Chapter 1

Introduction

1.1 Medicinal plants past, present

1.1.1 Medicinal plants and human health care

People have used plants for centuries as medicine or food additives with varying success to cure and prevent diseases. Written records about medicinal plants date back at least 5000 years to the Sumerians (Swerdlow, 2000). According to World Health Organization (WHO) around 80 % of the population in developing countries is dependent on herbal medicine for basic health care needs (Vines, 2004). This high demand for natural products is not limited only to the developing countries; the use of herbal medicine in developed countries is also increasing. For instance, 25 % of the population in the UK consumes herbal medicine regularly (Vines, 2004). In the 1970s, 25 % of all drugs dispensed in the USA contained compounds derived from flowering plants. Even at the dawn of the twenty-first century, 11 % of 252 drugs considered as basic and essential by WHO was exclusively of flowering plant origin (Rates, 2001). The recent findings in plant-derived antitumor drugs such as taxol, vinblastine, vincristine and camptothecin has dramatically improved the effectiveness of chemotherapy against some of the deadliest cancers (Raskin et al., 2002). Some of the most important drugs derived directly or as precursors from plants are listed in Table 1.1.

The strong relationship between plants and human health is not limited only to medicine. There is also a historic bond between food and the treatment of diseases such as “An apple a day keeps the doctor away”. Plant-derived products now have a considerable share in the market. These include phytopharmaceuticals, pharmaceuticals, herbal remedies, dietary supplements, homeopathics, medicinal and herbal teas, spirits, aromas and essences, perfumes, cosmetics, colouring agents, varnishes, and also detergents. As a consequence, there is a huge trade on the local, regional, national and international level, both for domestic use and commercial trade and this result in an enormous demand in botanicals.

Table 1. 1. Some of the most important pharmaceuticals (or their precursors) derived from plants (Raskin et al., 2002).

Name	Type	Source	Therapeutic use
Alkaloids:			
Atropine[a], hyoscyamine, scopolamine	Tropane alkaloid	*Solanaceous* spp.	Anticholinergic
Camptothecin[a]	Indol alkaloid	*Camptotheca acuminata* Decne	Antineoplastic
Capsaicin	Phenylalkyl-amine alkaloid	*Capsicum* spp.	Topical analgesic
Codeine, morphine	Opium alkaloid	*Papaver somniferum* L.	Analgesic, antitussive
Cocaine	Cocaine alkaloid	*Erythroxylum coca* Lamarck	Local anaesthetic
Colchicine	Isoquinoline alkaloid	*Colchicum autumnale* L.	Antigout
Emetine	Isoquinoline alkaloid	*Cephaelis ipecacuanha* (Brot.)A. Rich.	Antiamoebic
Galanthamine	Isoquinoline alkaloid	*Leucojum aestivum* L.	Cholinesterase inhibitor
Nicotine	Pyrrolidine alkaloid	*Nicotiana* spp.	Smoking cessation therapy
Physostigmine	Indole alkaloid	*Physostigma venenosum* Balfor	Cholinergic
Pilocarpine	Imidazole alkaloid	*Pilocarpus jaborandi* Holmes	Cholinergic
Quinine	Quinoline alkaloid	*Cinchona* spp.	Antimalarial
Quinidine	Quinoline alkaloid	*Cinchona* spp.	Cardiac depressant
Reserpine	Indole alkaloid	*Rauwolfia serpentina* L.	Antihypertensive, psychotropic
Tubocurarine	Bisbenzyl isoquinoline alkaloid	*Chondodendron tomentosum* Ruiz,	Skeletal muscle relaxant
		Strychnos toxifera Bentham	
Vinblastine, vincristine	Bis-indole alkaloid	*Catharanthus roseus* L.	Antineoplastic
Yohimbine	Indole alkaloid	*Apocynaceae, Rubiaceae* spp.	Aphrodisiac
Terpenes and steroids:			
Artemisinin	Sesquiterpene lactone	*Artemisia annua* L.	Antimalarial
Diosgenin[a], hecogenin[a], stigmastero	Steroids	*Dioscorea* spp.	Oral contraceptives and hormonal drugs
Taxol and other taxoids[a]	Diterpenes	*Taxus brevifolia* Nutt.	Antineoplastic
Glycosides:			
Digoxin, digitoxin	Steroidal glycosides	*Digitalis* spp.	Cardiotonic
Sennosides A and B	Hydroxy-anthracene glycosides	*Cassia angustifolia* Vahl.	Laxative
Others and mixtures:			
Ipecac	Mixture of ipecac alkaloids	*Cephaelis ipecacuanha* (Brot.)	Emetic
	and other components A. Rich.		
Podophyllotoxin[a]	Lignan	*Podophyllum peltatum* L.	Antineoplastic
[a]Most often used as precursors in chemical synthesis of final products.			

1.1.2 Medicinal plants in trade

Traded raw plant material consists mainly of dried roots, leaves, bark, wood, flowers, seeds or sometimes the whole plant (Lange, 1996). In the 1990s, the overall import trade amounted on average of 400 000 tons valued at USD 1 224 million. The international trade is dominated by only a few countries: 82 % of the world-wide imports are channelled to just 12 countries (Table 1.2), and 12 countries are also responsible for 80 % of the overall world exportation. Temperate Asia and Europe dominates the domestic import at 42 % and 33 % respectively. Hong Kong and China are the most important export and import countries (Table 1.2). On the other hand three countries, Hong Kong, USA and Germany are important trade centres for pharmaceutical plants.

Table 1. 2. List of the important countries for trading of medicinal plants (Lange, 1996).

Country of import	Quantity [t]	Value [USD]	Country of export	Quantity [t]	Value [USD]
Hong Kong	67,000	291,200,000	China	147,000	281,800,000
Japan	51,350	136,000,000	Hong Kong	63,150	228,800,000
USA	49,600	135,500,000	India	33,900	56,650,000
Germany	45,350	110,200,000	Germany	15,100	70,050,000
Rep. Korea	32,250	52,300,000	USA	13,500	115,500,000
France	21,350	52,000,000	Mexico	13,000	11,250,000
China	13,650	41,600,000	Egypt	11,750	13,850,000
Italy	11,700	42,850,000	Chile	11,600	28,200,000
Pakistan	11,050	11,150,000	Bulgaria	10,050	14,500,000
Spain	9,100	27,650,000	Singapore	9,600	56,600,000
United Kingdom	7,650	27,000,000	Morocco	8,000	13,300,000
Singapore	6,300	50,600,000	Pakistan	7,800	4,950,000
Total	**326,300**	**978,150,000**	**Total**	**344,400**	**893,400,000**

Fig. 1.1 shows that Japan is by far the most important consumer country, followed by the USA, Germany and the Republic of Korea. In these countries, the raw material is mainly processed as a industry of the country and then sold as finished products either on the domestic market or exported. In addition, China is the most important supplier of raw material to the world's medicinal and aromatic plant markets.

