($n = 12$) or with 900 mg/day SJW (LI160; $n = 13$) for another 10 days. No statistically significant changes were observed after the first dose of SJW extract. However, 10 days of treatment with the extract resulted in a 25 per cent decrease of digoxin AUC ($P = 0.0035$) and a reduction in trough concentrations and C_{max} of 33 per cent ($P = 0.0023$) and 26 per cent ($P = 0.0095$), respectively. SJW has recently been reported to induce Pgp. The administration of SJW extract to 8 healthy males over 14 days resulted in an 18 per cent decrease of digoxin concentration after a single dose of 0.5 mg (Dürr *et al.*, 2000).

HIV protease inhibitors

HIV patients are very likely to be taking a number of medications concurrently. They are also likely to be taking SJW for various reasons. Several reports suggest extreme caution should be used with respect to the potential for drug interactions in this sensitive group. Piscitelli and colleagues (2000) carried out a clinical study on the effects of SJW on plasma levels of the HIV protease inhibitor, indinavir, in healthy, non-HIV subjects. A baseline steady state with indinavir (3×800 mg) over 24 hours was established and, after a fourth dose on the next day, kinetic parameters were established. The same dosing regime was repeated after fourteen days of standardized SJW extract consumption at 3×300 mg/day. There was a large (57 per cent) reduction in the indinavir AUC after the SJW therapy. While the exact mechanism of this interaction is unclear, indinavir is a substrate of CYP3A4. However, as with cyclosporin, indinavir is also a substrate of Pgp (Choo *et al.*, 2000).

Warfarin

A crossover study examined the effect of SJW (LI160) extract on a single dose of phenprocoumon (an anticoagulant closely related to warfarin) in ten healthy males aged 18 to 50 (Maurer *et al.*, 1999). Subjects received SJW (300 mg t.i.d.) or placebo; on day 11 each received a single dose of phenprocoumon (12 mg). SJW resulted in a significant decrease (~17 per cent; $P = 0.007$) in the AUC of free phenprocoumon compared with placebo.

A letter to *The Lancet* from the Swedish Medical Products Agency reported seven cases where patients stabilized on warfarin had experienced reduced bleeding times during SJW consumption. No thromboembolic complications were noted, and either the SJW was discontinued or the warfarin dose was adjusted. The authors suggested the cause to be an interaction between SJW and CYP2C9 (the primary liver enzyme associated with warfarin metabolism) although there was no direct evidence for this (Yue *et al.*, 2000). Another possible explanation for the interaction is reduced intestinal absorption due to induction of Pgp. In support of this possibility, rats treated orally with SJW did not show changes in liver enzyme activity but did show reduced plasma levels of orally administered warfarin (Nöldner and Chatterjee, 2001).

Oral contraceptives

The same *Lancet* letter cited above (Yue *et al.*, 2000) also mentions reports of intermenstrual ($n = 8$) or changed ($n = 1$) menstrual bleeding in women (aged 23–31 years) who had been taking long-term oral contraceptives and had recently started taking SJW (within the previous week in five of the cases). No details are given but the authors suggest that induction of 3A4 by SJW is responsible, since steroids are known substrates of CYP3A4. These reports resulted in the Swedish MPA contacting marketers of SJW and requesting that a warning be added to the labelling and that studies on the extent and implications of these interactions be carried out. Isolated reports of irregular bleeding are still occasionally being reported (Ratz *et al.*, 2001) but the significance is undetermined. Exaggerated symptoms of low-dose oral contraceptives have been reported during concomitant administration with the antidepressant nefazodone, a CYP3A4 inhibitor (Adson and Kotlyar, 2001), so this effect is theoretically possible. To date, there have been no reports of decreased plasma levels of steroid hormones or of unwanted pregnancies associated with SJW.

Theophylline

Increased bioavailability of theophylline in human subjects (increased C_{max}, AUC and elimination half-life) has been reported when it is combined with certain food substances including piperine from black pepper (Bano *et al.*, 1991) and a high-carbohydrate, low-protein diet (Walter-Sack and Klotz, 1996). Theophylline has been reported to be metabolized (by demethylation) to a significant degree by CYP1A2 in human liver microsomes (Ha *et al.*, 1995; Lee *et al.*, 1998). As already noted, 1A2 enzymes are induced by tobacco, char-broiled meat, cruciferous vegetables, and a high-protein diet. There are no *in vivo* data that show an interaction between SJW and 1A2. On the contrary, eight days' treatment with SJW in 16 subjects showed no effects on 1A2 (Gewertz *et al.*, 1999). While an interaction between theophylline and SJW has

been cited dozens of times in the literature, the published report referred to is a letter to the editor regarding a single case of a 42-year-old woman (Nebel *et al.*, 1999). She smoked half a pack of cigarettes daily and took eleven other prescription medications, most of which affect CYP enzymes, in addition to taking SJW for the previous two months. On cessation of SJW, her plasma theophylline levels rose within seven days (Nebel *et al.*, 1999). This same paper also discussed unpublished *in vitro* data suggesting induction of 1A2 with pure hypericin at concentrations several hundred times greater than those found in plasma. This report is difficult to evaluate and does not constitute evidence for an SJW–theophylline interaction. CYP2E1 may also be involved in theophylline metabolism (Kharasch *et al.*, 1993). Alcohol is known to induce this enzyme, but no mention was made in the report of alcohol consumption or of the other dietary factors influencing 1A2. Smoking is a known inducer of CYP1A2 and has been reported to reduce plasma levels of the CYP1A2 substrate, clozapine, by 20–40 per cent, while cessation of smoking elevated clozapine levels by 72 per cent (Meyer, 2001). Until additional *in vivo* data are available for SJW and 1A2, little can be said about interaction potential.

Carbamazepine

Eight healthy volunteers received 100 mg of carbamazepine twice daily for 3 days, 200 mg twice daily for 3 days, and then 400 mg once daily for 14 days (Burstein *et al.*, 2000). On the last day, blood samples were taken. The subjects then took 300 mg SJW (0.3 per cent hypericin) 3 times daily with meals and with carbamazepine (400 mg) for an additional 14 days. On day 35, plasma samples were analysed for carbamazepine and its metabolite carbamazepine-10, 11-epoxide. There were no significant differences before and after the administration of SJW in carbamazepine concentrations at peak, trough or AUC. This suggests that SJW is either not a particularly powerful CYP3A4 inducer or that it cannot induce carbamazepine metabolism beyond the extent to which it induces itself.

Antidepressants

The concern about potential interactions between SJW and other antidepressants probably stems from reports about its ability to inhibit MAO and serotonin uptake. The theory goes that combination of an antidepressant and a MAO inhibitor could result in hypertensive crises or combination with an uptake inhibitor could result in serotonin syndrome. There have been no reports suggesting that MAO inhibitor side-effects have ever occurred with SJW and this is in line with current evidence suggesting that MAO inhibition may be an *in vitro* artefact (Cott, 1997). There are a few case reports of 'serotonin syndrome' in the United States, but, interestingly, none in Europe, where SJW has been used extensively for many years (Schulz, 2001). One report concerned four cases of elderly patients described as having 'mild serotonin syndrome' but were consistent with exaggerated side-effects of sertraline, namely, nausea, vomiting and restlessness (Lantz *et al.*, 1999). All patients were stable on sertraline and experienced these effects within 3 to 4 days of adding SJW. There are many conflicting literature references to drug metabolism, and sertraline is certainly an example. While most references do not list sertraline as a substrate of CYP3A4 (Xu *et al.*, 1999), there is a case report of a 12-year-old boy on sertraline who experienced a

serotonin syndrome when erythromycin, a known CYP3A4 inhibitor, was added (Lee and Lee, 1999). There is evidence, presented earlier, that acute doses of SJW may have a mild inhibiting action on CYP3A4. Since these patients were all stable on sertraline at the time they initiated SJW, this response can be explained by an increase in sertraline plasma levels – a pharmacokinetic effect, rather than a pharmacodynamic effect. A fifth elderly patient in the Lantz *et al.* (1999) report was stable on nefazodone when she added SJW. A similar exaggerated serotonergic response resulted which is consistent with increased blood levels of nefazodone due to acute inhibition of CYP3A4 (Feucht and Weissman, 2000). The opposite effect could be predicted if the SJW had been started first, followed by the antidepressant. This is in fact the result of a clinical trial of amitriptyline and SJW (Roots *et al.*, 2000). In this study, 12 depressed patients received 900 mg SJW extract along with 75 mg twice daily of amitriptyline for 14 days. Reductions in AUC of 21.7 per cent were seen for amitriptyline and 40.6 per cent for nortriptyline. Levels of amitriptyline and its metabolite continuously decreased over the 14-day period, consistent with enzyme induction. Amitriptyline is another drug where considerable contradiction exists in the literature regarding its metabolism. David Flockhart's website lists amitriptyline as a substrate for CYP1A2, 2C19, 2C9 and 2D6, while Feucht and Weissman (2000) also list it as a substrate for CYP3A4 and glucuronyl transferase.

Conclusion

Reports of interactions between medications and botanicals are becoming increasingly common and are often alarmist in tone. The application of a few pharmacological principles is necessary to determine the scientific validity and clinical relevance of these reports. Perhaps foremost among the herbal supplements, SJW is capable of weak inhibition of CYP3A4 acutely, and of moderate induction of intestinal CYP3A4 activity after repeated dosing. Chronic administration of SJW also induces the drug transporter protein, Pgp. Drugs that are substrates of *both* systems (e.g. indinavir and cyclosporin) are of particular concern as they would be predicted to be doubly affected by SJW. While the currently popular constituent, hyperforin, appears to be responsible for the enzyme and Pgp induction, it is not necessary for the therapeutic effect. This is evidenced by the low-hyperforin formulation, Ze 117, showing efficacy in major depression (Kaufeler *et al.*, 2001). Of particular interest for this review is that clinical pharmacokinetic studies have shown this formulation to lack interaction potential with either the CYP3A4 system or the Pgp transporter (Brattström, in press). One-year safety and efficacy data were recently made available (Woelk *et al.*, 2000). Normal dietary constituents, such as flavonoids, can also have major effects on drug availability. As the pharmacokinetics of drug–drug and herb–drug interactions become better understood, they become less threatening. They can even be put to use. For example, the combination of a CYP3A4 inhibitor like grapefruit juice with expensive 3A4 substrates such as the protease inhibitors, sildenafil or cyclosporin, would allow the use of smaller doses. The use of grapefruit has actually been reported to enhance the antipsychotic response to clozapine in a previously non-responsive patient (Özdemir *et al.*, 2001). In addition, since CYP3A helps to break down bile acids, 3A4 inducers such as SJW could be useful for treating hepatic cholestasis, and could even account for the historical use in liver diseases (Vogel, 2001b). It has also been demonstrated that inhibition of Pgp can greatly increase entry of Pgp substrates into

tissues where they would not otherwise go, potentially enhancing their efficacy (Choo *et al.*, 2000). Unexpected pharmacokinetic interactions that result in major plasma level alterations of medication are always undesirable, however. More *in vivo* data is badly needed to make sense of the plentiful, but often contradictory *in vitro* data. Lists of substrates, inducers and inhibitors of the various *hepatic* CYP450 enzymes systems are regularly updated by David Flockhart and can be found on the Internet at: http://medicine.iupui.edu/flockhart. Unfortunately, no such resource yet exists for Pgp, although several examples are found in recent reviews (Yu, 1999; Markowitz and DeVane, 2001).

Patients who are prescribed certain powerful medications with narrow therapeutic ratios should be monitored closely and should be cautioned about the use of any other medications as well as many foods. These patients would also be well advised to avoid experimenting with herbal medicines or other dietary supplements without the knowledge of their healthcare provider.

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9 Official and scientific information resources for botanical dietary supplements

Gail B. Mahady

Introduction

Over the past decade the sale of botanical dietary supplements, also known as herbal medicinal products, has increased dramatically worldwide (Eisenberg *et al*., 1998; Mahady, 1998, 2001; Tyler, 1999). In the United States, annual retail sales of botanical products rose from a meagre $200 million in 1988 (Mahady, 1999), to an estimated $5.1 billion in 1997, and consumer use of these products has increased dramatically (Eisenberg *et al*., 1998). In Europe, the herbal medicinal products market is estimated at $3.8 billion. In developing countries such as in Africa, China and India, botanicals have always played a central role in healthcare. Data from the World Health Organization suggest that 65 to 80 per cent of the populations in these countries depend on traditional medicine as the primary source of healthcare (Bennerman *et al*., 1983). Conservatively, the total global market for these products is of the order of $25 billion (Mahady, 2001).

Over the past ten years, herbal medicines have enjoyed a revival in many Western countries, including Australia, Canada, Europe and the United States (Eisenberg *et al*., 1998, Mahady, 2000). In the US, herbal medicinal products are now regulated as dietary supplements under the Dietary Health and Education Act of 1994, and this Act in essence removed herbals from the basic Food and Drug Administration's rules and regulations concerning safety and efficacy, as well as good manufacturing practices. As a result, there are now over 25,000 dietary supplement products available on the US market, and it is estimated that the use of botanicals alone by American consumers increased by 380 per cent, just in the period between 1990 and 1997 (Eisenberg *et al*., 1998). This dramatic increase is also fuelled by strong consumer interest in natural therapies and preventive medicine, as well as the perception that botanicals are safe and free from side-effects (Mahady, 1998). However, the widespread use of botanicals throughout the world has raised serious questions as to the quality, safety and efficacy of these products (Mahady, 1999; De Smet, 1995; Angell and Kassirer, 1998).

The first step towards the acceptance of botanicals depends on an accurate assessment of the available scientific and clinical information on quality, safety and efficacy (Angell and Kassirer, 1998). In the past, access to such information was difficult in the USA, as most of the scientific data were published in foreign journals and books, and this information was not considered to be very credible. This led many healthcare professionals to believe that there was no evidence to support the safety and efficacy of botanical dietary supplements. In fact, very little information about botanicals was

published in standard pharmaceutical, medical or nursing journals in the United States (Mahady, 2000).

This information void has dramatically changed over the last five years. Now most peer-reviewed pharmaceutical and medical journals in the US are publishing the results of well-designed, randomized controlled clinical trials investigating the safety and efficacy of botanical dietary supplements. In fact, for some of the most commonly used botanicals such as garlic, ginkgo, valerian, saw palmetto, *Echinacea* and St John's wort, there is now a great deal of scientific information published in peer-reviewed scientific journals, on the official websites of the Office of Dietary Supplements (ODS) at the National Institute of Health, and in official monographs published by various organizations throughout the world (ODS, 2001).

The purpose of this chapter is to provide an overview of the various scientific and official information resources available for botanical dietary supplements. The focus will be primarily on resources with some official standing, such as peer-reviewed monographs and scientific and research-oriented databases, as well as peer-reviewed books and journals. The chapter is not exhaustive and will not cover in detail every official and scientific information source, as this information could fill an entire book. It will also not deal with books or journals written for the layman, as they now number in the hundreds, and the information presented is sometimes based on speculation more than science, and thus may or may not be accurate.

Official information resources

The section on official sources of information includes reviews (monographs) by officially recognized experts on botanicals such as the United States Pharmacopoeia, World Health Organization Traditional Medicine Programme and the European Scientific Cooperative on Phytotherapy. It also includes information resources from governmental organizations such as the Office of Dietary Supplements at the National Institute of Health.

Monographs

United States Pharmacopoeia

Numerous governmental organizations have already begun to address the complex issues associated with quality, safety and efficacy by developing official monographs for botanicals. For example, from the period of 1998–2000 the United States Pharmacopoeia (USP) convened an *Ad Hoc Advisory Panel on Botanicals* to help revise the USP's drug information botanical monographs. In 2000, the USP Convention elected an official panel of botanical experts addressing quality issues. As a consequence of these efforts, the USP has now published a number of official standards monographs and have further botanical monographs in preparation (Table 9.1). All drafted monographs undergo extensive peer review and revision before publication. The finalized monographs are published in the USP National Formulary, while the new drafts are published for comment in the *Pharmacopoeial Forum*. Companies with botanical products that conform to the USP specifications are allowed to carry on their products the USP logo, which has historically been the symbol for quality products in the US (USP, 2001).