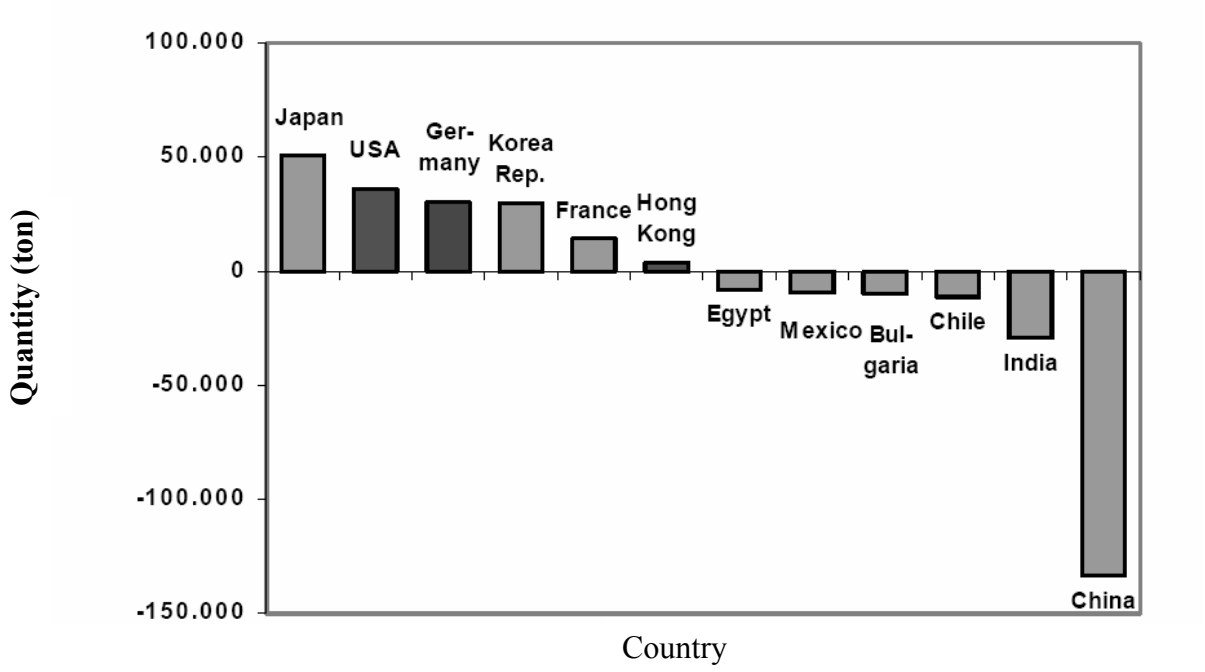

Fig. 1.1. Traded medicinal plants, cultivated or of wild origin (Lange, 1996).

In general, the cultivation of medicinal plant species has declined in the last thousand years. An example of cultivation would be the opium poppy (*Papaver somniferum*) which has been cultivated since 2700 B.C. (Heeger, 1989). Unfortunately, there is no exact information available of medicinal plant material produced under cultivation. On average over 80 000 tons of the annual exports of medicinal plants are wild-harvested. But, the botanicals exported are only a small part

of the annual domestic production which amounted to 700 000 tons in the early 1990s (Xiao, 1991).

In China, 60 % of the quantity in the trade are wild-collected (He and Sheng, 1997). In Nepal, every year more than 15 000 tons of herbs are taken from the wild (Bhattarai, 1997). Dozens of medicinal plant species are collected in large amounts from the wild in Canada, Mexico and the United States (Robbins, 1999; Fuller, 1991). In Europe, around 2 000 medicinal plant species are commercially used, of which 130-140 species are cultivated and the remainder are harvested from the wild (Lange, 1998). About 90 % of the 1 200 - 1 300 European native species are therefore primarily harvested from the wild (Lange, 1998). The quantity of wild-collected botanicals in east and southeast Europe in the late 1990s has been estimated to be at least 30 000 - 45 000 tons of dry plant material (the fresh material weighs two to three times more) (Lange, 2004). Harvesting from wild areas has resulted that some species are now threatened. Within Europe, for instance, at least 150 species are reported to be threatened. This is more than 10 % of the European native medicinal plant species and 1.2 % of the total European flora (Lange, 1998). Well-known medicinal plants that are threatened include *Arnica montana*, *Arctostaphylos uva-ursi* (bear-berry), *Gentiana lutea* (Lange, 1998), *Piper methysticum* (kava) and *Glycyrrhiza glabra* (liquorice) (Vines 2004). Another serious problem caused by wild harvesting is loss of genetic diversity, local extinctions and habitat destruction (Canter et al., 2005). Between 4 000 to 10 000 medicinal species may now be threatened globally (Edwards 2004).

According to the IUCN, WHO and WWF (1993) the cultivation of medicinal plants would be the best way to fulfill market demands. However, according to Lange (1998), cultivation has not proven to be useful for the majority of taxa in the trade due to the following reasons:

1- Many plants are difficult to cultivate due to their low germination potential or specific germination requirements. There is a lack of knowledge of the specific requirements for pollination, seed germination and growth, (Canter et al., 2005).

3- Cultivation of some species takes long time.

4- Many plants are only required in a small quantities.

5- In some cases the quality of wild-harvested material is better.

6- The cost of wild plant material is in general lower than for cultivated material (Lange, 1997).

1.1.3 Medicinal plants: opportunities

During the twentieth century, the emphasis gradually shifted from natural compounds obtained directly from plants to chemically synthesized compounds and their analogues. However, the natural products were widely considered as templates for the structure optimization programs designed to make perfect new drugs (Raskin et al., 2002). Although synthetic chemical drugs have a significant market share, the contribution of plants to disease treatment and prevention is still enormous. This is due to the fact that most herbal medicine is natural and therefore considered be safe for human consumption (Canter et al., 2005). In the twenty-first century the new focus on botanical pharmaceuticals as sources of human health products has became considerable. According to Raskin et al. (2002), this re-attention is based on some of the advantages of medicinal plants and these include:

- Potential of plants to synthesize mixtures of bioactive compounds with multiple therapeutic effects.
- Low-cost and better ability for scaling up the production of proteins and secondary metabolites.

- Changing the attention from chemically synthetic medicine to drug discovery and disease treatment and prevention.
- Cost limitation on the chemical synthesis of complex bioactive molecules.
- Public concern has indicated that plant phytochemicals provide a safer approach to disease treatment and prevention.

In 1999 the global market for herbal supplements exceeded US$15 billion, with a US$7 billion market in Europe, US$2.4 billion in Japan, US$2.7 in the rest of Asia and US$3 billion in North America (Glaser 1999). Many experts believe that the majority of plant-derived natural products remain undiscovered or unexplored for their pharmacological activity (Gentry, 1993; Mendelson and Balick, 1995).

The prospect of natural products to treat complex diseases is currently a good opportunity. In the twentieth century natural products have been used (applied) to treat complex diseases, such as diabetes, heart disease, cancer, and psychiatric disorders. However, there is no 'single golden molecular' solution as most of these diseases are not caused by a single genetic or environmental change, but are a result of interactions between genetics, environmental or behavioral factors (Kibertis, 2002). The traditional medicine systems are always based on the belief that a complex disease can only be effectively treated with a combination of botanical and non-botanical remedies (Raskin et al., 2002). For instance, the root extract of *Tripterygium wilfordii* has been traditionally used as a Chinese medicine to treat rheumatoid arthritis. The main active ingredient of this extract, a triptolide, is toxic unless given as a part of the root extract. This suggests that other unidentified extract components increase its safety and effectiveness (Su, et al., 1990).