Table 9.1 Current status of United States Pharmacopoeia National Formulary (USP-NF) and Dispensing Information (USP-DI) botanical monograph development

Botanical product	*Information development status*	*Standards development status*
BLACK COHOSH	*Actaea racemosa* (L.) Nutt.	
Rhizome with roots	Also known as *Cimicifuga racemosa*	
	Family Ranunculaceae	
Black cohosh		Draft under development
Black cohosh extract		Draft under development
Black cohosh, capsules and tablets		Draft under development
CAT'S CLAW	*Uncaria tormentosa* (Wild.) DC	
	Family Rubiaceae	
Cat's claw		Draft under development
Cat's claw extract		Draft under development
Cat's claw, capsules and tablets		Draft under development
CHAMOMILE	*Matricaria recutita* L.	
Flower heads	Family Asteraceae	
Chamomile		Official in *USP 24-NF19*
CHASTETREE	*Vitex agnus-castus* L.	
Berries	Family Verbenaceae	
Chastetree		Draft under preparation
Chastetree extract		Draft under preparation
Chastetree, capsules and tablets		Draft under preparation
CRANBERRY	*Vaccinium macrocarpon* Ait.;	*Vaccinium oxycoccos* L.
Juice from fruits	Family ericaceae	
Cranberry liquid preparation	Monograph under review by Advisory Panel	Official in *USP 24-NF19*
ECHINACEA ANGUSTIFOLIA	*Echinacea angustifolia* DC	
	Family Asteraceae	
Rhizome with roots		
Echinacea angustifolia,		In Process *PF 26(6)*; Nov–Dec 2000
Echinacea angustifolia, powdered		In Process *PF 26(6)*; Nov–Dec 2000
Enchinacea angustifolia, powdered extract		In Process *PF 26(6)*; Nov–Dec 2000
ECHINACEA PALLIDA	*Echinacea pallida* (Nutt.) Nutt.	
Rhizome with roots	Family Asteraceae	
Echinacea pallida		In Process *PF 26(6)*; Nov–Dec 2000
Echinacea pallida, powdered		In Process *PF 26(6)*; Nov–Dec 2000
Echinacea pallida, powdered extract		In Process *PF 26(6)*; Nov–Dec 2000

continued

Table 9.1 Continued

Botanical product	*Information development status*	*Standards development status*
ENCHINACEA PURPUREA Rhizome with roots and leaves with flowers	*Echinacea purpurea* (L.) Moench FamilyAsteraceae	
Echinacea purpurea, root		In Process *PF 26(6)*; Nov–Dec 2000
Echinacea purpurea, powdered		In Process *PF 26(6)*; Nov–Dec 2000
Echinacea purpurea, powdered extract		In Process *PF 26(6)*; Nov–Dec 2000
Echinacea purpurea, leaf with flower		Targeted for *PF 27(2)*; Mar–Apr 2001
FEVERFEW Leaves	*Tanacetum parthenium* (L.) Schultz-Bip. Family Asteraceae	
Feverfew	Final monograph February 1998 *USP-DI Update*	Official in *USP 24-NF19*
Feverfew, powdered		Official in *USP 24-NF19*
GARLIC Bulbs	*Allium sativum* L. Family Liliaceae	
Garlic	Monograph being developed	Official in *USP 24-NF19*
Garlic, powdered		Official in *USP 24-NF19*
Garlic fluid extract		In-Process Revision *PF 26(2)*; Mar–Apr 2000
Garlic extract, powdered		In Process *PF 26(5)*; Sep–Oct 2000
Garlic, delayed release tablet		In Process *PF 26(5)*; Sep–Oct 2000
Garlic, capsules		Draft under preparation
GINGER Rhizome	*Zingiber officinale* Roscoe Family Zingiberaceae	
Ginger		Official in *USP 24-NF19*
Ginger, powdered	Final monograph December 1997 *USP-DI Update*	Official in *USP 24-NF19*
Ginger tincture (oral solution)		In-Process Revision *PF 26(2)*; Mar–Apr 2000
Ginger, capsules		Previews in *PF 26(2)*; Mar–Apr 2000
GINKGO Leaves	*Ginkgo biloba* L. Family Ginkgoaceae	
Ginkgo		Official in *USP 24-NF19*
Ginkgo extract, powdered	Monograph under review by Advisory Panel	In-Process Revision *PF 26(4)*; July–Aug 2000
Ginkgo, capsules		In-Process Revision *PF 26(3)*; May–June 2000
Ginkgo, tablets		In-Process Revision *PF 26(3)*; May–June 2000

Table 9.1 Continued

Botanical product	*Information development status*	*Standards development status*
AMERICAN GINSENG Roots	*Panax quinquefolius* L. Family Araliaceae	
American ginseng		Previews *PF 26(5)* Sep–Oct 2000
American ginseng, powdered		Previews *PF 26(5)* Sep–Oct 2000
American ginseng, powdered extract		Previews *PF 26(5)* Sep–Oct 2000
ASIAN GINSENG Roots	*Panax ginseng* C. A. Meyer Family Araliaceae	
Asian ginseng		Official in *USP 24-NF19*
Asian ginseng, powdered		Official in *USP 24-NF19*
Asian ginseng, powdered extract		In-Process Revision *PF 26(2)*; March–April 2000
Asian ginseng, capsules/ tablets		In-Process Revision *PF 26(3)*; May–June 2000
ELEUTHERO (SIBERIAN GINSENG) Rhizome with roots	*Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim Family Araliaceae	
Eleuthero		In Process *PF 26(6)*; Nov–Dec 2000
Eleuthero, powdered		In Process *PF 26(6)*; Nov–Dec 2000
Eleuthero, powdered extract		In Process *PF 26(6)*; Nov–Dec 2000
GOLDENSEAL Rhizome with roots	*Hydrastis canadensis* L. Family Ranunculaceae	
Goldenseal		Previews *PF 26(4)* Jul–Aug 2000
Goldenseal, powdered		Previews *PF 26(4)* Jul–Aug 2000
Goldenseal, powdered extract		Previews *PF 26(4)* Jul–Aug 2000
HAWTHORN Leaves with flowers	*Crataegus laevigata* (Poir) DC; Family Rosaceae	*Crataegus monogyna* Jacq. emend Lindman
Hawthorn leaf with flower		In-Process Revision *PF* 26(5) Sep–Oct 2000
Hawthorn leaf with flower, powdered		In-Process Revision *PF* 26(5) Sep–Oct 2000
Hawthorn, tablets		Draft under preparation
Hawthorn, extract		Draft under preparation
HORSE CHESTNUT Seeds	*Aesculus hippocastanum* L. Family Hippocastanaceae	
Horse chestnut		Draft under preparation
Horse chestnut, extract		Draft under preparation
Horse chestnut, capsules and tablets		Draft under preparation

continued

Table 9.1 Continued

Botanical product	*Information development status*	*Standards development status*
KAVA	*Piper methysticum* G. Forst.	
Rhizome	Family Piperaceae	
Kava		In-Process Revision *PF 26(3)*; May–June 2000
Kava, powdered		In-Process Revision *PF 26(3)*; May–June 2000
Kava, powdered extract		In-Process Revision *PF 26(3)*; May–June 2000
Kava, semisolid extract		In-Process Revision *PF 26(3)*; May–June 2000
Kava, capsules		In-Process Revision *PF 26(3)*; May–June 2000
Kava, tablets		In-Process Revision *PF 26(3)*; May–June 2000
LIQUORICE	*Glycyrrhiza glabra* L.;	*Glycyrrhiza uralensis* Fisch. ex DC.
Rhizome, stolons and roots	FamilyFabaceae	
Licorice		In-Process Revision *PF 26(5)* Sep–Oct 2000
Licorice, powdered		In-Process Revision *PF 26(5)* Sep–Oct 2000
Licorice, powdered extract		In-Process Revision *PF 26(5)* Sep-Oct 2000
MILK THISTLE	*Silybum marianum* (L.) Gaertner	
Ripe fruit	Family Asteraceae	
Milk thistle	Monograph being developed	Official in *USP 24-NF19*
Milk thistle, powdered		Official in *USP 24-NF19*
Milk thistle, powdered extract		Previews in *PF 26(3)*; May–June 2000
Milk thistle, capsules/tablets		Previews in *PF 26(3)*; May–June 2000
STINGING NETTLE	*Urtica dioica* L. ssp. *dioica*	
Rhizome with roots	Family Urticaceae	
Stinging nettle		Previews in *PF 26(3)*; May–June 2000
Stinging nettle, powdered		Previews in *PF 26(3)*; May–June 2000
Stinging nettle, powdered extract		Previews in *PF 26(3)*; May–June 2000
RED CLOVER	*Trifolium pratense* L.	
Flower heads	Family Fabaceae	
Red clover		Draft under preparation
Red clover, extract		Draft under preparation
Red clover, capsules and tablets		Draft under preparation

Table 9.1 Continued

Botanical product	*Information development status*	*Standards development status*
SAW PALMETTO	*Serenoa repens* (Bartram) Small	
Ripe fruit	Family Arecaceae	
Saw palmetto	Final monograph Released 28 April 2000	Official in *USP 24-NF19*
Saw palmetto, powdered		Official in *USP 24-NF19*
Saw palmetto, extract		In Process *PF 26(6)*; Nov–Dec 2000
Saw palmetto, capsules		Draft under development
ST JOHN'S WORT (HYPERICUM)	*Hypericum perforatum* L. Family Hyperaceae	
Flowers or aerial parts		
St John's wort	Final monograph May 1998 *USP DI Update*	Official in *USP 24-NF19*
St John's wort, powdered		Official in *USP 24-NF19*
St John's wort, powdered extract		In-Process Revision *PF 26(2)*; Mar–Apr 2000
VALERIAN	*Valeriana officinalis* L.	
Rhizome, stolons and roots	Family Valerianaceae	
Valerian	Final monograph December 1997 *USP DI Update*	Official in *USP 24-NF19*
Valerian, powdered		Official in *USP 24-NF19*
Valerian, powdered extract		Official in *Supplement 1* (*USP 24-NF19*)
Valerian, capsules/tablets		Previews *PF 27(1)* Jan–Feb 2001
GENERAL TEST CHAPTER		
Botanical extracts		Official in *Supplement 1* (*USP 24-NF19*)
Disintegration and dissolution of nutritional supplements		In-Process Revision *PF 26(3)*; May–June 2000
Biological and chemical identification of articles of botanical origin		Draft under development

European Scientific Cooperative on Phytotherapy (ESCOP)

The ESCOP was founded in 1989, and is the European umbrella organization of national associations for phytotherapy (ESCOP, 2001). Its general aims include the advancement of the scientific status of herbal medicinal products, and promotion of harmonization of their regulatory status within the European Union. ESCOP, which is made up of scientists from both academia and industry in Europe, has published herbal monographs for the European market. Approximately 60 herbal medicine monographs are now available in five fascicules. All drafted monographs have undergone an extensive review process and discussion prior to publication. The ESCOP monographs summarize the medicinal uses of plant drugs, with particular emphasis on their safety aspects, which is an area of prime importance for scientific harmonization

(ESCOP, 2001). Approximately 15 of the monographs published in 1990 and 1992 were submitted to the Committee for Proprietary Medicinal Products (CPMP) for assessment. Under the recommendations of the CPMP, the format of the Summary of Product Characteristics (SPC) was adopted for subsequent documents. The SPC is an integral part of an application for authorization to market a medicinal product for human use in any of the 15 member states of the European Union. It is described as a 'definitive statement' between the competent authority and the marketing authorization holder and it is the common basis of communication between the competent authorities of all the member states. The data sheets for herbal medicinal products are based on the SPC, which is also the basis of information for the prescriber or supplier of the product (ESCOP, 2001). Since November 1992, the ESCOP Scientific Committee has been working on proposals for SPCs on individual plant drugs, primarily those for which European or national pharmacopoeial monographs exist. The sequence of topics in the SPC is designed to highlight clinical aspects and, compared to the earlier ESCOP monographs, contains more information on pharmacological properties, such as pharmacodynamics, pharmacokinetics and preclinical safety data (ESCOP, 2001). While SPC documents were prepared for assessment by regulatory authorities, the summaries are also available for wider distribution as published ESCOP monographs. For this purpose the SPC format has been slightly modified: specific product details have been omitted, while all the scientific data on plant drugs remains in exactly the same sequence. The original drafts are prepared by members of the subcommittees, and then circulated to an independent Board of Supervising Editors, including academic experts in phytotherapy and medicinal plant research for review. Comments and criticisms for each draft are collected and, where appropriate, these revisions are incorporated into the final version.

The first six fascicules, each containing ten ESCOP monographs, have been published (see Chapter 3). During the next year or so many of the monographs will be updated to include information on new clinical trials.

German Commission E monographs

In addition to the ESCOP monographs are the more familiar monographs of the German Commission E. The German Commission E monographs were prepared to facilitate the regulation and marketing of herbal medicinal products in Germany, but are also used in other parts of Europe. In order to promote the use of the German Commission E monographs, they have been translated from the original German language into English, in a project sponsored by the American Botanical Council. The translated monographs are now available as a book is entitled '*The Complete German Commission E Monographs: Therapeutic Guide to Herbal Medicines*' (Blumenthal *et al*., 1998), published by the American Botanical Council in cooperation with Integrative Medicine Communications, Austin, Texas. Of the original 300 monographs prepared by the Commission, approximately two-thirds were monographs with positive assessments covering herbs that have been found to be safe and effective; the remainder of the monographs were negative assessments due to the fact that the botanical has an unsatisfactory risk–benefit ratio (Blumenthal *et al*., 1998).

WHO monographs on selected medicinal plants

For the global herbal market, the World Health Organization's Traditional Medicine Programme (WHO-TRM) in collaboration with the WHO Collaborating Center for Traditional Medicine at the College of Pharmacy, University of Illinois at Chicago has reviewed the quality, safety and efficacy of 90 widely used medicinal plants (botanical medicines). These monographs were prepared according to the *Guidelines for the Assessment of Herbal Medicines* published by WHO-TRM (2002). The monographs were drafted based on a systematic review of the scientific literature of 1900–2001, including various national pharmacopoeias; monographs such as the German Commission E; information from Medline, Napralert and Toxline; reference texts; and peer-reviewed scientific journals from around the world. Approximately 120 botanical experts in over 40 different countries reviewed and commented on the draft monographs. These experts included members of WHO's Expert Advisory Panels for Traditional Medicine, the International Pharmacopoeia and Pharmaceutical Preparations, and Drug Evaluation and National Drug Policies, as well as drug regulatory authorities of 16 countries. Revisions to each monograph were based on the comments received, where appropriate. Each herbal monograph is composed of two parts. Part one includes pharmacopoeial-style summaries of quality assurance including a definition, a description of botanical features, geographical distribution, identity tests, purity requirements, chemical assays and a listing of the major chemical constituents. The second part summarizes medical uses, pharmacology, contraindications, warnings, precautions, adverse reactions and dosage. Each pharmacopoeial-style summary includes a 'Definition' section, which provides the correct Latin binomial, probably the most important criterion in quality assurance. Listings of Latin synonyms and vernacular names are also provided. The detailed botanical description is intended for quality assurance during collection and production, whereas the detailed description of the drug material is provided to facilitate manufacturing of products and commerce. General identity tests, purity criteria and chemical assays are all normal compendial components included under their own heading. Since each medicinal plant, and the specific plant part used, contains a characteristic chemical profile that can be used for chemical quality control and quality assurance, the constituents are described in the section 'Major chemical constituents'.