1.1.4 Medicinal plants: limitations

There are certain limitations in the usage of medicinal plants. One of the major problems is the lack of reproducibility of the activity. In more than 40 % of plant extracts tested the activities could not be repeated when the extracts were re-sampled and re-extracted (Cordell, 2000). In addition, the biochemical profiles of plants can be changed due to the time and the location of harvesting (Raskin et al., 2002). Moreover, the current popular interest in drug discovery is to search for an active single compound rather than for a mixture. However, these valuable bioactive molecules are often associated with pigments and poly-phenols that interfere with their medicinal properties. A further limitation is the lack of an efficient and rapid strategy with a high-throughput screen to isolate and characterize a natural product from a plant extract. Poorly proven efficacy and safety, lack of standardization and quality standards are also a current limitation (Osowski et al., 2000, Kressmann et al., 2002).

1.1.5 Production of medicinal compounds

1.1.5.1 Traditional cultivation

Domestic cultivation is an alternative to overcome the problems that are inherent when working with medicinal plants. Problems include: misidentification, genetic and phenotypic variability, extract variability and instability, toxic components and contaminants. The use of controlled environments can overcome cultivation difficulties and could be a means to manipulate phenotypic variation in bioactive compounds and toxins. Improvement of domestic cultivation of medicinal plants requires using traditional and modern techniques such as biotechnological plant-breeding technologies. The techniques can be applied at the genetic level for improving the yield through seed uniformity, modifying the potency and toxicity, which are all agronomical traits of medicinal plants. Extracts of *Ginko biloba* are for example standardized to contain 24-27 %

flavonoid glycosides and 6-7 % terpene lactones while the toxic component, ginkgolic acid, is kept below 5 ppm (Canter et al., 2005). This is done during the characterization of the plant extracts. If these kinds of traits could be successfully identified at early stages, it would translate into a more cost-effective extraction process. The technique can be applied for the selection of desirable genotypes at early stages with molecular markers, and could speed up the selection of desirable plants at an early stage. There are several publications on the use of one particular type of molecular technique (RAPDs) for population analyses (Sangwan et al., 1999; Bai et al., 1997).

Controlled growth systems can also make it feasible to increase potency, reduce toxin levels and increase uniformity and predictability of plant extracts. This is due to the fact that the target compounds in plants frequently serve as adaptations to fluctuating temperature and light conditions (e.g. antioxidants), stress (e.g. proline), infection (e.g. flavanoids) or herbivory (e.g. alkaloids) (Canter et al., 2005). According to McChesney (1999) for example Caucasian-grown *Atropa belladonna* has an alkaloid content of 1.3%, compared to 0.3% in plants grown in Sweden. Shade-grown *Mentha piperata* has a lower essential oil content (1.09% v 1.43%) and lower menthol content within the oil (57.5% v 61.8%) compared to those grown in the light (McChesney, 1999). *Papaver somniferum* (poppy) grown in cool conditions contains more morphine but has a lower alkaloid content than *P. somniferum* grown in warmer conditions (Mc Chesney, 1999). These examples indicate the dependence of secondary metabolite accumulation on environmental conditions such as water availability, exposure to soil microorganisms and variations in soil pH and nutrients (McChesney, 1999).

1.1.5.2 Tissue and cell suspension cultures

Tissue culture experimental approaches used for the propagation of medicinal plants can be divided into three broad categories. The most common approach is the propagation of whole

plants through meristematic tissues such as shoot tips and auxiliary buds. This process is referred to as micropropagation. It can also be used for the conservation of valuable biodiversity (Augustine and D'Souza, 1997; Purohit et al., 1994). These techniques have a potentially significant role to play in establishing breeding material from wild populations (Kintzios and Michaelakis, 1999) and for mass-producing material for selection or engineering (Lakshmanan et al., 2002).

In the second approach, adventitious shoots are initiated from leaf, root or stem segments or from a callus derived from these organs, and this is known as organogenesis.

The third system of propagation involves induction of somatic embryogenesis in cell and callus cultures. Tissue cultures often promote genetic disturbances, which result in somaclonal variation, greatly extending the range of useful variation available to the breeder (Rout et al., 2000; Sevon and Oksman-Caldentey, 2002). Plant cell culture also provides an alternative approach, which is the production of secondary metabolites. Metabolite yield in cell suspension cultures may significantly exceed that observed in the parent plants. By using this technology therefore, the metabolites can be produced under controlled and reproducible conditions, independent of geographical and climatic factors. Table 1.3 shows some plant cell suspension cultures with higher production of secondary metabolites than when compared to their production in the parent plants. Although tissue and root cultures offer genetic stability (Flores and Curtis, 1992), as well as, in some cases, superior metabolic performance over suspension cultures of the same lines, cell suspension culture still has more potential for industrial application than plant tissue or organ cultures (Kieran et al., 1997).

Table 1.3. List of some cell suspension cultures with higher production of secondary metabolites as compared to what is present in intact plant (Ramachandra and Ravishankar, 2002).

Product	Plant species	Yield (%D.W.)	Reference
Rosmarinic acid	*Sa. officinalis*	36	Hippolyte et al. (1992)
Rosmarinic acid	*Col. blumei*	21.4	Ulbrich et al. (1985)
Anthroquinones	*M. citrifolia*	18	Zenk et al. (1975)
Shikonin	*L. erythrorhizon*	12.4	Fujita (1988)
Berberine	*Th. minus*	10.6	Kobayashi et al. (1988)
Jatrorhizine	*Berberis wilsonae*	10	Breuling et al. (1985)
Anthocyanins	*Pe. frutescens*	8.9	Zhong et al. (1994)
Berberine	*C. japonica*	7.5	Matsubara et al. (1989)
Diosgenin	*Diosc. deltoidea*	3.8	Sahai and Knuth (1985)
Sanguinarine	*P. somniferum*	2.5	Park et al. (1992)
Serpentine	*Cath. roseus*	2.2	Zenk et al. (1977)

1.1.5.3 Genetic manipulation of medicinal plants

DNA manipulation to change the gene expression in medicinal plants is now expanding. The modification of a desirable trait, which is related to one or a small number of genes, is now feasible (Canter et al., 2005). Although the primary target for trait manipulation in medicinal plants is the content of the active compounds, the other traits, including basic agronomic characters related to uniformity, stability, growth and development, and resistance to the pests, diseases and stresses (biotic and abiotic), must also be improved (Bajaj, 1998; Bernath, 2002). There are a considerable number of publications about the manipulation of plant biosynthetic pathways to produce drug precursors, food components or pesticides (Stevenson, 2004). In *Mentha spp* (mints), for example the biosynthetic pathway has been engineered to modify the essential oil production in the trichomes and to enhance the resistance of the plant to fungal infection and abiotic stresses (Veronese, et al., 2001). There is a long history of commercial production of high-valued phytochemicals by genetic modification through gene transfer into the

plant genome. An excellent example would be where the modification is carried out by infecting cells with *Agrobacterium rhizogenes*, carrying the gene of interest. The hairy root technique has been successfully applied for transformation of several important medicinal plants, including *Papaver somniferum* (Park and Facchini, 2000), *Artemisia spp*. (wormwoods) (Chen et al., 1999), members of the Solanaceae family (Moyano et al., 1999) and *Taraxacum platycarpum* (Lee et al., 2004).

A second modification technique is based on the transformation of the plants with *Agrobacterium tumefaciens*. This has been used for *Taxus* spp. (yew) (Han et al., 1994), *Echinacea spp.* (Wang and To, 2004), *Scrophularia spp.* (figwort) (Park et al., 2003), *Digitalis spp.* (foxglove) (Sales et al., 2003), *Thalictrum spp.* (meadow rues) (Samanani et al., 2002) and *Artemisia spp.* (Chen et al., 2000). A general problem however is where some important species still remain recalcitrant, notably *Ginko biloba* to regenerating the whole plant from transformed cells (Balz et al., 1999).

1.1.5.4 Pathway engineering in medicinal plants

Recent advances in metabolic engineering of both native and heterologous secondary metabolite producing organisms have allowed higher levels of production, direct synthesis of desired products, and the biosynthesis of novel products (Mijts and Schmidt-Dannert, 2003). Increasing the production of active phytochemical compounds is targeted through genetic manipulation of the metabolic biosynthetic pathways of the active compounds. Metabolic engineering utilizes knowledge of cellular metabolism to alter biosynthetic pathways and has many advantages over traditional methods of strain improvement through extensive screening. Although the metabolic pathways of active compounds are mostly poorly understood, and relatively few key genes have been isolated, there are some successful reports with regards to the engineering of biosynthetic

pathways leading to improved breeding of medicinal plants (Ferreira and Duke, 1997; Charlwood and Pletsch, 2002). An example is the nine-fold enhancement in the production of the sedative compound scopolamine in hairy root cultures of *Hyoscyamus niger* (black henbane). This has been performed by the simultaneous overexpression of two genes encoding the rate-limiting upstream and downstream biosynthetic enzymes (Zhang et al., 2004). A threefold enhancement has also been reported in the production of artemisinin, with anti-malarial and anti-cancer activity, in transgenic *Artemisia annua* plants through the overexpression of farnesyl diphosphate synthase, the enzyme involved in the process in the first biosynthetic step (Chen et al., 1999; Chen et al., 2000). In another example a 78-fold increase in flavonoid levels in the tomato peel was achieved by the overexpression of the *Petonia* chalcone isomerase (CHI) gene, an early enzyme of the flavonoid pathway, which was found to be a key enzyme in the increase of flavonol production (Muir et al., 2001). This technique can also be used for the induction of new compounds in plants. Isoflavones in legumes, for instance act as phytoalexins, that is, the biosynthesis of these antimicrobially active compounds are induced by microbial infection. By overexpression of isoflavone synthase, a cytochrome P450 enzyme, these compounds could be produced in *Arabidopsis*, tobacco plants and maize, which normally lack the ability to synthesize these compounds (Jung et al., 2000; Yu et al., 2000).

Another approach in the engineering pathway is combinatorial engineering by which genes responsible for individual metabolic pathway steps from different source organisms are combined to generate novel branches in the metabolic pathways and biosynthesize products that were previously inaccessible (Mijts and Schmidt-Dannert, 2003). Metabolic and combinatorial engineering of isoprenoid compound pathways includes those synthesizing many important industrial and pharmaceutical products such as terpenes and carotenoids. They provide excellent examples of the utility of this approach in secondary metabolite production. Overexpression of the

dxr (1-deoxy-D-xylulose- 5-phosphate reductoisomerase) and dxs (1-deoxy- xylulose- 5-phosphate synthase) genes (from non-mevalonate pathway), individually and in combination improves the yield of carotenoids when combined with engineered carotenoid biosynthetic pathways in *E. coli* (Matthews and Wurtzel, 2000; Jones et al., 2000).

1.2 Medicinal plants in South Africa

South Africa is rich in plant biodiversity, with almost 10 % (about 25 000 plant species) of all the plants known to humankind and is home to one of the 6 richest plant kingdoms in the world. About 70 % of these species are endemic to South Africa. (Torssel, 1997). It is estimated that there are more than 100 000 practising traditional healers in the country, with a contingent industry worth about R500 million per annum (Mander and Le Breton, 2005). More than 80% of South Africans rely on indigenous medicinal plants for their health care. Mander (1998) estimated that there are 27 million indigenous medicine consumers in South Africa with a large supporting industry. Up to 700 000 tons of plant materials are consumed annually which most of these plant species are collected in the wild for local use (Cunningham, 1988) and international trade (Lange, 1997). The current demand for numerous plant species resulting in intensive harvesting of indigenous plant stocks, and this is in association with the lack of major resource management and plant production, has resulted in the paucity of numerous indigenous medicinal plants. According to the South Africa Trade Directory of Indigenous Natural Products, this situation has already "killed more than 80% of the country's highly-valued medicinal trees". Twenty thousand harvested tons of more than 1 000 plant species are harvested with a street-value of R270 million and traded annually in the informal and commercial sectors for indigenous medicinal use (Technology for sustainable livelihood, Department of Science and Technology, Republic of South Africa.

1.3 The genus *Helichrysum*

The genus *Helichrysum* belongs to the family Asteraceae and is represented by approximately 600 species in Africa with about 244 species indigenous to South Africa (Drewes and van Vuuren, 2008). The name *Helichrysum* is derived from the Greek *helios*, the sun, and *chrysos,* gold, as there are many species with yellow flower heads.

Bioactivities of different species of *Helichrysum* such as antioxidant, antimicrobial, antifungal, antimalarial, antiviral and antidiabetic, have been widely reported in literature (Tomas-Barberan et al., 1988; Tomas-Lorente et al., 1989; Tomas-Barberan et al., 1990; Meyer et al., 1996; Tape et al., 2005; Drewes et al., 2006; van Vuuren et al., 2006; Lall et al., 2006; Aslan et al., 2006). Ethanol extracts of *Helichrysum armenium*, *H. graveolens* and *H. plicatum* have been reported to be active against *Staphylococcus albus* and *S. aureus* (Cosar and Cubukcu, 1990). The dichloromethane extract of *H. stoechas* was found to be active against Gram positive bacteria (Rios et al., 1991) and extracts of *H. decumbens* and *H. nitens* showed antifungal activity (Tomas-Barberan et al., 1988; Tomas- Lorente et al., 1989). An antimalarial study has shown that the essential oil of *H. cymosum* is active against the chloroquine-resistant *Plasmodium falciparum* FCR-3 strain (Van Vuuren et al., 2006).

1.3.1 Compounds isolated and identified from the genus *Helichrysum*

Many *Helichrysum* species have been studied for their chemical components. These include 38 species from South Africa (Jakupovic et al., 1986; Jakupovic et al., 1989b; Meyer et al., 1997) eight from Madagascar (Randriaminahy et al., 1992), several from Spain (Tomas-Baberan et al., 1988; Tomas-Barberan et al., 1990) and many species from Australia (Jakupovic et al., 1989a) The fact that different *Helichrysum* species produce different secondary metabolites (acetophenones, favonoids, phloroglucinols) as a biochemical defense mechanism against bacteria

and fungi is of great interest, since it indicates the use of different metabolic pathways (Mathekga et al., 2000). Figure 1.2 indicates some of isolated and identified compounds from different parts of *Helichrysum* species.