Part two of each monograph contains information necessary for the practising healthcare professional. The section begins with medicinal uses, which are categorized as the following: uses supported by clinical data; uses described in pharmacopoeias and traditional systems of medicine (not supported by clinical data, but having some experimental pharmacology to support the use); and uses described in folk medicine, not supported by experimental or clinical data. This section is written specifically for a busy healthcare practitioner, and will enable one to quickly ascertain which clinical uses are supported by clinical data, without having to read through all of the pharmacology. The rest of part two includes contraindications, warnings, precautions (general, drug interactions, drug and laboratory tests, carcinogenesis/mutagenesis/impairment of fertility, pregnancy (teratogenic or non-teratogenic effects), nursing mothers and paediatric use. The section also covers adverse reactions, and a dosage section entitled 'Posology'. Each monograph is fully referenced, enabling the reader to access further information when necessary.

Volume I of the *WHO Monographs on Selected Medicinal Plants* was published in

1999, and includes 28 monographs on 41 species of plants (WHO, 1999). This volume includes monographs for the following botanicals:

Allium cepa
Allium sativum
Aloe vera gel
Aloe vera juice
Astragalus membranaceus
Brucea javanica
Bupleurum falactum
Bupleurum falcatum
var. *scorzonerifolium*
Cassia senna (leaf)
Cassia senna (fruit)
Centella asiatica
Chamomilla recutita
Cinnanomum verum
Coptis chinensis
Coptis deltoides
Coptis japonica
Curcuma longa
Echinacea angustifolia
Echinacea angustifolia
var. *strigosa*
Echinacea pallida
Echinacea purpurea
Ephedra sinica
Ginkgo biloba
Glycyrrhiza uralensis
Paeonia lactiflora
Plantago afra
Plantago indica
Plantago ovata
Plantago asiatica
Platycodon grandiflorum
Thymus vulgaris
Thymus zygis
Valeriana officinalis
Zingiber officinale

Volume II of the *WHO Monographs on Selected Medicinal Plants*, published in 2001, contains monographs for the following botanicals:

Aesculus hippocastanum
Althaea officinalis
Andrographis paniculata
Angelica polymorpha var. *sinensis*
Arctostaphylos uva-ursi
Calendula officinalis
Cimicifuga racemosa
Crataegus monogyna
Crataegus laevigata
Eleutherococcus senticosus
Eucalyptus globulus (2 monographs, one leaf, one oil)
Hamamelis virginiana
Harpagophytum procumbens
Hypericum perforatum
Melaleuca alternifolia
Melissa officinalis
Mentha piperita (2 monographs, one leaf, one oil)
Ocimum sanctum
Oenothera biennis
Piper methysticum
Polygala senega
Prunus (Pygeum) africana
Rhamnus frangula
Rhamnus purshiana
Sambucus nigra
Serenoa repens
Silybum marianum
Tanacetum parthenium
Urtica dioica, U. urens

Volume III is currently in the final draft stages, and has been sent out for review. This volume currently contains the following monographs:

1. Fructus Ammi majoris
2. Fructus Ammi visnagae
3. Fructus Anethi
4. Aetheroleum Anisi
5. Fructus Anisi
6. Semen Armeniacae
7. Flos Arnicae
8. Folium Azadirachtii
9. Olelum Azadriachtii
10. Flos Carthami
11. Stigma Croci
12. Fructus Foeniculi
13. Radix Gentiana luteae
14. Radix Gentiana scabrae
15. Gummi Gugguli
16. Radix Harpagophytii
17. Rhizoma Hydrastis
18. Radix Ipecacuanha
19. Aetheroleum Lavandulae
20. Flos Lavandulae
21. Strobilus Lupuli
22. Folium Mate
23. Gummi Myrrha
24. Herba Passiflorae
25. Testa Plantaginis
26. Radix Rehmanniae
27. Fructus Schisandrae
28. Radix Scutellariae
29. Radix cum herba Taraxaci
30. Semen Trigonellae foenumgraecui
31. Cortex Uncariae
32. Ramulus cum unics Uncariae
33. Fructus Ziziphi

The purpose of the WHO monographs is to provide accurate scientific information on the safety, efficacy and quality control or quality assurance of widely used medicinal plants to facilitate the harmonization of common uses of herbal medicines or phytomedicines in the member states. The contents of the monographs, including the sections on microbiological quality, are in compliance with well-established standards for safety, efficacy and quality control. In particular, there should be no toxicity of such plant preparations within the range of the recommended dose. The monographs were not written to replace other compilations such as those found in pharmacopoeias, formularies or legislative documents, but to provide further accurate scientific information to facilitate regulation and usage. The importance and impact of these officially published monographs should not be underestimated. In 1998, the *Ad Hoc Working Group on Herbal Medicinal Products*, within the European Agency for the Evaluation of Medicinal Products, recommended that the scientific monographs drafted by both ESCOP and WHO-TRM be used in the support of the demonstration of the safety and efficacy of herbal medicines (EMEA, 1998).

Databases

Office of Dietary Supplements, National Institute of Health

In 1999 the Office of Dietary Supplements (ODS) within the National Institute of Health (NIH) of the United States completed one of the mandated activities found in the Dietary Supplement Health and Education Act of 1994 (ODS, 2001). This mandate was to collect and compile into a database, the results of scientific research relating to dietary supplements, including scientific data from foreign sources or the Office of Alternative Medicine (now known as the National Center for Complementary and Alternative Medicine (NCCAM). The database is now known as IBIDS, which stands for the International Bibliographic Information on Dietary Supplements. The

purpose of this database is to assist researchers, physicians and the general public in locating scientific literature on dietary supplements. IBIDS contains published, peer-reviewed, international, scientific literature on dietary supplements that draws upon numerous medical and scientific databases already in existence (ODS, 2001). The first phase of IBIDS includes published, peer-reviewed, international, scientific literature on dietary supplements from 1985 to the present. Phase II of the project will involve the addition of monographs, serials, and reports as well as peer-reviewed literature from 1975–1985 into IBIDS. It contains approximately 328,000 citations and abstracts. Although IBIDS does not provide full-text articles on the Web, a journal list of over 1,500 publications, including links to their websites is provided to assist users in obtaining full-text journal articles.

The IBIDS search engine is accessible through the ODS Internet home page for general use, see http://dietary-supplements.info.nih.gov. For a more complete description of the current contents of the IBIDS database, go to 'About IBIDS'. The database is updated quarterly with new citations and abstracts. In addition, new search terms are added to reflect emerging areas in dietary supplement research. IBIDS is available to the public free of charge through a search engine on the ODS Internet home page. The search engine for IBIDS was designed to be user-friendly, thereby allowing users with all levels of expertise to quickly search the database for information. In addition, for those unfamiliar with dietary supplement terminology, a keyword list is available. This enables the searcher to find a word that is located in the abstract or title of the paper. The information available to the user (i.e. the abstracts) is dependent upon the availability of information from the individual databases accessed and the current copyright policies of each journal (ODS, 2001).

Computer Access to Research on Dietary Supplements (CARDS)

Another database available through the ODS home page is CARDS. The purpose of this database is to provide information on research in the field of dietary supplements and individual nutrients that is currently being supported by the Federal government (ODS, 2001). With information from CARDS, reports can be generated to determine the focus areas of dietary supplement research, as well as identifying which agencies of the Federal government and Institutes of the NIH are supporting this research. This information is useful to the US Congress, agencies of the Federal government, and the NIH for budgetary considerations. In addition, the database will provide useful information for researchers, healthcare providers, industry and the general public. CARDS is available through the ODS home page (see above). The search engine, which is the method used to search the database, is being designed to be user-friendly and will utilize an interface similar to that of IBIDS. The database design again allows individuals, at all levels of expertise, to quickly and effectively search the database (ODS, 2001).

Another information service provided by the Office of Dietary Supplements (ODS) at the National Institutes of Health (NIH) is the *Annual Bibliography of Significant Advances in Dietary Supplement Research*. This publication was developed as a joint effort between the ODS and the Consumer Healthcare Products Association (CHPA). The purpose of the publication is to promote sound scientific research in this field, especially of dietary supplements. To develop the bibliography, ODS and CHPA contacted editors of peer-reviewed journals asking them to nominate original research papers from their respective journals in 1999. In response to this request, over 200 nominations were received and

then forwarded to scientific experts to review and identify the top 25. It is envisioned that the bibliography will serve as a useful reference source for nutrition and health professionals, educators and health communicators, as well as the scientists who conduct the research (ODS, 2001). Copies of the first Annual Bibliography of Significant Advances in Dietary Supplement Research may be downloaded from the Office of Dietary Supplements website at http://ods.od.nih.gov/publications/publications.html. Single copies may also be requested by contacting the ODS office (ods@nih.gov).

Along with the ODS, National Center for Complementary and Alternative Medicine (NCCAM), http://nccam.nih.gov, a division of the NIH, facilitates research and evaluation of unconventional medical practices and disseminates information to both health professionals and the public. This index consists of more than 180,000 bibliographic citations from 1963 to 1998 extracted from the Medline database (NCCAM, 2001).

Scientific information resources

Journals and books

JOURNALS

The number and quality of journals publishing original research works for botanical dietary supplements has dramatically increased in the past five years. Some of the journals publishing original research, including *in vitro*, *in vivo* and clinical trials, are listed. All listed journals are English-language and peer-reviewed. Below is a partial listing of US journals; as they now number in the hundreds and a complete listing is beyond the scope of this chapter.

1 *Alternative Therapies in Clinical Practice*
2 *Archives of Internal Medicine*
3 *New England Journal of Medicine*
4 *Pharmacotherapy*
5 *American Journal of Gastroenterology*
6 *The Lancet*
7 *British Medical Journal*
8 *Nutrition in Clinical Care*
9 *Journal of Nutrition*
10 *Alternative Therapies in Health and Medicine*
11 *Complementary Therapies in Medicine*
12 *European Journal of Herbal Medicine*
13 *Focus on Alternative and Complementary Therapies*
14 *Phytomedicine*
15 *Herbalgram*, the journal of the American Botanical Council and Herb Research Foundation (http://herbs.org) gives a listing of articles from major medical journals.
16 *Journal of Alternative and Complementary Medicine*
17 *Journal of Herbal Pharmacotherapy*

BOOKS

For official and scientific resources for botanical dietary supplements and herbal medicinal products, there are also numerous books available. All are written by experts in

the field and are reasonably good information sources for researchers, as well as for healthcare and regulatory professionals.

1 *WHO Monographs on Selected Medicinal Plants*, Volume I, World Health Organization, Geneva, Switzerland, 1999 (also mentioned under monographs)
2 *WHO Monographs on Selected Medicinal Plants*, Volume II, World Health Organization, Geneva, Switzerland, 2001
3 Mahady, G.B., Fong, H.H.S. and Farnsworth, N.R., *Botanical Dietary Supplement: Quality, Safety and Efficacy*, Swets & Zeitlinger, Amsterdam, 2001
4 *Monographs on the Medicinal Uses of Plant Drugs*, European Scientific Cooperative on Phytotherapy, Exeter, UK, 1996 (also mentioned under monographs)
5 *Herbal Medicines: A Guide for Health-Care Professionals*. C.A. Newall, L.A. Anderson and J.D. Phillipson. The Pharmaceutical Press, London, 1996
6 *Tyler's Herbs of Choice: The Therapeutic Use of Phytomedicinals*. J.E. Robbers and V.E. Tyler. The Haworth Herbal Press, New York, 1999
7 *Tyler's Honest Herbal: A Sensible Guide to the Use of Herbs and Related Remedies*, 4th edition. S. Foster and V.E. Tyler. The Haworth Herbal Press, New York, 1999
8 *Herbal Medicinals: A Clinician's Guide*. L.O. Miller and W.J. Murray. The Haworth Herbal Press, New York, 1998
9 *Rational Phytotherapy: A Physician's Guide to Herbal Medicine*. V. Schulz, R. Hansel and V.E. Tyler. The Haworth Herbal Press, New York, 1998
10 *The Review of Natural Products. Facts and Comparisons*, St Louis, MO, various dates
11 *PDR for Herbal Medicines*. Medical Economics Company, Montvale, NJ, 1998
12 *PDR for Nonprescription Drugs and Dietary Supplements*, 20th edition. Medical Economics Company, Montvale, NJ, 1999
13 *The Complete German Commission E Monographs: Therapeutic Guide to Herbal Medicines*, M. Blumenthal *et al*., editors, published by the American Botanical Council in cooperation with Integrative Medicine Communications, Austin, Texas, 1998

For more detailed reviews on information resources from journals and books see the reviews by Jackson (Jackson, 2001; Jackson and Tanmaz, 2001).

Databases and websites

Numerous databases and websites with scientific information concerning botanical research are available. Some, such as Napralert have been around for over 25 years, while many are relatively new. Probably the oldest and most comprehensive database is the Napralert[SM] database. Napralert, an acronym for natural products alert, is the largest relational database of world scientific literature on the ethnobotanical uses, pharmacology, pharmacokinetics, toxicology and chemistry of botanicals. The database was started by Dr Norman Farnsworth in 1975, and is currently housed within the UIC/NIH Center for Botanical Dietary Supplements, Program for Collaborative Research in the Pharmaceutical Sciences, College of Pharmacy at the University of Illinois in Chicago. Napralert currently contains data extracted from over 150,000 scientific articles collected from over 48 countries throughout the world. Many articles written in other languages, have been translated into English and are in the form of either an abstract or the entire translated article. Napralert contains data on over 52,000 species of organisms, 151,000 chemical substance of natural origin and has more than 75,000 ethnomedical records and 1.5 million pharmacological records that

associate these previous record types with biological activity. Approximately 700 international journals are perused on a monthly basis by Ph.D.-level scientists for inclusion of pertinent articles into the database. Data from over 600 articles are added monthly to the database, which appears to be sufficient to cover the world literature of botanical research. Since the structure of the Napralert database is relational, multiple-level queries are available to provide specific answers to specific questions. The language used to query Napralert is SQL (Structured Query Language), which has a simple syntax and has been widely adopted for relational database functions.

Generally speaking, the Napralert database encodes five kinds of records: ethnomedical data (folkloric uses), organism data (plant description), activity data (pharmacology activity), chemical data (chemical constituents) and bibliographical data. A referencing system to standardize all botanical nomenclature, and yet retain the author's nomenclature, as well as a single standard nomenclature have been implemented. This is to instil consistency, so that a given plant will always be referred to by one name. This has been accomplished by determining a correct scientific name (Latin binomial) for each plant species and the possible synonyms of a plant Latin binomial, using the best taxonomic literature available and taxonomic specialists. The database also maintains lists of common names for each plant species, though all searches are based on the correct Latin name of the genus and species. The ethnomedical records contain information on the folkloric uses of the plant species, while the biological activity records contain information on the pharmacology of the plant or plant extract or isolated chemical constituents. All activities are uniquely coded through a system of 'major pharmacological activities/specific pharmacological activities (mpa/spa)'. All of the data are tied together via the worktype table which indicates what type of work is described in each article. For example, experiment type (*in vitro*, *in vivo*, clinical trials, biosynthetic studies), isolation and identification methods (HPLC, IR, UV, NMR, etc.). Currently, queries of Napralert are restricted to three-part profiles that list all of the known chemistry, ethnomedical uses and pharmacological activities for a given genus and species, all completely referenced. However, the user may also generate just the chemistry of a given plant, or just the pharmacology if desired. Other searches can generate lists of biological activities for an isolated natural compound, percentage of compound found in the plant, or where the compound is found in nature.

Napralert is available online through the Scientific and Technical Network, or by contacting mlquinn@uic.edu. See http://info.cas.org/ONLINE/DBSS/napralertss.html for summary information. Over the next six months Napralert will undergo a data conversion process in preparation for an Internet version of the database. This site will be an interactive on-line access, and allow the user to manipulate the data to allow for specific answers to specific questions and further enhance the utility of this underutilized resource.

Alternative and Allied Medicine Database (AMED) is produced and updated monthly by the British Library's Medical Information Centre. This database of citations contains over 65,000 references from 400 international journals (Jackson and Tanmaz, 2001).