1.3.2 *Helichrysum aureonitens*

Helichrysum aureonitens Sch. Bip. is a perennial, with many slender, erect, stems about 300 mm high, and arising from a creeping rootstock. The stems and leaves are covered with white woolly hairs. The leaves are narrowly oblong to oblong-spathulate, with margins flat or a little revolute (rolled to the underside of the leaf). Each leaf has a tiny, hard, hairless tip (Ready, 2007). Globose flower heads compact clusters with bracts around each head, and bright yellow, often tinged with brown (Fig. 1.2). Flowering time is from September to February. The fruits are one-seeded and each bears a number of fine bristles. *H. aureonitens* is widespread in the grasslands of Limpopo, North-West, Gauteng, and Mpumalanga in the north, and south through Swaziland, eastern Free State, Kwazulu-Natal and western Lesotho to the Eastern Cape, as far as King William's Town. It is therefore found in the summer rainfall region of South Africa. It grows in full sun in moist to well-drained sandy loam soils (Ready, 2007). Seeds of *H. aureonitens* are usually difficult to grow as they need to be stimulated by fire to germinate (Ready, 2007).

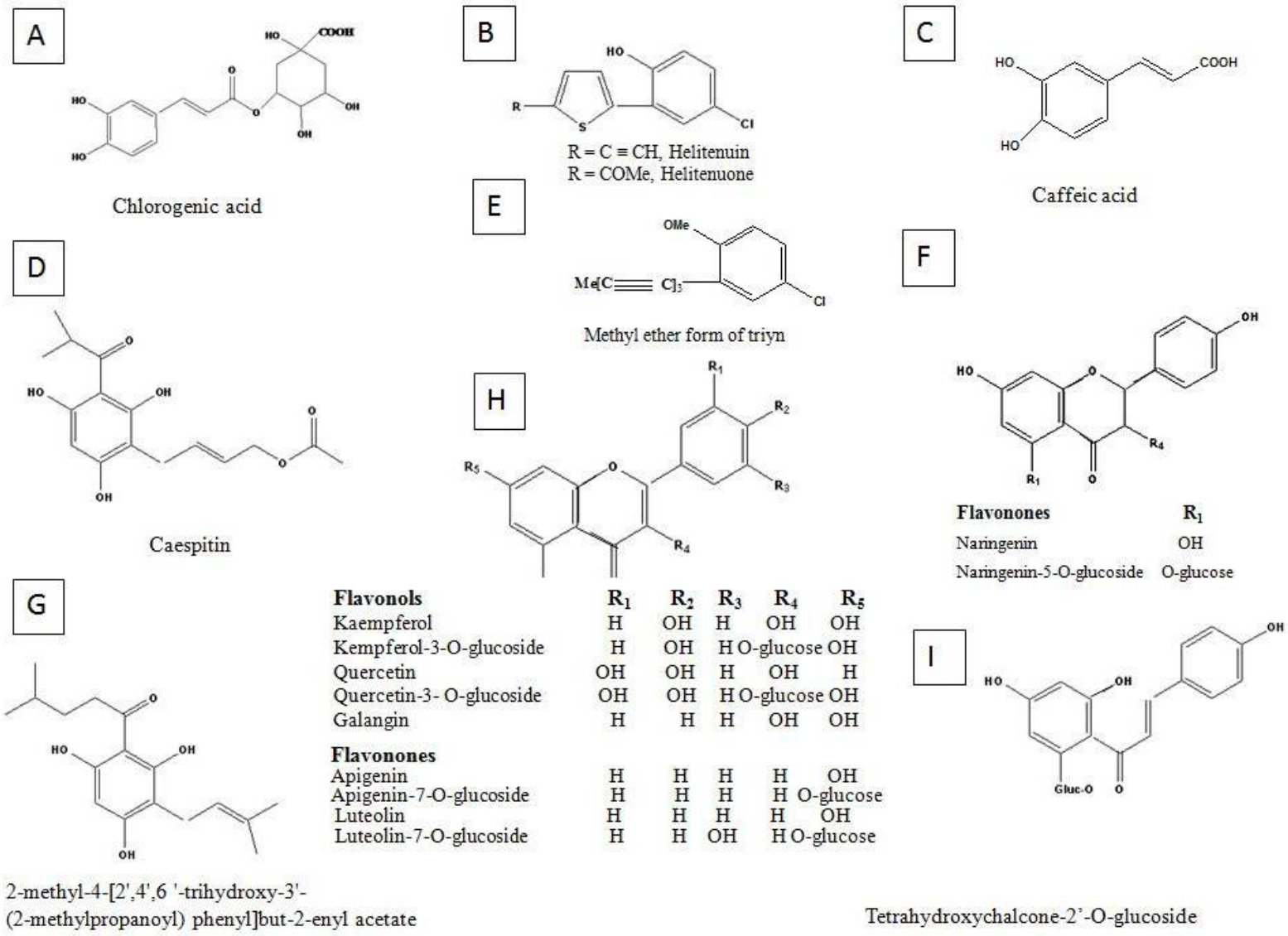

Fig. 1. 2. Isolated compounds from different parts of *Helichrysum* species. **A:** *H. arenarium* (Czinner et al., 2000); **B:** *H. tenuifolium* (Bohlmann et al., 1979); **C:** *H. arenarium* (Czinner et al., 2000); **D:** *H. caespitium* (Mathekga et al., 2000); **E:** *H. coriaceum* (Bohlmann et al., 1984); **F:** *H. arenarium* (Czinner et al., 2000); **G:** *H. caespitium* (Dekker et al., 1983); **H:** *H. arenarium* (Czinner et al., 2000); **I:** *H. stoechas* (Carini et al., 2001).

1.3.3 Medicinal properties of *H. aureonitens*

H. aureonitens is used 'to invoke the goodwill of ancestors, and by diviners to induce trances' (Pooley, 1998). It is also used as a remedy for bed-wetting in children and for treating a wide range of infections (Ready, 2007). In rural areas it is used to kindle fires by friction. It can be used to keep red mites away (Ready, 2007). It is also widely used in folk medicine (Phillips, 1917). Extracts from *H. aureonitens* are used topically by the indigenous people of South Africa against infections such as *Herpes zoster* (Meyer et al., 1996; Afolayan and Meyer, 1997). Antimicrobial and antiviral activity has been also reported (Meyer et al., 1996; Afolayan and Meyer, 1997). The dichloromethane extract has been found to be active against five Gram-positive bacteria and the methanol extract is active against *Bacillus cereus, B. pumilus* and *Micrococcus kristinae* (Meyer and Afolayan, 1995). Essential oils of the aerial parts of *H. aureonitens* has also shown a significant inhibitory effect on Gram-positive and Gram-negative bacteria (Yani et al., 2005).

Fig. 1. 3. Aerial parts of *Helichrysum aureonitens*.(www.plantzafrica.com).

1.4 Aims of the study

Galangin (3,5,7-trihyroxyflavonone) is one of the compounds that has been isolated from the aerial parts of *H. aureonitens*. Galangin has shown inhibitory activity against bacteria, fungi and viruses (Afolayan and Meyer, 1997; Meyer et al., 1997). Based on these results, this study was planned initially to investigate how the production of galangin can be enhanced in *Helichrysum aureonitens*? For that purpose the strategy of elicitation of galangin in different systems including hairy root induction and cell suspension cultures was planned. The intention was to establish a cell suspension culture system as well as the induction of a hairy root culture with *Agrobacterium sp*., and then investigate the elicitation of galangin using biotic and abiotic elicitors. Based on the initial negative results of the hairy root induction experiments it was found that hairy root induction is not feasible and the experiment was terminated.

After the establishment of the cell suspension culture of *H. aureonitens*, it was found that galangin was not detected in these samples, but surprisingly some other compounds which were not observed in intact plants of *H. aureonitens*.

These preliminary results lead to the following hypotheses for this study:

Hypotheses:

1- Cells of *H. aureonitens* cell suspension culture might produce interesting and perhaps novel compounds when growing in the dark, while the PAL enzyme is not activated.

2- Since previous results on *H. aureonitens* in our laboratory have shown the presence of the kaempferol, but none of its 4'-OH precursors, it is hypotesised that kaempferol is probably synthesized via galangin by the C4H enzyme.

1.5 Objectives of this study

According to the defined hypotheses, the objectives of this study were as follows:

1- Investigation on the reasons for the absence of galangin in the cell suspension cultures of *H. aureonitens.*

2- Isolation and identification of the major compound in the cell suspension culture of *H. aureonitens.*

3- The bioactivity of the isolated major compound against *Mycobacterium tubercolusis* and a human cancer cell line, prostate epithelial carcinoma (DU145).

4- Investigation on the biosynthetic pathway for galangin and subsequently kaempferol production in *H. aureonitens* and its relation with the (4'-OH) biosynthetic pathway of flavonols.

Chapter 2

Production of galangin in the cell suspension cultures of *Helichrysum aureonitens*

2.1 Abstract

In *Helichrysum aureonitens* galangin is one of the flavonol compounds with good medicinal properties. *H. aureonitens* was targeted to be enhanced via cell suspension culture as a potential system to produce valuable natural products. In ethanol extracts of the cell suspension cultures, galangin was however not detected, even though it was present in the leaves of intact plants. Other compounds were induced to be synthesised in higher amounts in the cells of the *H. aureonitens* suspension cultures than in the intact plants. According to the known biosynthetic pathway for the production of flavonols, some intermediates were searched for by GC-MS, including cinnamic acid, *p*-coumaric acid and naringenin as well as kaempferol. None of the mentioned intermediates was detected in the *H. aureonitens* cells suspension cultures. It was therefore concluded that the biosynthetic pathway of 4'-OH flavonol compounds is not functioning in the *H. aureonitens* cells suspension cultures.

2.2 Introduction

According to the medicinal properties of *H. aureonitens* described in the previous chapter in general and those of galangin specific, the enhancement of pharmaceutical compounds in cell suspension cultures of *H. aureonitens* seems to be important. *H. aureonitens* is only found in the wild and the negative effect of unsustainable collection from the wild has been mentioned before. Therefore it is important that other strategies should be considered to promote conservation of the plants and to try to increase the quality and quantity of medicinal compounds through different methods. Plant cell culture systems represent a potential renewable source of valuable medicinal compounds such as flavours, essences and colourants that cannot be produced by microbial cells or chemical syntheses. Recent advances in molecular biology, enzymology, physiology and fermentation technology of plant cell cultures suggest that these systems will become a viable source of important natural products (DiCosmo and Misawa, 1995). According to Ramachandra Rao and Ravishankar (2002) the advantages of this technology over the conventional agricultural production are as follows:

1- Independence of geographical and seasonal variations and various environmental factors.
2- Offering a defined production system, that ensures the continuous supply of products uniform in quality and yield.
3- Producing novel compounds that are not normally found in the parent plant.

Optimizing the cultural conditions, such as media components, phytohormones, pH, temperature, aeration, agitation and light affects the production of secondary metabolites (Mulabagal and Tsay, 2004). Through optimization of cultural conditions several products were found to be accumulating in the cultured cells at higher levels than compared to the native plants. Manipulation of the physical aspects mentioned before and the nutritional elements in a

culture is perhaps the most efficient way for the optimization of culture productivity. Good examples are the production of ginsenosides by *Panax ginseng* (Furuya et al., 1984; Furuya, 1988; Choi et al., 1994; Franklin and Dixon, 1994), rosmarinic acid by *Coleus bluemei* (Ulbrich et al., 1985), shikonin by *Lithospermum erythrorhizon* (Takahashi and Fujita, 1991), ubiquinone-10 by *Nicotiana tabacum* (Fontanel and Tabata, 1987), berberin by *Coptis japonica* (Matsubara et al., 1989), all which accumulated at higher levels in the cultured cells than in the intact plants. Moreover, secondary pathways are activated in response to stress by induction of secondary metabolites as a defence to the stress condition. This response can be used for increasing the production of secondary metabolites by the application of biotic and abiotic elicitors in the cell suspension culture system (DiCosmo and Tallevi, 1985; Eilert, 1987; Barz et al., 1988).

There is little information with regards to the yield and biological activity of compounds in *Helichrysum* species in cell suspension culture systems. This part of the study was therefore carried out to establish a cell suspension culture system of *H. aureonitens* with the purpose of the enhancement of galangin using elicitors as well as the chemical analysis of some intermediates from the galangin biosynthetic pathway.

2.3 Materials and methods

2.3.1 Induction of plant callus and establishment of cell suspension cultures

Cell suspension cultures were induced from fully expanded leaves, which were harvested from *H. aureonitens* growing in the greenhouse. Leaves were surface sterilized for 30 seconds with 70 % (v/v) ethanol, followed by treatment with 5 % sodium hypochlorite plus 1 drop Tween-20 for 10 minutes and then washed three times with sterile distilled water. Callus induction was carried out by culturing the leaf explants on Murashige and Skoog (1962) (MS) medium supplemented with MS vitamins, 3 % (w/v) sucrose, 5.37 µM α-naphthalenacetic acid (NAA) and 0.85 µM 6-benzylaminopurine (BA). The pH was adjusted to 5.7 with NaOH (1 M) and the cultures were incubated at 25 °C in the dark. Suspension cultures of *H. aureonitens* were established from the leaf-derived callus in the liquid medium with the same medium composition as that used for callus induction medium and incubated at 25 °C in dark conditions on a rotary shaker at 110 rpm. Subcultures were created at 20-day intervals by transferring 200 mg of fresh cells into a 100-ml Erlenmeyer flask containing 20 ml fresh medium and incubated on a rotary shaker at 110 rpm at 25 °C in the dark.

2.3.2 Extraction and thin layer chromatography (TLC)

In a preliminary experiment the fresh leaves from greenhouse grown plants and the cells from the cell suspension cultures of *H. aureonitens* were extracted with different solvents including methanol, ethyl acetate, acetone, ethanol and dichloromethane, but for the rest of study only 100 % ethanol was used for extraction. Detection of compounds, including galangin in the extracts and fractions were initially carried out by using thin layer chromatography (TLC). The samples were spotted on TLC plates, developed in a hexane, dichloromethane, methanol

system (10: 10: 0.5) followed by exposure to short wave UV light to detect the conjugated compounds. The TLC plates were then sprayed with a 0.34 % vanillin in a 3.5 % sulphuric acid in methanol solution to visualize all the compounds on the TLC plates.