The Cochrane Library provides access to regularly updated systematic reviews of the effect of healthcare interventions (Cochrane Database of Systematic Reviews, CDSR), structured abstracts of quality-assessed, previously published reviews (Database of Abstracts of Reviews of Effectiveness, DARE), and references to controlled trials (Cochrane Controlled Trials Register, CCTR). More detailed information about the library and how to access it can be accessed at www.cochrane.co.uk. The abstracts are free off the Internet; however, for full text one must subscribe. The Cochrane

Complementary Medicine Field is a specific section of the Cochrane Library only available to subscribers; it summarizes randomized controlled trials of complementary medicine interventions published in peer-reviewed journals (Jackson and Tanmaz, 2001)

The American Botanical Council (ABC) home page, www.herbalgram.org, provides access to information on *Herbalgram*, the complete German Commission E monographs, and a book catalogue on herbs and botanicals.

The Natural Medicines Comprehensive Database, F. Batz and K. Hitchens (associate editors), Pharmacist's Letter, Stockton, CA, is available in both printed and Web versions. This database will include 15 columns of information on approximately 1,000 herbs and dietary supplements, the Web version will allow more sophisticated searching for information as well as constant updating. For a more complete description of this database and its utility see the extensive review by Jackson (2001).

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Appendix
Dietary supplements of plant origin
Plants, diet and cancer prevention

In order to survive, plants have developed sophisticated mechanisms including an elaborate chemical arsenal of toxic substances, such as terpenes and alkaloids, that inhibit the growth of other plants and make them unattractive to predators (da Rocha *et al.*, 2001; see also chapter by Maffei). The ability of plants to produce bioactive compounds has been exploited by humans for thousands of years, and plant extracts and plant parts have been used since ancient times to cure various diseases. The knowledge of these plants was passed on through the generations and thus humans gathered considerable experience of drugs which could be obtained from the plants in their surroundings (Samuelsson, 1992). Despite major scientific and technological progress in combinatorial chemistry, drugs derived from natural products still make an enormous contribution to drug discovery today. Some examples of agents derived from natural sources that are currently used in clinical practice are given in Table A.1. The number of diseases that can be cured through the use of plant-derived bioactive compounds is incredibly high and several plant-derived compounds are currently successfully

Table A.1 Drugs developed from plant natural sources. Reprinted from *Current Opionion in Pharmacology*, da Rocha *et al*., 2001, with permission from Elsevier Science

Drug	*Medical use*	*Mechanism of action*
Aspirin	Analgesic, anti-inflammatory, antipyretic	Inhibition of cyclo-oxygenase
Atropine	Pupil dilator	Antagonist of acetylcholine at muscarinic receptors at post-ganglionic parasympathetic neuroeffector sites
Caffeine	Stimulant	Adenosine receptor antagonist
Codeine	Analgesic, antitussive	Opioid receptor agonist
Digoxin	For atrial fibrillation and congestive heart failure	Inhibition of the Na^+/K^+ ATPase membrane pump
Eugenol	Toothache	Reduces excitability of sensory nerves (increased K^+ efflux and reduced Ca^{2+} influx)
Morphine	Analgesic	Opioid receptor agonist
Pilocarpine	Glaucoma	Muscarinic receptor agonist
Quinine	Malaria prophylaxis	Inhibition of protein synthesis in the malaria parasite
Taxol	Anticancer agent	Antimitotic agent (binds to and stabilizes microtubules)

employed in cancer treatment and prevention. Furthermore, a number of additional plant-derived agents are currently under investigation (da Rocha *et al.*, 2001).

Epidemiology suggests that cancer is largely an unavoidable disease and that more than two-thirds of cancers might be prevented through lifestyle modification (Ferguson, 1999). According to some authors the four main causes of cancer deaths are smoking, diet, chronic infections (especially in developing countries) and hormonal factors (Ames and Gold, 1998). One of the major influences on cancer risk appears to be diet. Dietary imbalances are important – especially the lack of sufficient amounts of dietary fruits and vegetables (Ames and Gold, 1997). Despite disagreement between different authors on details, there is agreement on the considerable number of modifying factors in the carcinogenic process and that there are very good prospects for dietary interventions (Ferguson, 1999). Epidemiological studies, supported by preclinical data from animal and *in vitro* experiments and by clinical findings, have contributed immensely in providing insights into links between diet and cancer prevention and to the development of diet and cancer hypotheses for testing in clinical trials (Greenwald *et al.*, 2001).

Overall, available data support an inverse relationship between cancer risk and intakes of vegetables, fruits, whole grains, dietary fibre, certain micronutrients and certain types of fat (e.g. *n*-3 fatty acids, particularly *n*-3/*n*-6 ratios), as well as physical activity. The data also support a direct relationship between cancer risk and intakes of total fat or certain types of fat (e.g. saturated fat) and alcohol, as well as obesity and certain food preparation methods such as smoking, salting and pickling foods, and high-temperature cooking of meats (World Cancer Research Fund, 1997; Heber *et al.*, 1999; Greenwald *et al.*, 2001).

Plant-derived foods, including vegetables, fruits and whole grains, contain thousands of chemically diverse phytochemicals. Table A.2 lists some phytochemical classes, along with examples of specific compounds, food sources and representative cancer-prevention-related activities.

The European Prospective Investigation into Cancer and Nutrition (EPIC), a prospective study with approximately 460,000 subjects, which is being carried out in 22 centres across nine European countries, is designed to investigate the relationship between diet, nutritional and metabolic characteristics, various lifestyle characteristics and cancer risk (Riboli and Kaaks, 1997). Studies, such as EPIC, that include a molecular epidemiology component, can help to assess variations in risk across populations more accurately and to identify particularly cancer-susceptible subgroups within populations, thus facilitating the development of effective approaches to cancer prevention (Greenwald *et al.*, 2001). For example, data from the EPIC-Italy study show strong negative associations between levels of leucocyte DNA adducts, a biological marker that might be predictive of cancer risk (Perera, 1997) and consumption of fresh vegetables and fruits, particularly green leafy vegetables (Palli *et al.*, 2000). In addition, evidence exists that increased vegetable and fruit consumption can reduce oxidative damage to DNA and influence the activity of xenobiotic-metabolizing enzymes (Greenwald *et al.*, 2001).

According to Greenwald and co-workers (2001) several constituents found in vegetables and fruits (including dietary fibre, micronutrients and various phytochemicals) and interactions between these constituents might contribute to the ability of these foods to reduce cancer risk. Determining which constituents are most effective and how they exert their effects pose significant challenges for the cancer research community.

Table A.2 Selected phytochemicals associated with cancer prevention. Reprinted from *European Journal of Cancer*, Greenwald *et al*., 2001, with permission from Elsevier Science

Phytochemical class	*Typical compounds*	*Food sources*	*Cancer prevention-related activities*
Carotenoids	α-Carotene, β-carotene, lycopene, β-cryptoxanthin, lutein, astaxanthin	Yellow-red and dark-green	Antioxidant activity, vegetables and fruits modulation of carcinogen metabolism, inhibition of cell proliferation, inhibition of oncogene expression, beneficial effects on immune function, beneficial effects on cell transformation and differentiation, enhancement of cell-to-cell communication
Organosulphur compounds	Diallyl sulphide, diallyl disulphide, allyl methyl trisulphide, dithiolthiones	Sulphides: *Allium* vegetables (e.g. garlic, onion); dithiolthiones: cruciferous vegetables (e.g. broccoli, cabbage)	Increase phase II enzyme activity, inhibit cell proliferation, induce cell differentiation, alter steroid hormone metabolism, inhibit ornithine decarboxylase activity
Polyphenols	Phenolic acids (e.g. caffeic acid), hydroxycinnamic acids (e.g. curcumin), flavanols (e.g. quercetin, apigenin), flavanones (e.g. naringin, hesperidin), catechins (e.g. epigallocatechin gallate), thea flavins, resveratrol	Vegetables and fruits; catechins: green tea; thea flavins: black tea; resveratrol: red wine	Reduce carcinogen–DNA adduct formation, inhibit cell proliferation, induce cell cycle arrest and apoptosis, inhibit signal transduction pathways, enhance cell-to-cell communication, improve immune function
Phyto-oestrogens	Isoflavones (e.g. genistein, daidzein), lignans (e.g. matairesinol, secoisolariresinol)	Isoflavones: soybeans, soy-based foods; lignans: vegetables, flaxseed, rye	Alter ooestrogen metabolism, decrease tyrosine kinase activity, induce cell cycle arrest and apoptosis, induce topoisomerase II-mediated DNA breakage
Glucosinolates, isothiocyanates, indoles	Glucobrassicin, sulphorophane, indole-3-carbinol	Cruciferous vegetables	Increase phase II enzyme activity, induce cell cycle arrest and apoptosis, inhibit cell adhesion and invasion
Terpenes	Monoterpenes (e.g. limonene, perillyl alcohol, geraniol), Sesquiterpenes (e.g. farnesol)	Vegetables and fruits (e.g. citrus)	Increase phase II enzyme activity, influence cell cycle progression, induce apoptosis

Ethnomedical plant-use data in many forms has been heavily utilized in the development of formularies and pharmacopoeias, providing a major focus in global healthcare, as well as contributing substantially to the drug development process (Graham *et al.*, 2000). Table A.3 lists the results of a query of the Napralert database for plants which have been reported to have been used ethnomedically against cancer.

The future for understanding linkages between diet and cancer will be expanded by the ability of the biomedical research community to use newly available technological advances to conduct basic research studies in molecular biology and genetics. This will take motivation, dedication, collaboration and education and training across disciplines, as well as a concerted effort by nutritional scientists, molecular biologists, geneticists and clinical cancer researchers to achieve this vision (Greenwald *et al.*, 2001).

According to Greenwald and co-workers (2001), during the early decades of the 21st century researchers in diet and cancer will keep the best of the 'old' science and combine it with the best of the 'new' science, to design effective, targeted cancer prevention strategies that will benefit both the general population and those at high risk for cancer.

Table A.3 List of plants reported to be used against cancer, but not found in Hartwell's *Plants Used against Cancer*: A Survey (cited in Graham et al., 2000). Reprinted from *Journal of Ethnopharmacology*, Graham et al., 2000, with permission from Elsevier Science

Family/Species	*Part*	*Country*	*Uses*	*Reference*
Acanthaceae				
Acanthus ilicifolius L.	Root	China	Used for cancer.	Duke and Ayensu, 1985a,b
Acanthus ilicifolius L.	Stem	China	Used for cancer.	Jongsuwat, 1981
Acanthus ilicifolius L	Stem	Thailand	Used for cancer.	Duke and Ayensu, 1985a,b
Hygrophila auriculata (Schumach.) Heine	Part not specified	India	Used against cancer.	Jain, 1970
Ruellia tuberosa L.	Bark	India	Used for cancer in stomach.	Reddy *et al.*, 1991
Thunbergia laurifolia Lindl.	Entire plant	Thailand	Used for cancer.	Wasuwat, 1967
Aceraceae				
Acer pseudoplatanus L.	Bark	England	Used to treat tumors.	Culpeper, 1950
Acer pseudoplatanus L.	Fruit	England	Fruit dissolves tumors.	Culpeper, 1950
Actinidiaceae				
Actinidia arguta (Seib. and Zucc.) Planch. ex Miq.	Entire plant	Korea	Used for stomach cancer.	Han *et al.*, 1984
Actinidia chinensis Planch.	Entire plant	China	Used for cancer.	Duke and Ayensu, 1985a,b
Agavaceae				
Dracaena draco (L.) L.	Sap	Canary Islands	Used as an anticarcinogen.	Darias *et al.*, 1989
Amaranthaceae				
Achyranthes longifolia Makino	Entire plant	China	Used for nipple tumors.	Duke and Ayensu, 1985a,b
Achyranthes longifolia Makino	Entire plant	China	Used to treat tumors.	Duke and Ayensu, 1985a,b
Deeringia amaranthoides (Lam.) Merr.	Fruit	India	Used for cancer.	Sati *et al.*, 1990
Pfaffia paniculata (Mart.) Kuntze	Root	Brazil	Used for cancer and leukaemia.	Takemoto *et al.*, 1983
Amaryllidaceae				
Lycoris radiata (L'Her.) Herbert	Entire plant	China	Used against cancer.	Huang *et al.*, 1983
Anacardiaceae				
Anacardium giganteum Hancock ex Engl.	Bark	Guyana	Used for cancerous ulcers.	Wijisekera, 1978
Anacardium giganteum Hancock ex Engl.	Fruit	Guyana	Used for cancerous ulcers.	Wijisekera, 1978
Lannea stuhlmannii (Engl.) Engl.	Root	Tanzania	Used for cancer.	Chhabra *et al.*, 1991

continued

Table A.3 continued

Family/Species	*Part*	*Country*	*Uses*	*Reference*
Lannea stuhlmannii (Engl.) Engl.	Stembark	Tanzania	Used to treat cancer.	Chhabra *et al.*, 1987
Metopium brownei (Jacq.) Urb.	Bark	Mexico	Used for warts.	Flores and Ricalde, 1996
Rhus longipes Engl.	Root	Tanzania	Used for cancer.	Chhabra *et al.*, 1984
Rhus longipes Engl.	Root	Tanzania	Used for cancer.	Chhabra *et al.*, 1987
Rhus vernicifera DC.	Resin	Korea	Used for cancer.	Cha, 1977
Rhus vernicifera DC.	Resin	Korea	Used to treat tumors.	Cha, 1977
Schinus lentiscifolius Marchand	Leaf	Argentina	Used for cancer.	Graziano *et al.*, 1970
Annonaceae				
Annona cherimola Mill.	Seed	Colombia	Used to treat malignant tumors.	Garcia-Barriga, 1974
Annona senegalensis Pers.	Rootbark	Nigeria	Used for cancer treatment.	Adesogan and Durodola, 1976
Annona senegalensis Pers.	Rootbark	Nigeria	Used to treat cancer.	Durodola, 1975a
Annona senegalensis Pers.	Rootbark	Nigeria	Used to treat cancer.	Durodola, 1975b
Cyathostemma argentium (Blume) J. Sincl.	Root	Malaysia	Used to treat breast cancer.	Khamis *et al.*, 1997
Fissistigma oldhamii Merr.	Stem	Taiwan	Used as an antitumor agent.	Wu *et al.*, 1994
Fissistigma oldhamii Merr.	Stem	Taiwan	Used for antitumor purposes.	Wu *et al.*, 1993a,b
Apocynaceae				
Adenium obesum (Forssk.) Roem. and Schult.	Leaf	Kenya	Used for skin lumps.	Omino and Kokwaro, 1993
Alstonia scholaris (L.) R. Br.	Bark	Admiralty Islands	Used to treat lung cancer.	Holdsworth and Wamoi, 1982
Alstonia scholaris (L.) R. Br.	Bark	India	Used to treat tumors.	Wasuwat, 1967
Alstonia scholaris (L.) R. Br.	Entire plant	India	Used for cancer.	Gupta, 1979
Alstonia scholaris (L.) R. Br.	Part not specified	India	Used to treat tumors.	Anon., 1985
Alstonia scholaris (L.) R. Br.	Root	Thailand	Used for cancer.	Vashi and Patel, 1989
Aspidosperma polyneuron Mull. Arg.	Bark and leaf	Colombia	Used to reduce tumors.	Schultes, 1979
Cerbera manghas L.	Root	Samoa	Used as a treatment for cancer.	McCuddin, 1974
Echites yucatanensis (Millspex.) Stand.	Latex (leaf)	Mexico	Used for corns.	Flores and Ricalde, 1996
Echites yucatanensis (Millspex.) Stand.	Latex (leaf)	Mexico	Used for warts.	Flores and Ricalde, 1996
Himatanthus sucuuba (Spruce ex Mull. Arg.) Woodson	Latex (unspec.)	Brazil	Used as an antitumor agent.	Van den Berg, 1984
Himatanthus sucuuba (Spruce ex Mull. Arg.)	Stembark	Peru	Used to treat tumors.	Persinos-Perdue and Blomster, 1978
Rauwolfia vomitoria Afzel.	Root	Guinea	Used to treat tumors.	Vasileva, 1969
Rhazya stricta Decne.	Entire plant	Pakistan	Used against tumors.	Atta-Ur-Rahman and Fatima, 1982

Rhazya stricta Decne.	Entire plant	Pakistan	Used as an antitumor agent.	Ahmad *et al.*, 1971
Rhazya stricta Decne.	Leaf	India	Used to treat tumors.	Jewers *et al.*, 1980
Tabernaemontana amygdalaefolia Jacq.	Latex (unspec.)	Colombia	Used to treat tumors.	Schultes, 1979
Tabernaemontana amygdalaefolia Jacq.	Leaf	Colombia	Used to treat tumors.	Garcia-Barriga, 1975
Tabernaemontana elegans Stapf	Rootbark	Tanzania	Used for cancer.	Chhabra *et al.*, 1987
Thevetia ahouia (L.) A.DC.	Leaf and stem	Mexico	Used for warts.	Flores and Ricalde, 1996
Thevetia gaumeri Hemsley	Leaf and stem	Mexico	Used for warts.	Flores and Ricalde, 1996
Thevetia peruviana (Pers.) K. Schum.	Leaf	Mexico	Used to destroy tumors.	Mendez, 1937
Thevetia peruviana (Pers.) K. Schum.	Leaf and stem	Mexico	Used for warts.	Flores and Ricalde, 1996
Trachelospermum jasminoides (Lindl.) Lem.	Leaf	China	Used for carcinomatous growths.	Duke and Ayensu, 1985a,b
Araceae				
Acorus gramineus Sol. in Aiton	Entire plant	China	Used as a cancer folk remedy.	Duke and Ayensu, 1985a,b
Arisaema consanguineum Schott	Entire plant	China	Used for cancer.	Duke and Ayensu, 1985a,b
Arisaema consanguineum Schott	Root	China	Used for cancer.	Duke and Ayensu, 1985a,b
Arisaema erubescens (Wall.) Schott	Root	China	Used to treat cancers.	Ducki *et al.*, 1995
Arisaema lobatum Engl.	Corm	China	Used on malignant sores.	Duke and Ayensu, 1985a,b
Epipremnum pinnatum (L.) Engl.	Leaf	Singapore	Used to treat cancer.	
Rhaphidophora korthalsii Schott	Leaf	Singapore	Used as a remedy for cancer.	Wong and Tan, 1996
Aristolochiaceae				
Aristolochia mollissima Hance	Entire plant	China	Used for cancer.	Duke and Ayensu, 1985a,b
Aristolochia tagala Cham.	Leaf	Philippines	Used to treat cancer.	Masilungan *et al.*, 1971
Asclepiadaceae				
Asclepias speciosa Torrey	Latex (unspec.)	USA–NV	Used to remove corns.	Train *et al.*, 1957
Cynanchum hancockianum (Maxim.) Iljinski	Entire plant	Mongolia	Used to treat cancer.	Konda *et al.*, 1990
Cynanchum hancockianum (Maxim.) Iljinski	Root	China	Used for cancer.	Lou *et al.*, 1991
Cynanchum taiwanianum Yamazaki	Root	Taiwan	Used to treat tumors.	Lin *et al.*, 1997a,b
Heterostemma brownii Hay	Aerial parts	Taiwan	Used to treat tumors.	Lin *et al.*, 1997a,b
Janakia arayalpathra J. Joseph and V. Chandrasekaran	Root	India	Used as a remedy for tumors.	Subramonian *et al.*, 1996
Leptadenia hastata (Pers.) Decne.	Bark	Senegal	Used as an antitumor drug.	Aquino *et al.*, 1995
Marsdenia tenacissima (Roxb.) Wight and Arn.	Entire plant	China	Used as a folkloric antitumor drug.	Yang *et al.*, 1981
Marsdenia tenacissima (Roxb.) Wight and Arn	Entire plant	China	Used as an antitumor folkdrug.	Miyakawa *et al.*, 1986
Marsdenia tenacissima (Roxb.) Wight and Arn.	Stem	China	Used for cancer.	Luo *et al.*, 1993a,b

continued

Table A.3 continued

Family/Species	*Part*	*Country*	*Uses*	*Reference*
Marsdenia tenacissima (Roxb.) Wight and Arn.	Stem	China	Used for treatment of cancer.	Luo *et al.*, 1993a,b
Marsdenia tenacissima (Roxb.) Wight and Arn.	Stem	China	Used for treatment of cancer.	Zhou *et al.*, 1980
Marsdenia tenacissima (Roxb.) Wight and Arn.	Stem	China	Used to treat tumors.	Duke and Ayensu, 1985a,b
Raphionacme hirsuta (E. Mey.) R.A. Dyer	Bulb	South Africa	Used as an anticancer remedy.	Charlson, 1980
Raphionacme hirsuta (E. Mey.) R.A. Dyer	Part not specified	South Africa	Used to treat cancer.	Charlson, 1979
Raphionacme hirsuta (E. Mey.) R.A. Dyer	Tuber	South Africa	Used for the treatment of tumors.	Watt and Breyer-Brandwijk, 1962
Sarcostemma australe R.Br.	Latex (unspec.)	Australia	Used to treat cancer.	Reid and Betts, 1979
Tylophora floribunda Miq.	Entire plant	China	Used as an anticancer agent.	Duke and Ayensu, 1985a,b
Balanaphoraceae				
Sarcophyte piriei Hutch.	Entire plant	Tanzania	Used for cancer.	Chhabra *et al.*, 1987
Begoniaceae				
Begonia crassirostris Irmscher	Entire plant	China	Used for cancer.	Duke and Ayensu, 1985a,b
Begonia glabra Aubl.	Entire plant	Panama	Used as an anticancer agent.	Gupta *et al.*, 1996
Berberidaceae				
Berberis amurensis Maxim.	Entire plant	China	Used as an anticancer agent.	Duke and Ayensu, 1985a,b
Dysosma aurantiocaulis (Hand. Mazz.) Hu	Root	China	Used to treat tumors.	Duke and Ayensu, 1985a,b
Dysosma pleiantha (Hance) Woodson	Root	Taiwan	Used to treat condylomata.	Kao *et al.*, 1992
Dysosma pleiantha (Hance) Woodson	Root	Taiwan	Used to treat tumors.	Kao *et al.*, 1992
Epimedium hunanense (Hand. Mazz.) Hand. Mazz.	Aerial parts	China	Used for leukopenia.	Liang *et al.*, 1997
Mahonia aquifolium (Pursh.) Nutt.	Root	USA	Used for cancer.	Nebelkopf, 1981
Mahonia bealei (Fortune) Carr.	Leaf	China	Used for cancer.	Duke and Ayensu, 1985a,b
Mahonia fortunei (Lindl.) Fedde	Leaf	China	Used for cancer.	Duke and Ayensu, 1985a,b
Mahonia japonica (Thunb.) DC.	Leaf	China	Used for cancer.	Duke and Ayensu, 1985a,b
Nandina domestica Thunb.	Fruit	Japan	Used for pharyngeal tumors.	Shoji *et al.*, 1984
Betulaceae				
Alnus japonica (Thunb.) Steud.	Wood	South Korea	Used to treat cancer.	Lee *et al.*, 1992
Alnus japonica (Thunb.) Steud.	Wood	South Korea	Used to treat cancer.	Rhan *et al.*, 1992
Betula platyphylla Sukaczev	Bark	China	Used for mammary carcinoma.	Duke and Ayensu, 1985a,b
Betula platyphylla Sukaczev	Rootbark	China	Used for cancer.	Duke and Ayensu, 1985a,b

Bignoniaceae				
Kigelia africana (Lam.) Benth.	Rootbark	Malawi	Used to treat cancer of the uterus.	Msonthi and Magombo, 1983
Tabebuia rosea (Bertol.) DC.	Bark	Mexico	Used for uterine cancer.	Gentry, 1980
Tabebuia serratifolia (Vahl.) Nicholson	Bark	Bolivia	Used for cancer.	Gentry, 1980
Zeyheria tuberculosa (Vell.) Bureau	Trunkwood	Brazil – Minas Gerais	Used for cancer.	De *et al.*, 1976
Bombacaceae				
Bombax brevicuspe Sprague	Root	Guinea	Used as an antitumor agent.	Vasileva, 1969
Boraginaceae				
Echium arenarium Guss.	Leaf	Sudan	Used to treat tumors and abscesses.	Yousif *et al.*, 1983
Buddlejaceae				
Buddleja globosa Hope	Leaf	Bolivia	Used to treat warts.	Houghton, 1984
Buddleja globosa Hope	Leaf	Chile	Used to treat warts.	Houghton, 1984
Buddleja incana R. and P.	Flower and leaf	Chile	Used to treat warts.	Houghton, 1984
Buddleja officinalis Maxim.	Flowers	China	Used for cancer.	Houghton, 1984
Campanulaceae				
Codonopsis lanceolata (Siebold and Zucc.) Trautv.	Root	China	Used for cancer.	Duke and Ayensu, 1985a,b
Codonopsis nervosa (Chipp.) Nannf.	Root	China	Used for cancer of the cervix.	Duke and Ayensu, 1985a,b
Codonopsis pilosula (Franch.) Nannf.	Root	China	Used for cancer.	Duke and Ayensu, 1985a,b
Laurentia longiflora (L.) Petermann	Entire plant	Indonesia	Used as a cancer cure.	Hsu, 1967
Pratia nummularia A. Br. and Ascher	Entire plant	Taiwan	Used against tumors.	Ho *et al.*, 1995
Capparidaceae				
Cadaba glandulosa Forsk.	Leaf	Sudan	Used to treat tumors and abscesses.	Yousif *et al.*, 1983
Cadaba rotundifolia Forsk.	Leaf	Sudan	Used to treat tumors and abscesses.	Yousif *et al.*, 1983
Cadaba rotundifolia Forsk.	Root	Sudan	Used for tumor cure.	Yousif *et al.*, 1984
Capparis tomentosa Lam.	Entire plant	Mozambique	Used for treatment of cancer.	Amico, 1977
Caprifoliaceae				
Lonicera etrusca G. Santi	Seed	Madeira	Used to treat warts.	Rivera and Obon, 1995
Sambucus racemosa L.	Bark	USA – WA	Used to treat tumors.	Forlines *et al.*, 1992

continued

Table A.3 continued

Family/Species	*Part*	*Country*	*Uses*	*Reference*
Caryophyllaceae				
Dianthus zonatus Fenzl	Flowers	Turkey	Used to treat warts on hands.	Ishikura, 1982
Drymaria gracilis Cham. and Schltdl.	Leaf and stem	Mexico	Used to treat tumors.	Woo and Woo, 1989
Melandrium firmum (Siebold and Zucc.) Rohrb.	Entire plant	South Korea	Used for breast cancer.	Duke and Ayensu, 1985a,b
Celastraceae				
Celastrus orbiculatus Thunb.	Entire plant	China	Used as an anticancer agent.	Gupta *et al.*, 1996
Maytenus blepharodes (Pitt.) A. Gentry	Branches	Panama	Used as an antitumor agent.	Darias *et al.*, 1989
Maytenus canariensis (Loes.) Kunkel	Fruitjuice	Canary Islands	Used to remove papillomas.	Shirota *et al.*, 1995
Maytenus chuchuhuasca R. Hamet and Col.	Bark	Brazil	Used for skin cancer.	Itokawa *et al.*, 1992
Maytenus ebenifolia Reiss.	Stembark	Peru	Used as an antitumor agent.	Anon., 1985
Maytenus emarginata (Willd.) Ding Hou	Entire plant	India	Used for cancer.	Shirota *et al.*, 1996
Maytenus krukovii A.C. Smith	Stembark	Brazil	Used to treat skin cancer.	Chavez *et al.*, 1998
Maytenus krukovii A.C. Smith	Part not specified	Brazil	Used to treat skin cancer.	Honda *et al.*, 1997
Maytenus macrocarpa (R. and P.) Briquet	Stembark	Peru	Used for skin cancer.	Kokwaro, 1976
Maytenus obscura (A. Rich.) Cufod.	Leaf	East Africa	Used against cancer.	Chhabra *et al.*, 1991
Maytenus senegalensis (Lam.) Excell	Bark	Ethiopia	Used to treat cancer.	Wilson and Mariam, 1979
Maytenus senegalensis (Lam.) Excell	Rootbark	Tanzania	Used to treat cancer.	Ulubelen and Cole, 1965
Maytenus trichotoma Turcz.	Entire plant	Mexico	Used as an antitumor agent.	Shen and Zhou, 1992
Tripterygium regelii Sprague and Take.	Root	China	Used for cancer.	Takaishi *et al.*, 1991
Tripterygium wilfordii Hook.f.	Fruit	Japan	Used for cancer.	Anon., 1976
Cephalotaxaceae				
Cephalotaxus fortunei Hook.f.	Part not specified	China	Used for tumor treatment.	Tai and Hung, 1981
Chloranthaceae				
Chloranthus glaber Makino	Aerial parts	China	Used for cancer.	Duke and Ayensu, 1985a,b
Sarcandra glabra (Thunb.) Nakai	Entire plant	China	Used to treat cancers.	Jain, 1970
Sarcandra glabra (Thunb.) Nakai	Aerial parts	Hong Kong	Used to treat cancer.	Takeda *et al.*, 1993
Sarcandra glabra (Thunb.) Nakai	Entire plant	China	Used as an antitumor agent.	Tsui and Brown, 1996
Combretaceae				
Anogeissus latifolia (Roxb. ex DC.) Wall. ex Bedd.	Part not specified	India	Used for cancer on the face.	Pettit *et al.*, 1982
Combretum caffrum (Eckl. and Zeyh.) Kuntze	Rootbark	South Africa	Used to treat cancer.	Abela and Santos, 1974

Commelinaceae				
Cyanotis vaga (Lour.) Roem. and Schult.	Entire plant	Philippines	Used for control of breast cancer.	Garcia-Barriga, 1975
Compositae				
Acanthospermum australe (Loefl.) Kuntze	Entire plant	Colombia	Used to treat cancer.	Garcia-Barriga, 1975
Achyrocline lehmanii Heiron.	Entire plant	Colombia	Used to treat cancer.	Garcia-Barriga, 1975
Achyrocline saturoides (Lam.) DC.	Entire plant	Colombia	Used to treat cancer.	Morton, 1975
Ageratum houstonianum Mill.	Leaf	Venezuela	Used as a poultice for tumors.	Salie *et al.*, 1996
Arctotis auriculata Jacq.	Leaf	South Africa	Used for uterine cancer.	Lao *et al.*, 1984
Artemisia argyi H. Lév. and Vaniot	Entire plant	China	Used as an antitumor agent.	Duke and Ayensu, 1985a,b
Aster amellus L.	Entire plant	China	Used for inguinal tumors.	Hussain *et al.*, 1993
Aster amellus L.	Flowers	India	Used to treat tumors.	Hussain *et al.*, 1993
Aster amellus L.	Root	India	Used in inguinal tumors.	Cha, 1977
Aster tataricus L.f.	Entire plant	China	Used as a cancer folk remedy.	Cha, 1977
Aster tataricus L.f.	Root	Korea	Used to treat tumors.	Duke and Ayensu, 1985a,b
Aster tataricus L.f.	Root	Korea	Used to treat tumors.	Han *et al.*, 1984
Cirsium maackii Maxim	Entire plant	Korea	Used for mammary cancer.	Duke and Ayensu, 1985a,b
Crassocephalum bojeri (DC.) Robyns	Entire plant	Tanzania	Used as a cancer remedy.	Lin and Lin, 1993
Echinops grijisii Hance	Root	Taiwan	Used for bone tumors.	Lin and Lin, 1993
Echinops grijisii Hance	Root	Taiwan	Used for hepatoma.	Lin *et al.*, 1992
Echinops grijisii Hance	Root	Taiwan	Used to treat cancers.	Duke and Ayensu, 1985a,b
Echinops latifolius Tausch.	Root	Taiwan	Used to treat cancers.	Hsu, 1967
Echinops latifolius Tausch.	Root	China	Used to treat tumors.	Lin *et al.*, 1992
Eclipta alba (L.) Hassk.	Leaf	Indonesia	Used as an anticancer agent.	Hsu, 1967
Eclipta alba (L.) Hassk.	Leaf	Indonesia	Used to treat cancers.	Lee *et al.*, 1972
Eupatorium formosanum Hayata	Leaf and stem	Taiwan	Used for leukemia.	Garcia-Barriga, 1975
Gnaphalium elegans Kunth.	Entire plant	Colombia	Used to treat cancer.	Torrenegra *et al.*, 1980
Gnaphalium elegans Kunth.	Flower and leaf	Colombia	Used to treat cancer	
Gnaphalium elegans Kunth.	Flowers	Colombia	Used for cancer.	Duke and Ayensu, 1985a,b
Inula britannica L.	Flowers	China	Used for cancer of the esophagus.	Duke and Ayensu, 1985a,b
Inula britannica L.	Flowers	China	Used for cancer.	Chien *et al.*, 1983
Inula linariaefolia Turcz.	Flowers	China	Used for malignant tumors.	Zani *et al.*, 1995
Jungia floribunda Less.	Aerial parts	Brazil	Used for warts.	Duke and Ayensu, 1985a,b
Laggera pterodonta (DC.) Benth.	Entire plant	China	Used as an anticancer agent.	Ma *et al.*, 1997
Ligularia intermedia Nakai	Root	China	Used as an antitumor agent.	Sarg, 1975
Pulicaria crispa (Forsk.) Sch.-Bip.	Aerial parts	Saudi Arabia	Used for vaginal tumors.	Duke and Ayensu, 1985a,b