2.3.3 GC-MS analysis

GC-MS analysis was carried out under the following conditions:

A mass spectrometer (JMS-AM SUN200, JEOL) connected to a gas chromatograph (6890A, Agilent Technologies), with the following parameters were used, EI (70 eV), source temperature 250 °C, HP-5 column (30 m x 0.25 mm, 0.32 µm film thickness, J&W Scientific), injection temperature 250 °C, column temperature program: 80 °C for 1 min, then raised to 300 °C at a rate of 10 °C min^{-1}, and held on this temperature for 10 min; interface temperature 280 °C, carrier gas He, flow rate 1.2 mL min^{-1}, splitless injection.

The chemical structure of the isolated compound was identified by applying different spectroscopic techniques including NMR (1D and 2D) IR, MS and UV.

2.4 Results

Results showed that galangin is present in all leaf extracts, but was not observed in the extracts of cell suspension cultures (Fig. 2.1). However, two other major compounds were produced in the cell suspension cultures.

In addition to TLC, GC-MS analysis was carried out for the detection of galangin in the cell suspension cultures of *H. aureonitens*. GC-MS results also confirmed that galangin is not produced in the cell suspension cultures, although it is present in leaves of *H. aureonites* (Fig. 2.2). To investigate any blockage of galangin biosynthesis, some intermediates from the known galangin biosynthetic pathway which includes cinnamic acid, *p*-coumaric acid, naringenin as well as kaempferol were GC-MS analyzed in the cell suspension culture sample. Figures 2.3 to 2.6 show that these intermediates were also not detected in the cell suspension of *H. aureonitens*.

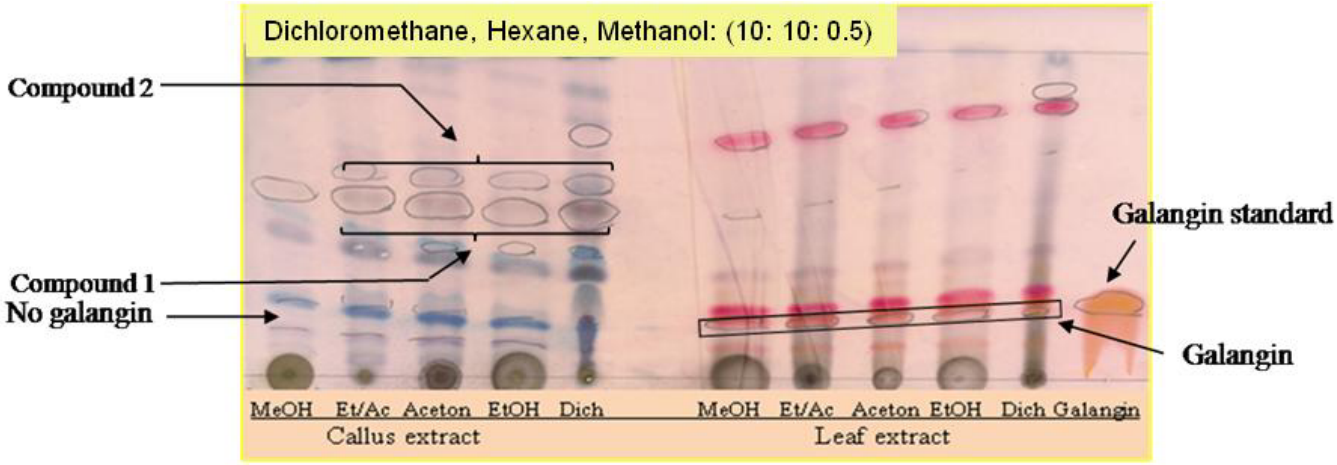

Fig. 2.1: Comparison of the galangin production in the leaf and in the cell suspension cultures of *H. aureonitens* with different solvents. Compounds **1** and **2** are the compounds produced in the cell suspension cultures of *H. aureonitens*, but not observed in its leaves.

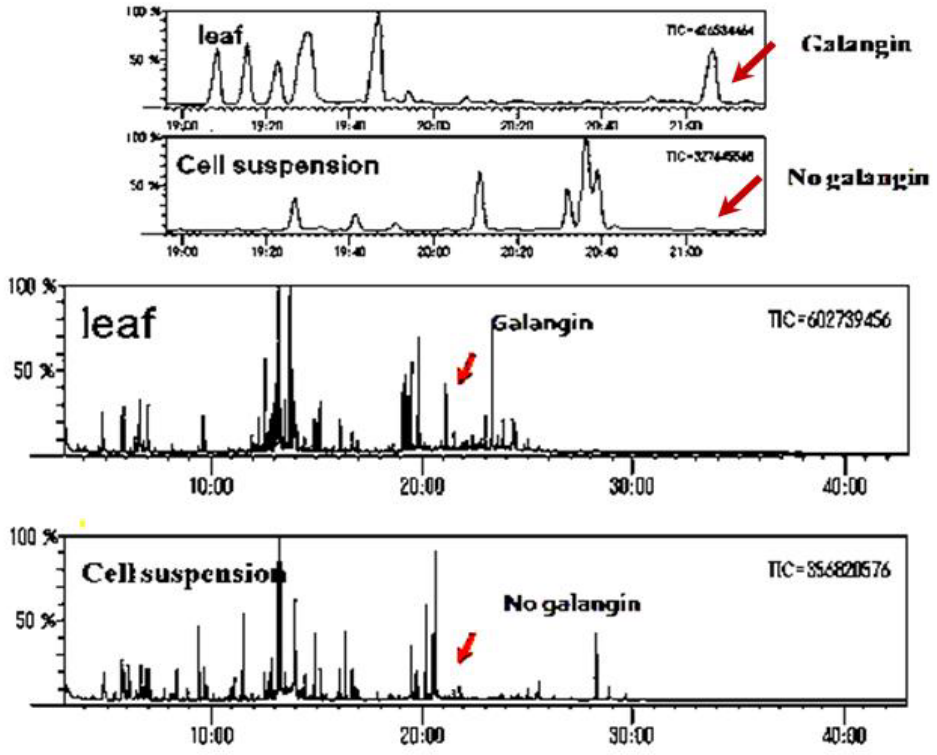

Fig. 2.2: GC-MS analysis comparison of the galangin production in the leaf and in cell suspension cultures of *H. aureonitens.* Arrow show the position of galangin picks.