continued

Table A.3 continued

Family/Species	*Part*	*Country*	*Uses*	*Reference*
Saussurea lappa (Decne.) Sch.-Bip.	Root	India	Used for treatment of cancer.	Saxena and Dixit, 1993
Senecio integrifolius (L.) Clairv.	Entire plant	China	Used as an anticancer agent.	Duke and Ayensu, 1985a,b
Senecio oryzetorum Diels	Entire plant	China	Used as an anticancer agent.	Duke and Ayensu, 1985a,b
Spilanthes acmella (L.) Murray	Entire plant	Indonesia	Used as an anticancer agent.	Hsu, 1967
Spilanthes acmella (L.) Murray	Entire plant	Indonesia	Used as an anticancer agent.	Hsu, 1967
Spilanthes acmella (L.) Murray	Entire plant	Indonesia	Used as an anticancer agent.	Hsu, 1967
Wedelia chinensis (Osbeck) Merr.	Entire plant	China	Used as an anticancer agent.	Duke and Ayensu, 1985a,b
Xanthium sibiricum Patrin ex Widder	Fruit	China	Used for cancer.	Duke and Ayensu, 1985a,b
Xanthium sibiricum Patrin ex Widder	Entire plant	China	Used as an anticancer agent.	Duke and Ayensu, 1985a,b
Convolvulaceae				
Calystegia sepium (L.) R.Br.	Entire plant	Italy	Used to treat corns.	De Feo *et al.*, 1992
Cuscuta chilensis Ker Gawl.	Entire plant	Chile	Used to treat tumors.	San Martin, 1983
Cuscuta chinensis Lam.	Part not specified	India	Used for cancer.	Jain, 1970
Cuscuta chinensis Lam.	Entire plant	India	Used for malignancy.	Nisa *et al.*, 1985
Crassulaceae				
Aeonium arboreum (L.) Webb and Berth.	Leaf	Madeira	Used as a corn salve.	Rivera and Obon, 1995
Aeonium glandulosum (Ait.) Webb and Berth.	Leaf	Madeira	Used as a corn salve.	Han *et al.*, 1984
Sedum alboroseum Baker	Entire plant	Korea	Used for lymphoma.	Fujita *et al.*, 1995
Sempervivum armenum Boiss. and Huet	Leaf	Turkey	Used for warts.	Novaretti and Lemordant, 1990
Sempervivum arvense Lec and Lamot.	Leaf	France	Used to treat corns.	Novaretti and Lemordant, 1990
Sempervivum montanum L.	Leaf	France	Used to treat corns.	Garcia-Barriga, 1974
Cruciferae				
Draba litamo L.	Entire plant	Colombia	Used against cancer.	Duke and Ayensu, 1985a,b
Draba nemorosa L.	Entire plant	China	Used for leukemia.	Yu *et al.*, 1994a,b
Cucurbitaceae				
Bolbostemma paniculatum (Max.) Franquet	Bulb	China	Used for treatment of tumors.	Xu *et al.*, 1986
Bolbostemma paniculatum (Max.) Franquet	Bulb	China	Used to treat tumors and warts.	Kong *et al.*, 1986
Bolbostemma paniculatum (Max.) Franquet	Tuber	China	Used for treatment of carcinoma.	Abebe, 1986
Cucumis prophetarum L.	Root	Ethiopia	Used for skin cancer.	Pan, 1960
Trichosanthes japonica Regel	Fruit	China	Used for vaginal cancer.	Duke and Ayensu, 1985a,b

Trichosanthes japonica Regel	Fruit	China	Used for vaginal cancer.	Pan, 1960
Trichosanthes uniflora Hao	Entire plant	China	Used for carcinomatous sores.	Chiang *et al.*, 1994
Cupressaceae				
Juniperus recurva Buch.-Ham. ex D. Don	Part not specified	Nepal	Used to prevent and cure cancer.	Lechner-Knecht, 1982
Juniperus squamata Buch.-Ham. ex Lamb	Part not specified	Nepal	Used to prevent and cure cancer.	Lechner-Knecht, 1982
Cyatheaceae				
Cyathea fauriei (Christ.) Copel	Shoots	Taiwan	Used to treat tumors.	Chiang *et al.*, 1994
Cycadaceae				
Cycas revoluta Thunb.	Leaf	China	Used for cancer.	Hsu, 1967
Cycas revoluta Thunb.	Leaf	China	Used for liver cancer.	Duke and Ayensu, 1985a,b
Cycas rumphii Miq.	Resin	India	Used for malignant ulcers.	Holdsworth *et al.*, 1983
Cyperaceae				
Bolboschoenus maritimus (L.) Palla	Tuber	Korea	Used for abdominal tumors.	Cha, 1977
Bolboschoenus maritimus (L.) Palla	Tuber	Korea	Used for vaginal indurations.	Cha, 1977
Bolboschoenus maritimus (L.) Palla	Tuber	Korea	Used to treat tumors.	Cha, 1977
Kyllinga brevifolia Rott	Part not specified	India	Used against tumors.	Jain, 1970
Scirpus holoschoenus L.	Inflorescence	Spain	Used to treat warts.	Gonzalez-Tejero *et al.*, 1995
Davalliaceae				
Davallia mariesii T. Moore ex Bak.	Protoplasts	South Korea	Used for stomach cancer.	Cui *et al.*, 1990
Dichapetalaceae				
Dichapetalum toxicarium (G. Don) Baill.	Bark	Guinea	Used to treat tumors.	Vasileva, 1969
Dilleniaceae				
Dillenia suffruticosa (Griff.) Martelli	Fruit	Malaysia	Used to treat cancerous growths.	Ahmad and Holdsworth, 1995
Dioscoreaceae				
Dioscorea collettii Hook.f.	Rhizome	China	Used for carcinoma of the bladder.	Hu *et al.*, 1996
Dioscorea collettii Hook.f.	Rhizome	China	Used for carcinoma of the bladder.	Hu *et al.*, 1996
Dioscorea collettii Hook.f.	Rhizome	China	Used for renal tumors.	Hu *et al.*, 1996
Dioscorea collettii Hook.f.	Rhizome	China	Used to treat cervical carcinoma.	Hu *et al.*, 1996
Dioscorea esculenta (Lour.) Burkill	Tuber	Indonesia	Used for swellings and tumors.	Hirschhorn, 1983
Dipsacaceae				
Dipsacus asper Wall.	Root	China	Used for cancer of the breast.	Duke and Ayensu, 1985a,b

continued

Table A.3 continued

Family/Species	*Part*	*Country*	*Uses*	*Reference*
Droseraceae				
Drosera anglica Huds.	Leaf	England	Juice destroys warts and corns.	Culpeper, 1950
Drosera anglica Huds.	Leaf	England	Used to erode the skin.	Culpeper, 1950
Ebenaceae				
Diospyros tomentosa Poir.	Part not specified	India	Used to treat tumors.	Jain, 1970
Euclea crispa (Thunb.) Gurke	Rootbark	Zimbabwe	Used to treat melanomas.	Sibanda *et al.*, 1992
Elaeagnaceae				
Hippophae salicifolia D. Don	Fruit	India	Used as an anticancer drug.	Uniyal, 1990
Elaeocarpaceae				
Aristotelia chilensis (Molina) Stuntz	Leaf and stem	Chile	Used to treat tumors as a plaster.	He *et al.*, 1997
Ericaceae				
Vaccinium bracteatum C.P. Thunb.	Entire plant	China	Used as an anticancer agent.	Duke and Ayensu, 1985a,b
Eriocaulaceae				
Eriocaulon sieboldianum Siebold and Zucc. ex Steud.	Stembark	China	Used for cancer.	Duke and Ayensu, 1985a,b
Euphorbiaceae				
Acalypha californica Benth.	Leaf	Mexico	Used for cancer.	Ishikura, 1982
Cnidoscolus chayamansa McVaugh	Thorns	Mexico	Used for warts.	Flores and Ricalde, 1996
Croton draco Schltdl.	Aerial parts	Panama	Used as an antitumor agent.	Gupta *et al.*, 1996
Croton draconoides Mull. Arg.	Latex (unspec.)	Peru	Used for cancer.	Piacente *et al.*, 1998
Croton erythrochilus Mull. Arg.	Latex (unspec.)	Peru	Used for cancer.	Piacente *et al.*, 1998
Croton flavens L.	Leaf	Mexico	Used for callouses.	Flores and Ricalde, 1996
Croton flavens L.	Leaf	Mexico	Used for warts.	Flores and Ricalde, 1996
Croton lechleri Mull. Arg.	Latex (unspec.)	Peru	Used for cancer.	Cai *et al.*, 1993
Croton lechleri Mull. Arg.	Sap	Brazil	Used against cancer.	Pieters *et al.*, 1992
Croton lechleri Mull. Arg.	Sap	Ecuador	Used for treatment of cancer.	Cai *et al.*, 1993
Croton urucurana Baill.	Bark	Brazil	Used to treat some types of cancer.	Peres *et al.*, 1997
Croton urucurana Baill.	Stembark	Brazil	Used to treat cancer.	Peres *et al.*, 1997
Euphorbia australis Boiss.	Latex (unspec.)	Australia	Used as an embrocation for skin sores.	Reid and Betts, 1979

Euphorbia caducifolia Haines	Latex (unspec.)	Pakistan	Used as an antitumor agent.	Ahmad *et al.*, 1977
Euphorbia caducifolia Haines	Root	Pakistan	Said to have antitumor properties.	Ahmad *et al.*, 1977
Euphorbia ebracteolata Hayata	Aerial parts	South Korea	Used in treatment of lymphadenoma.	Ahn *et al.*, 1996
Euphorbia heterophylla L.	Stem	Mexico	Used for warts.	Flores and Ricalde, 1996
Euphorbia kansui Liou ex S.B. Ho	Root	China	Used as an antitumor drug.	Cha, 1977
Euphorbia kansui Liou ex S.B. Ho	Root	Korea	Used to treat abdominal tumors.	Cha, 1977
Euphorbia kansui Liou ex S.B. Ho	Root	Korea	Used to treat tumors.	Wu *et al.*, 1991
Euphorbia kansui Liou ex S.B. Ho	Root	Taiwan	Used to treat cancer.	Ding and Jia, 1992
Euphorbia marginata Pursh.	Entire plant	Italy	Used to treat warts and verrucas.	De Feo *et al.*, 1992
Euphorbia micractina Boiss.	Entire plant	China	Used as an antitumor agent.	Shi *et al.*, 1994
Euphorbia micractina Boiss.	Entire plant	China	Used as an antitumor agent.	Shi *et al.*, 1995
Euphorbia parvifolia E. Mey.	Part not specified	Colombia	Used locally for cancer.	Hirschhorn, 1982
Euphorbia pekinensis Rupr.	Root	Korea	Used to treat tumors.	Cha, 1977
Euphorbia poissonii Pax	Latex (unspec.)	Nigeria (Northern)	Used for skin papillomas.	Evans and Kinghorn, 1975
Euphorbia prolifera Buch. Ham	Latex (unspec.)	China	Used for treatment of tumors.	Wu *et al.*, 1995
Euphorbia prolifera Buch. Ham	Root	China	Used to treat tumors.	Dagang *et al.*, 1994
Euphorbia serrata L.	Latex (unspec.)	Spain	Used for warts.	Martinez-Lirola *et al.*, 1996
Glochidion concolor Mull. Arg.	Leaf	Tonga	Used to treat breast carcinoma.	Singh *et al.*, 1984
Hura polyandra Baill.	Leaf	Mexico	Used for warts.	Flores and Ricalde, 1996
Jatropha dioica Sesse	Root	Mexico	Used to treat cancer.	
Jatropha elliptica Mull. Arg.	Tuber	Brazil	Used for neoplasms.	Calixto and Sant'ana, 1987
Sapium macrocarpum Muell.	Stem	Mexico	Used for warts.	Flores and Ricalde, 1996
Suregada multiflora (A. Juss.) Baill.	Wood	Thailand	Used for cancer.	Wasuwat, 1967
Tragia involucrata L.	Part not specified	India	Used for cancer.	Jain, 1970
Flacourtiaceae				
Casearia sylvestris Sw.	Leaf and stem	Colombia	Used as an antitumor agent.	Garcia-Barriga, 1975
Hydnocarpus anthelmintica Pierre ex Laness.	Seed	China	Used for cancer.	Duke and Ayensu, 1985a,b
Hydnocarpus anthelmintica Pierre ex Laness.	Seed	Thailand	Used for cancer.	Wasuwat, 1967
Fouquieriaceae				
Fouquieria fasciculata Nash	Stem	USA – NM	To help remove benign tumors.	Moore, 1979
Ganodermataceae				
Ganoderma applanata (Pers.) Patouillard	Fruitbody	South Korea	Used to treat cancers.	Kim *et al.*, 1994
Ganoderma lucidum (Leyss. Fr.) Karst.	Fruitbody	Japan	Used for cancer.	Huang *et al.*, 1991
Ganoderma lucidum (Leyss. Fr.) Karst.	Fruitbody	Taiwan	Used as an antitumor remedy.	Furusawa *et al.*, 1992

continued

Table A.3 continued

Family/Species	*Part*	*Country*	*Uses*	*Reference*
Gentianaceae				
Gentiana loureiroi (D. Don) Griseb.	Entire plant	China	Used to treat tumors.	Duke and Ayensu, 1985a,b
Gentiana rhodantha Franch.	Entire plant	China	Used for cancer.	Duke and Ayensu, 1985a,b
Gentiana scabra Bunge	Root	China	Used for cancer.	Duke and Ayensu, 1985a,b
Geraniaceae				
Pelargonium graveolens L'Her	Essential oil	China	Used for cervical cancer.	Duke and Ayensu, 1985a,b
Globulariaceae				
Globularia alypum L.	Leaf	Tunisia	Used to treat cancer.	Boukef *et al.*, 1982
Gramineae				
Sasa palmata (hort. ex Burb.) E.G. Camus	Leaf	Japan	Used as an anticancer agent.	Namba and Bae, 1982
Sorghum bicolor (L.) Moench.	Entire plant	Korea	Used for cancer.	Han *et al.*, 1984
Guttiferae				
Hypericum japonicum Thunb.	Entire plant	China	Used for treatment of tumors.	Ishiguro *et al.*, 1994a,b
Hypericum japonicum Thunb.	Entire plant	China	Used for treatment of tumors.	Ishiguro *et al.*, 1984
Hypericum japonicum Thunb.	Entire plant	China	Used to treat tumors.	Ishiguro *et al.*, 1990
Hypericum japonicum Thunb.	Entire plant	China	Used to treat tumors.	Ishiguro *et al.*, 1991
Hypericum japonicum Thunb.	Entire plant	China	Used to treat tumors.	Ishiguro *et al.*, 1993
Hypericum japonicum Thunb.	Entire plant	China	Used to treat tumors.	Ishiguro *et al.*, 1994a,b
Hypericum japonicum Thunb.	Entire plant	Japan	Used to treat tumors.	Ishiguro *et al.*, 1987
Gyrostemonaceae				
Codonocarpus cotinifolius (Desf.) F. Muell.	Leaf	Australia	Infusion taken as a cancer cure.	Reid and Betts, 1979
Codonocarpus cotinifolius (Desf.) F. Muell.	Rootbark	Australia	Infusion used as a cancer cure.	Reid and Betts, 1979
Hernandiaceae				
Gyrocarpus americanus Jacq.	Bark	Tonga	Used for breast cancer.	Singh *et al.*, 1984
Hymenochaetaceae				
Inonotus obliquus (Pers.: Fr.) Pilát	Fruitbody	Area not stated	Used for cancer.	Konopa *et al.*, 1961
Inonotus obliquus (Pers.: Fr.) Pilát	Fruitbody	France	Used to treat cancer.	Reinach-hirtzbach and Ourisson, 1971

Hypoxidaceae				
Hypoxis nyasica Baker	Rhizome	Malawi	Used for intestinal cancer.	Vinesi *et al.*, 1990
Hypoxis nyasica Baker	Tuber	Malawi	Used for uterine cancer.	Kamwendo *et al.*, 1985
Hypoxis rooperii S. Moore	Tuber	Swaziland	Used to treat uterine cancer.	Amusan *et al.*, 1995
Icacinaceae				
Icacina mannii Oliver	Root	Zaire	Used to treat fibrous tumors.	On'Okoko *et al.*, 1985
Iridaceae				
Crocosmia crocosmiiflora (Lem. ex Morren) N.E. Br.	Corm	Japan	Used as an antitumor agent.	Asada *et al.*, 1988
Iris pallasii Fisch. ex Trev.	Seed	China	Used in folk remedies for cancer.	Li *et al.*, 1981
Juglandaceae				
Juglans mandshurica Maxim.	Cotyledons	China	Used for cancer.	Duke and Ayensu, 1985a,b
Juglans mandshurica Maxim.	Root	South Korea	Used for treatment of cancer.	Son, 1995
Juglans mandshurica Maxim.	Root	South Korea	Used to treat cancer.	Joe *et al.*, 1996
Juncaceae				
Juncus acutus L.	Leaf	Spain	Used for warts.	Martinez-Lirola *et al.*, 1996
Krameriaceae				
Krameria cytisoides Cav.	Root	Mexico	Used for stomach cancer.	Achenbach *et al.*, 1987
Labiatae				
Ajuga genevensis L.	Twig	China	Used for vaginal cancer.	Pan, 1960
Ajuga genevensis L.	Twig	China	Used to treat cancer.	Pan, 1960
Glechoma hederacea L.	Fruitjuice (unripe)	England	Used to treat cancer.	Culpeper, 1950
Isodon wikstroemioides (Hand. Mazz.) H. Hara	Leaf	China	Used to treat tumors.	Wu *et al.*, 1993a,b
Leonurus artemisia (Lour.) S.Y. Hu	Shoots	China	Used for whitlow.	Hu, 1976
Leonurus artemisia (Lour.) S.Y. Hu	Shoots	China	Used to treat cancer.	Hu, 1976
Leonurus heterophyllus Sweet	Entire plant	China	Used for breast tumors.	Duke and Ayensu, 1985a,b
Leonurus heterophyllus Sweet	Stem	China	Used for cancer.	Duke and Ayensu, 1985a,b
Lepechinia spicata Willd.	Part not specified	Mexico	Used for uterine tumors.	Ishikura, 1982
Leucas flaccida R. Br.	Leaf	Rarotonga	Used for abdominal tumor.	Holdsworth, 1991
Rabdosia rubescens (Hemsl.) Hara	Aerial parts	China	Used for the treatment of tumors.	Sun *et al.*, 1982
Rabdosia rubescens (Hemsl.) Hara	Leaf	China	Used for the treatment of tumors.	Li *et al.*, 1987
Rabdosia rubescens (Hemsl.) Hara	Leaf	China	Used for the treatment of tumors.	Sun *et al.*, 1982, 1992
Salvia chinensis Benth.	Entire plant	China	Used for cancer.	Qian and Li, 1992

continued

Table A.3 continued

Family/Species	*Part*	*Country*	*Uses*	*Reference*
Salvia hiptoides Mart. and Gal.	Entire plant	El Salvador	Used as a cataplasm for tumors.	Hirschhorn, 1982
Salvia plebeia R. Br.	Aerial parts	South Korea	Used to treat tumors.	Um *et al.*, 1996
Scutellaria barbata D. Don	Aerial parts	China	Used for cancers.	Wong *et al.*, 1993
Scutellaria barbata D. Don	Entire plant	China	Used for cancers.	Ducki *et al.*, 1996
Scutellaria barbata D. Don	Entire plant	China	Used to treat tumors.	Wong *et al.*, 1992a,b
Scutellaria barbata D. Don	Part not specified	China	Used for liver, lung and rectal cancers.	Wong *et al.*, 1992a,b
Scutellaria barbata D. Don	Root	China	Used for liver, lung and rectal cancers.	Wang *et al.*, 1996
Scutellaria indica L.	Root	South Korea	Used for cancer.	Bae *et al.*, 1994
Scutellaria rivularis Wall. ex Benth.	Aerial parts	Taiwan	Used to treat tumors.	Lin, 1987
Scutellaria rivularis Wall. ex Benth.	Entire plant	China	Used to treat tumors.	
Scutellaria rivularis Wall. ex Benth.	Root	China	Used to treat tumors.	
Zhumeria majdae Rech. f. and Wendelbo	Entire plant	Iran	Applied on topical cancer lesions.	Zargari, 1992
Lauraceae				
Ocotea caparrapi (Nates) Dugand	Essential oil	Colombia	Used to treat cancerous tumors.	Palomino *et al.*, 1996
Ocotea caparrapi (Nates) Dugand	Sap	Colombia	Used to treat tumors.	Garcia-Barriga, 1974
Ocotea foetens Benth. and Hook.	Branchlets	Madeira	Used for malignant diseases.	Rivera and Obon, 1995
Ocotea usambarensis Engl.	Bark	East Africa	Used against cancer.	Kokwaro, 1976
Leeaceae				
Leea guineense G. Don	Leaf	Guinea	Used against cancer.	Vasileva, 1969
Leguminosae				
Acacia xanthophloea Benth.	Fruit	Tanzania	Used to treat cancer.	Chhabra *et al.*, 1991
Albizia julibrissin Durazz.	Stembark	South Korea	Used for lung cancer.	Woo, 1985
Albizia schimperiana Oliver	Stembark	Tanzania	Used to treat warts.	Chhabra *et al.*, 1984
Astragalus membranaceus (Fisch. ex Link.) Bunge	Root	China	Used as an adjunct to cancer therapy.	Wong *et al.*, 1992a,b
Caesalpinia coriaria (Jacq.) Willd.	Fruit	Colombia	Used to treat malignant ulcers.	Garcia-Barriga, 1974
Caesalpinia coriaria (Jacq.) Willd.	Pod	Domin. Repub.	Acclaimed as a cure for cancer.	Morton, 1975
Caesalpinia volkensii Harms	Part not specified	Kenya	Used for retinoblastoma.	
Caragana cuneata Moench	Leaf	India	Used to treat gastic cancer.	Singh *et al.*, 1996a,b
Cassia obtusifolia L.	Leaf	Nepal	Used to treat tumors.	Singh *et al.*, 1979

Cassia obtusifolia L.	Seed	Nepal	Used to treat tumors.	Singh *et al.*, 1979
Centrosema pubescens Benth.	Flowers	Mexico	Used against cancer.	Hastings, 1990
Crotalaria assamica Benth.	Entire plant	China	Used as an anticancer agent.	Duke and Ayensu, 1985a,b
Crotalaria ferruginea Graham	Entire plant	China	Used as an anticancer agent.	Duke and Ayensu, 1985a,b
Crotalaria sessiliflora L.	Entire plant	China	Used for skin and cervical carcinomas.	Duke and Ayensu, 1985a,b
Dalea leporina (Aiton) Bullock	Part not specified	Mexico	Used against tumors.	Hastings, 1990
Dalea lutea Willd.	Part not specified	Mexico	Used against tumors.	Hastings, 1990
Entada glandulosa Pierre ex Gagnepain	Seed	Thailand	Used for cancer.	Wasuwat, 1967
Entada scandens Benth.	Seed	Thailand	Used for cancer.	Wasuwat, 1967
Enterolobium schomburgkii (Benth.) Benth.	Leaf	Panama	Used for 'inflamed tumors'.	Esposito-Avella *et al.*, 1985
Erythrophleum guineense G. Don	Bark	Mozambique	Used as a cancer remedy.	Amico, 1977
Glycyrrhiza uralensis Fisch. in DC.	Root	China	Used for cancer.	Duke and Ayensu, 1985a,b
Maackia tenuifolia (Hemsl.) Hand.-Mazz.	Root	China	Used as an antitumor drug.	Martin-Cordero *et al.*, 1995
Maackia tenuifolia (Hemsl.) Hand.-Mazz.	Root	China	Used as an antitumor drug.	Zeng *et al.*, 1996
Maackia tenuifolia (Hemsl.) Hand.-Mazz.	Root	China	Used as an antitumor drug.	Zeng *et al.*, 1997
Mucuna collettii Lace	Seed	Thailand	Used for cancer.	Wasuwat, 1967
Parkinsonia aculeata L.	Twig	India	Used to treat tumors.	Sharma *et al.*, 1992
Piliostigma thonningii (Schumach.) Milne-Redh.	Root	Tanzania	Used against malignant ulcers.	Chhabra *et al.*, 1987
Pterocarpus erinaceus Poiret	Stembark	Senegal	Used to treat tumors of the glands.	Ayensu, 1978
Sophora japonica L.	Entire plant	China	Used as an anticancer agent.	Duke and Ayensu, 1985a,b
Sophora subprostrata Chun and T.C. Chen	Root	China	Used as an antitumor agent	Kyogoku *et al.*, 1975
Sophora subprostrata Chun and T.C. Chen	Root	China	Used for cancer.	Sakamoto *et al.*, 1992
Sophora subprostrata Chun and T.C. Chen	Root	China	Used to treat tumors.	Duke and Ayensu, 1985a,b
Sophora tomentosa L.	Leaf	Rarotonga	Used to treat cancer of the breast.	Holdsworth, 1991
Vigna radiata (L.) R. Wilczek	Entire plant	China	Used for carcinomatous swellings.	Duke and Ayensu, 1985a,b
Liliaceae				
Hosta japonica Trattinick	Entire plant	Korea	Used to treat lymphoma.	Han *et al.*, 1984
Hosta longipes L.H. Bailey	Entire plant	Korea	Used to treat lymphoma.	Han *et al.*, 1984
Merendera caucasica M. Bieb.	Bulb	Turkey	Used for malignant disease.	Ulubelen and Tanker, 1975
Smilax menispermoidea A.DC.	Rhizome	China	Used as an anticancer drug.	Ju and Jia, 1992
Smilax sieboldii Miq.	Entire plant	South Korea	Used to treat tumors.	Suh *et al.*, 1996
Smilax sieboldii Miq.	Root	China	Used to treat tumors.	Woo *et al.*, 1992

continued

Table A.3 continued

Family/Species	*Part*	*Country*	*Uses*	*Reference*
Linaceae				
Linum virginianum L.	Seed	USA	Used to treat tumors.	Hussey, 1974
Loranthaceae				
Plicosepalus sagittifolius (Sprague) Danse	Branches	Tanzania	Used to treat cancer.	Chhabra *et al.*, 1991
Plicosepalus sagittifolius (Sprague) Danse	Branch and leaf	Tanzania	Used to treat cancer.	Chhabra *et al.*, 1984
Viscum calcaratum S. Balle	Part not specified	India	Used for skin tumors.	Tiwari, 1995
Lycoperdaceae				
Lycoperdon bovista L.	Entire plant	Malaysia	Applied to tumors.	Burkill, 1966
Melastomataceae				
Medinilla crassinervia Blume	Entire plant	Papua-New Guinea	Used to treat nasal cancer.	Holdsworth and Sakulas, 1986
Melastoma malabathricum L.	Flowers	India	Used for treatment of cancer.	Mohandoss and Ravindran, 1993
Meliaceae				
Aglaia elliptica Blume	Bark	Thailand	Used against tumors.	Pannell, 1992
Aphanamixis polystachya (Wall.) N.R. Park	Root	Geog. area not stated	Used to treat tumors.	Jain and Srivastava, 1985
Aphanamixis polystachya (Wall.) N.R. Park	Stembark	India	Used for spleen and liver tumors.	Srivastava and Agnihotri, 1985
Aphanamixis polystachya (Wall.) N.R. Park	Stembark	India	Useful for tumors.	Rabi and Gupta, 1995
Azadirachta indica Adr. Juss.	Leaf	Nigeria	Used to treat whitlow.	Bhat *et al.*, 1990
Carapa guianensis Aubl.	Seed oil	Brazil	Used to treat uterine cancer.	Hammer and Johns, 1993
Swietenia humilis Zucc.	Seed	Mexico	Used to treat cancer.	Segura-Correa *et al.*, 1993
Moraceae				
Ficus pretoriae Burtt Davy	Sap	Oman	Used for warts.	Ghazanfar and Al-Sabahi, 1993
Maclura cochinchinensis (Lour.) Corner	Stemwood	Thailand	Used as an antineoplastic.	Karnjanapee and Natori, 1966
Maclura cochinchinensis (Lour.) Corner	Wood	Thailand	Used for cancer	Wasuwat, 1967
Myristicaceae				
Knema tenuinervia W.J.J.O. De Wilde	Stembark	Thailand	Said to be popular cancer remedy.	Banerji *et al.*, 1994
Knema tenuinervia W.J.J.O. De Wilde	Stembark	Thailand	Used as a cancer remedy.	Kijjoa *et al.*, 1991
Virola bicuhyba Warb.	Seed	Brazil	Used to treat tumors of the joints.	Plotkin and Schultes, 1990

Myrtaceae				
Leptospermum scoparium Forst. and Forst.	Aerial parts	Australia	Used for treatment of cancers.	Mayer, 1993
Nyssaceae				
Camptotheca acuminata Decne.	Root	Taiwan	Used as an antitumor folk medicine.	Wu *et al.*, 1980
Nyssa sinensis Oliv.	Rootbark	China	Used as a herbal medicine for cancer.	Luo and Xiong, 1991
Olacaceae				
Minquartia guianensis Aubl.	Bark	Ecuador	Used to treat lung cancer.	El-Seedi *et al.*, 1994
Ximenia americana L.	Root	Kenya	Used for cancer.	Suh *et al.*, 1996
Oleaceae				
Forsythia koreana Nakai	Fruit	South Korea	Used to treat tumors.	Moon *et al.*, 1985
Ligustrum lucidum Aiton f.	Seed	China	Used as an adjunct to cancer treatment.	Wong *et al.*, 1992a,b
Onagraceae				
Epilobium hirsutum L.	Entire plant	Egypt	Used for adenoma.	Barakat *et al.*, 1997
Epilobium hirsutum L.	Entire plant	Egypt	Used for prostate tumors.	Barakat *et al.*, 1997
Orchidaceae				
Pleione bulbocodioides Rolfe	Tuber	China	Used to treat tumors.	Bai *et al.*, 1997
Pandaceae				
Microdesmis puberula Hook.f. ex Planch.	Entire plant	Guinea	Used against tumors.	Vasileva, 1969
Papaveraceae				
Chelidonium sinense DC.	Entire plant	Korea	Used for stomach cancer.	Han *et al.*, 1984
Pedaliaceae				
Rogeria adenophylla J. Gay ex Delile	Stem	Sudan	Used for abscesses and tumors.	Yousif *et al.*, 1983
Phytolaccaceae				
Phytolacca bogotensis Kunth	Leaf	Colombia	Used to treat tumors.	Garcia-Barriga, 1974
Phytolacca esculenta Van Houtte	Root	China	Used as an antitumor remedy.	Hua, 1992
Phytolacca esculenta Van Houtte	Root	China	Used for treatment of tumors.	Yi and Dai, 1991
Phytolacca esculenta Van Houtte	Root	China	Used to treat tumors.	Yi, 1992
Phytolacca rivinoides Kunth and Bouche	Leaf	Colombia	Used to treat tumors.	Garcia-Barriga, 1974
Phytolacca sanguinea H. Walter	Leaf	Colombia	Used to treat tumors.	Garcia-Barriga, 1974

continued

Table A.3 continued

Family/Species	*Part*	*Country*	*Uses*	*Reference*
Pinaceae				
Pinus parviflora Siebold and Zucc.	Strobilus	Japan	Used for cancer.	Sakagami *et al.*, 1992
Pinus parviflora Siebold and Zucc.	Strobilus	Japan	Used for gastric cancer.	Lai *et al.*, 1990
Pinus parviflora Siebold and Zucc.	Strobilus	Japan	Used to treat gastric carcinoma.	Sakagami *et al.*, 1989
Piperaceae				
Piper boehmerifolium Wall.	Root	India	Used for the treatment of tumors.	Mahanta *et al.*, 1974
Piper latifolium Forst.f.	Leaf	Rarotonga	Used to treat breast cancer.	Holdsworth, 1991
Piper methysticum Forst.f.	Leaf	Rotuma	Used for warts.	
Piper sylvaticum Roxb.	Root	India	Used for the treatment of tumors.	Mahanta *et al.*, 1974
Plantaginaceae				
Plantago asiatica L.	Leaf	Easter Island	Used for internal cancers.	Holdsworth, 1992
Plantago hirtella Kunth	Entire plant	Mexico	Used against cancerous diseases.	Jiu, 1966
Plantago paralias Decne.	Leaf	Argentina	Used as an antitumor agent.	Bandoni *et al.*, 1976
Plumbaginaceae				
Plumbago toxicaria Bertol.	Root	South Africa	Used against cancerous diseases.	Ko, 1933
Polygalaceae				
Monnina obtusifolia H.B.K.	Aerial parts	Ecuador	Used as an antitumor agent.	Pinto *et al.*, 1994
Polypodiaceae				
Phymatosorus diversifolium (Willd.) Pich.	Root	Easter Island	Used for cancer.	Holdsworth, 1992
Polypodium maritimum Heiron.	Rhizome	Panama	Used against cancer.	Gupta *et al.*, 1979
Polypodium subpetiolatum Hook.	Rhizome	Guatemala	Used for tumors of the skin.	Anderson *et al.*, 1979
Polyporaceae				
Antrodia cinnamomea Chang et Chou	Fruitbody	Taiwan	Used for cancer.	Cherng *et al.*, 1996
Coriolus versicolor (L.: Fr.) Quelet	Fruitbody	Taiwan	Used to treat tumors.	Liu *et al.*, 1993
Grifola frondosa (Dicks.: Fr.) Gray	Fruitbody	Japan	Used for cancer.	Ohno *et al.*, 1985
Pteridaceae				
Adiantum macrophyllum Sw.	Part not specified	Colombia	Used for tumors.	Laferriere, 1994
Cheilanthes contracta (Kunze) Mett. ex Kuhn	Part not specified	South Africa	Used to treat cancer.	Charlson, 1979
Cheilanthes sulphurea (Cav.) Mickel and Beitel	Rhizome	Peru	Used for tumors.	Ramirez *et al.*, 1988

Ranunculaceae				
Anemone rivularis Buch. Ham ex DC.	Root	India	Used in the treatment of tumors.	Jain and Puri, 1984
Coptis japonica Makino	Rhizome	Korea	Used to treat abdominal tumors.	Cha, 1977
Coptis japonica Makino	Rhizome	Korea	Used to treat tumors.	Cha, 1977
Pulsatilla cernua (Thunb.) Bercht. and Presl	Root	Japan	Used as a remedy against cancer.	Shimizu *et al.*, 1978
Ranunculus glaberrimus Hook.	Entire plant	Canada	Used for cancers on skin.	Turner, 1984
Semiaquilegia adoxoides (DC.) Makino	Root	China	Used for hepatoma.	Duke and Ayensu, 1985a,b
Thalictrum faberi Ulbr.	Root	China	Used for treatment of stomach cancer.	Lin *et al.*, 1994a,b
Thalictrum faberi Ulbr.	Root	China	Used to treat stomach cancer.	Lin *et al.*, 1983
Thalictrum faberi Ulbr.	Root	China	Used to treat stomach cancer.	Wagner *et al.*, 1984
Rhamnaceae				
Pomaderris kumeraho A. Cunn	Leaf	New Zealand	Used for skin cancer.	Brooker *et al.*, 1989
Rhizophoraceae				
Rhizophora brevistyla Salvosa	Bark	Colombia	Used to treat esophageal cancer.	Garcia-Barriga, 1975
Rosaceae				
Duchesnea indica (Andrews) Focke	Entire plant	China	Used for cancers.	Peng *et al.*, 1995a,b
Duchesnea indica (Andrews) Focke	Part not specified	China	Used as an anticancer herb.	Murakami *et al.*, 1985
Prunus africana (Hook.f.) Kalm	Bark	Kenya	Used for retinoblastoma.	
Rosa laevigata Michx.	Pericarp	China	Used for skin tumors.	Yoshida *et al.*, 1989
Rubiaceae				
Galium aparinoides Forsk.	Entire plant	East Africa	Used against cancer.	Kokwaro, 1976
Galium aparinoides Forsk.	Entire plant	East Africa	Used to cure throat cancer.	Kokwaro, 1976
Hedyotis chrysotricha (Pallib.) Merrill	Entire plant	China	Used to treat cancer.	Peng *et al.*, 1995a,b
Hedyotis chrysotricha (Pallib.) Merrill	Entire plant	China	Used to treat cancer.	Peng *et al.*, 1995a,b
Morinda citrifolia L.	Fruit	Hawaii	Used in the treatment of breast cancer.	
Morinda citrifolia L.	Root	Rarotonga	Used for external cancerous lesions.	Holdsworth, 1991
Nauclea diderrichii (De Wild.) Merr.	Leaf	Guinea	Applied to tumors.	Vasileva, 1969
Oldenlandia affinis (Roem. and Schult.) DC.	Entire plant	China	Used for intestinal cancer.	Hsu, 1967
Oldenlandia diffusa (Willd.) Roxb.	Aerial parts	China	Used against tumors.	Hsu, 1967
Oldenlandia diffusa (Willd.) Roxb.	Aerial parts	China	Used to treat cancers.	Wong *et al.*, 1992a,b
Oldenlandia diffusa (Willd.) Roxb.	Aerial parts	Taiwan	Used in tumor therapy.	Hsu, 1967

continued

Table A.3 continued

Family/Species	*Part*	*Country*	*Uses*	*Reference*
Oldenlandia diffusa (Willd.) Roxb.	Entire plant	China	Used for liver cancer.	Huang, 1981
Oldenlandia diffusa (Willd.) Roxb.	Entire plant	Malaysia	Used to treat cancer.	Wong *et al.*, 1992a,b
Oldenlandia diffusa (Willd.) Roxb.	Entire plant	Singapore	Used to treat cancer.	Wong *et al.*, 1993
Oldenlandia diffusa (Willd.) Roxb.	Part not specified	China	Used for liver, lung and rectal cancer.	Hsu, 1967
Rubia discolor Turcz	Root	Ethiopia	Used against cancer.	Kloos *et al.*, 1978.
Rubia discolor Turcz.	Root	Ethiopia	Used for cancer.	Kloos, 1977
Uncaria tomentosa (Willd. ex Roem. and Schult.) DC.	Bark	Peru	Used against tumors.	Tirillini, 1996
Uncaria tomentosa (Willd. ex Roem. and Schult.) DC.	Bark	Peru	Used to treat cancer.	Wirth and Wagner, 1997
Uncaria tomentosa (Willd. ex Roem. and Schult.) DC.	Rootbark	Peru	Used to treat cancer.	Stuppner *et al.*, 1992
Uncaria tomentosa (Willd. ex Roem. and Schult.) DC.	Rootbark	Peru	Used to treat cancer.	Stuppner *et al.*, 1993
Xeromphis obovata (Hochst.) Keay	Rootbark	Zimbabwe	Used for melanoma.	Sibanda *et al.*, 1989
Rutaceae				
Haplophyllum dauricum (L.) G. Don	Aerial parts	Russia	Used to treat tumors.	Bessonova *et al.*, 1984
Murraya koenigii (L.) Sprengel	Leaf and stem	India	Used for leprosy.	Philip, 1981
Zanthoxylum acanthopodium DC.	Fruit	India	Used to remove tumors.	Bose and Bose, 1939
Zanthoxylum armatum DC.	Fruit	India	Claimed to be good for tumors.	Chopra *et al.*, 1960
Zanthoxylum oxyphyllum Edgew.	Fruit	Nepal	Used against tumors.	Suwal, 1970
Santalaceae				
Iodina rhombifolia Hook. and Arn.	Leaf	Brazil	Used for carcinoma.	Spitzer *et al.*, 1994
Osyris quadripartita Salzm. ex Decne.	Root	Tanzania	Used for cancer.	Chhabra *et al.*, 1991
Scrophulariaceae				
Castilleja tenuiflora Benth.	Part not specified	Mexico	Used for cancer.	Hirschhorn, 1982
Pedicularis resupinata L.	Aerial parts	South Korea	Used to treat malignant abscesses.	Yoo *et al.*, 1993
Picria fel-terrae Lour.	Entire plant	China	Used against cancer.	Huang *et al.*, 1988
Sibthorpia peregrina L.	Leaf	Madeira	Used as a corn salve.	Rivera and Obon, 1995
Veronica persica Poir.	Entire plant	Peru	Used for cancer.	Ramirez *et al.*, 1988

Selaginellaceae				
Selaginella doederleinii Hieron.	Entire plant	China	Used as an anticancer agent.	Lin *et al.*, 1994a,b
Selaginella tamariscina (P. Beauv.) Spring	Entire plant	South Korea	Used as a folk medicine to treat cancer.	Rhan *et al.*, 1992
Selaginella tamariscina (P. Beauv.) Spring	Entire plant	South Korea	Used to treat cancer.	Lee *et al.*, 1992
Simaroubaceae				
Harrisonia abyssinica Oliv.	Root	Tanzania	Used as a cytotoxic agent.	Chhabra *et al.*, 1993
Solanaceae				
Fabiana imbricata R. and P.	Aerial parts	Chile	Used to treat tumors.	Brown and Shill, 1994
Solanum dubium Dunal	Fruit	Sudan	Used to treat tumors.	El Kheir and Salih, 1979
Solanum hispidum Pers.	Part not specified	India	Used to treat skin tumors.	
Solanum lyratum Thunb. in Murr.	Aerial parts	Taiwan	Used to treat cancers.	Lee *et al.*, 1997
Solanum lyratum Thunb. in Murr.	Entire plant	China	Used for cancers.	Yu *et al.*, 1994a,b
Solanum lyratum Thunb. in Murr.	Entire plant	China	Used to treat cancers.	Murakami *et al.*, 1985
Wedelia chinensis V. Tackh.	Leaf	Sudan	Used for abscesses and tumors.	Yousif *et al.*, 1983
Sparganiaceae				
Sparganium stoloniferum (Graebn.) Buch.-Ham	Rhizome	China	Used for abdominal tumors.	Anon., 1977
Stemonaceae				
Stemona collinsae Craib	Root	Thailand	Used for cancer.	Wasuwat, 1967
Sterculiaceae				
Waltheria americana L.	Branches	Mexico	Used for cancer.	Dimayuga *et al.*, 1987
Waltheria americana L.	Leaf	Mexico	Used for cancer.	Dimayuga *et al.*, 1987
Waltheria americana L.	Root	Mexico	Used for cancer.	Dimayuga *et al.*, 1987
Taxaceae				
Taxus brevifolia Nutt.	Bark	USA – WA	Used to treat skin cancer.	Forlines *et al.*, 1992
Taxodiaceae				
Glyptostrobus pensilis (Staunton) K.K.	Stem	China	Used to treat tumors.	Duke and Ayensu, 1985a,b
Tremellaceae				
Tremella fuciformis Berk.	Entire plant	China	Used for cancer.	Lin *et al.*, 1984a,b
Tropaeolaceae				
Tropaeolum majus L.	Leaf and stem	Mexico	Used for lung cancer.	Morton, 1977

continued

Table A.3 continued

Family/Species	*Part*	*Country*	*Uses*	*Reference*
Ulmaceae				
Holoptelea integrifolia (Roxb.) Planch.	Bark	India	Used for treating intestinal cancer.	Sabnis and Bedi, 1983
Ulmus davidiana Planch.	Rootbark	South Korea	Used for gastric cancer.	Son *et al.*, 1989
Ulmus davidiana Planch.	Rootbark	South Korea	Used to treat gastric cancer.	Kim *et al.*, 1996a,b
Ulmus davidiana Planch.	Rootbark	South Korea	Used to treat gastric cancer.	Kim *et al.*, 1996a,b
Ulmus manschurica Nakai	Bark	South Korea	Used as a folk medicine to treat cancer.	Rhan *et al.*, 1992
Umbelliferae				
Cnidium monnieri (L.) Cusson	Fruit	China	Used to treat tumors.	Duke and Ayensu, 1985a,b
Hydrocotyle sibthorpioides Lam.	Entire plant	China	Used for hepatoma.	Duke and Ayensu, 1985a,b
Oenanthe javanica (Blume) DC.	Entire plant	China	Used for cancerous swellings.	Duke and Ayensu, 1985a,b
Sanicula coerulescens Franch.	Entire plant	China	Used on malignant sores.	Duke and Ayensu, 1985a,b
Urticaceae				
Myrianthus arboreus Beauv.	Leaf	Guinea	Used against tumors.	Nqounou *et al.*, 1990
Myrianthus arboreus Beauv.	Trunkwood	Cameroon	Used to treat tumors.	Vasileva, 1969
Valerianaceae				
Patrinia scabra Bunge	Root	China	Used to treat cervical erosion.	Kouno *et al.*, 1994
Verbenaceae				
Callicarpa rubella Lindl.	Bark	India	Used for cancer.	Jamir, 1990
Clerodendrum serratum (L.) Moon	Leaf	India	Used for swellings or tumor growth.	Bhandary *et al.*, 1995
Nyctanthes arbor-tristis L.	Part not specified	India	Used for cancer.	Jain, 1970
Pygmaeopremna herbacea (Roxb.) Moldenke	Root	India	Used to treat tumors.	Sankaram and Rao, 1978
Vitex negundo L.	Leaf	Philippines	Used to treat cancer.	Masilungan *et al.*, 1971
Violaceae				
Viola diamantica Nakai	Aerial parts	South Korea	Used for cancer.	Yook *et al.*, 1989

Table A.3 continued

Family/Species	*Part*	*Country*	*Uses*	*Reference*
Zingiberaceae				
Alpinia katsumadai Hayata	Seed	China	Used for cancer-like symptoms.	Saiki *et al.*, 1978
Alpinia parpurtura Sch.	Leaf	Cook Islands	Used to treat tumors.	Holdsworth, 1990
Zygophyllaceae				
Fagonia indica Burm.f.	Aerial parts	Pakistan	Used as a remedy for tumors.	Atta-Ur-Rahman *et al.*, 1984
Fagonia indica Burm.f.	Leaf and twigs	Pakistan	Claimed to be a remedy for tumors.	Ali Ansari *et al.*, 1982
Fagonia indica Burm.f.	Leaf and twigs	Pakistan	Used for cancer.	Atta-Ur-Rahman, 1982

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