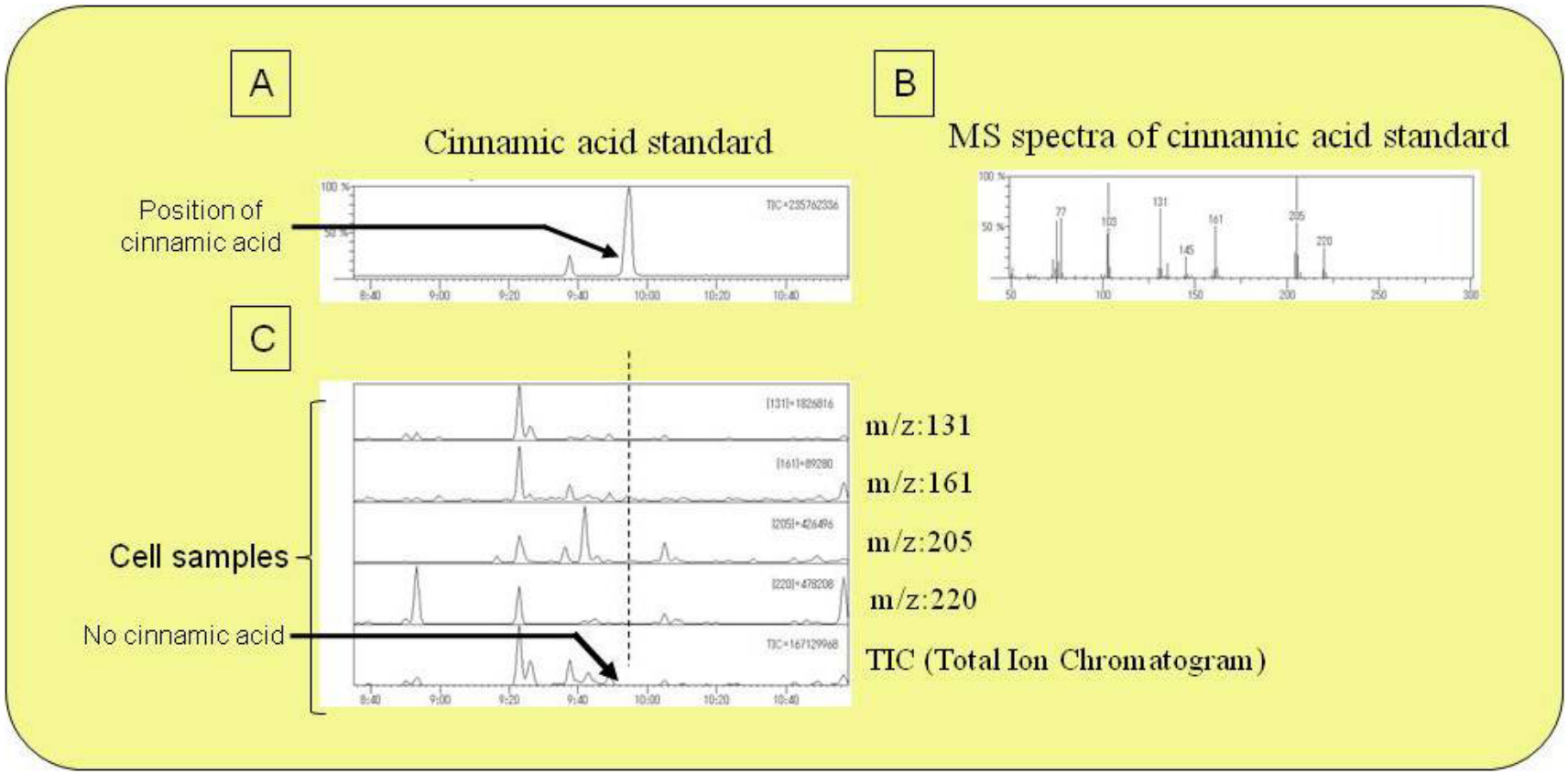

Fig. 2.3: GC-MS analysis of the cell suspension culture extracts for the detection of cinnamic acid. **A:** Cinnamic acid standard chromatograph; **B:** MS chromatograph of the cinnamic acid standard; **C:** Total ion chromatogram.

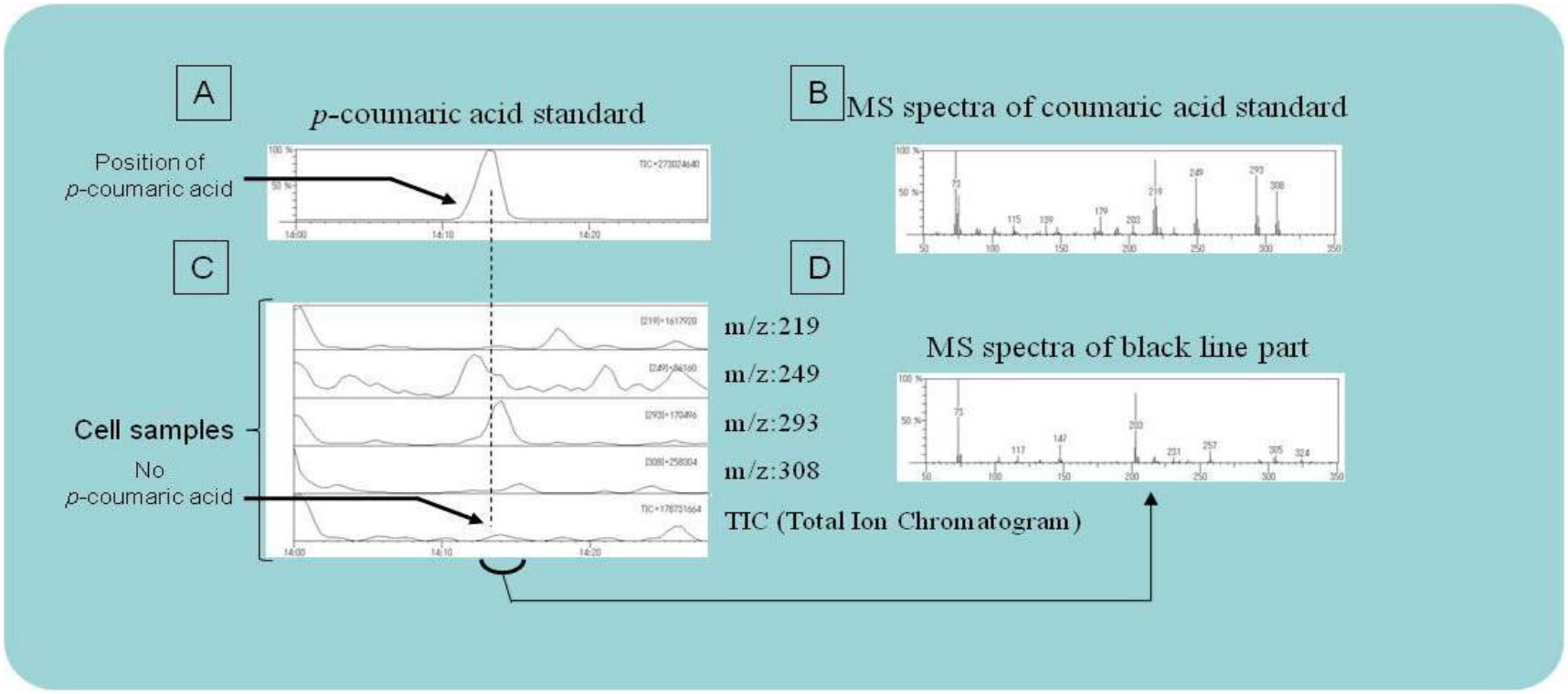

Fig. 2.4: GC-MS analysis of the cell suspension culture extracts for the detection of *p*-coumaric acid. **A:** *p*-coumaric acid standard chromatograph; **B:** MS chromatograph of the *p*-coumaric acid standard; **C:** Total ion chromatogram. **D:** MS chromatograph of the arrowed pick in TIC chromatogram.

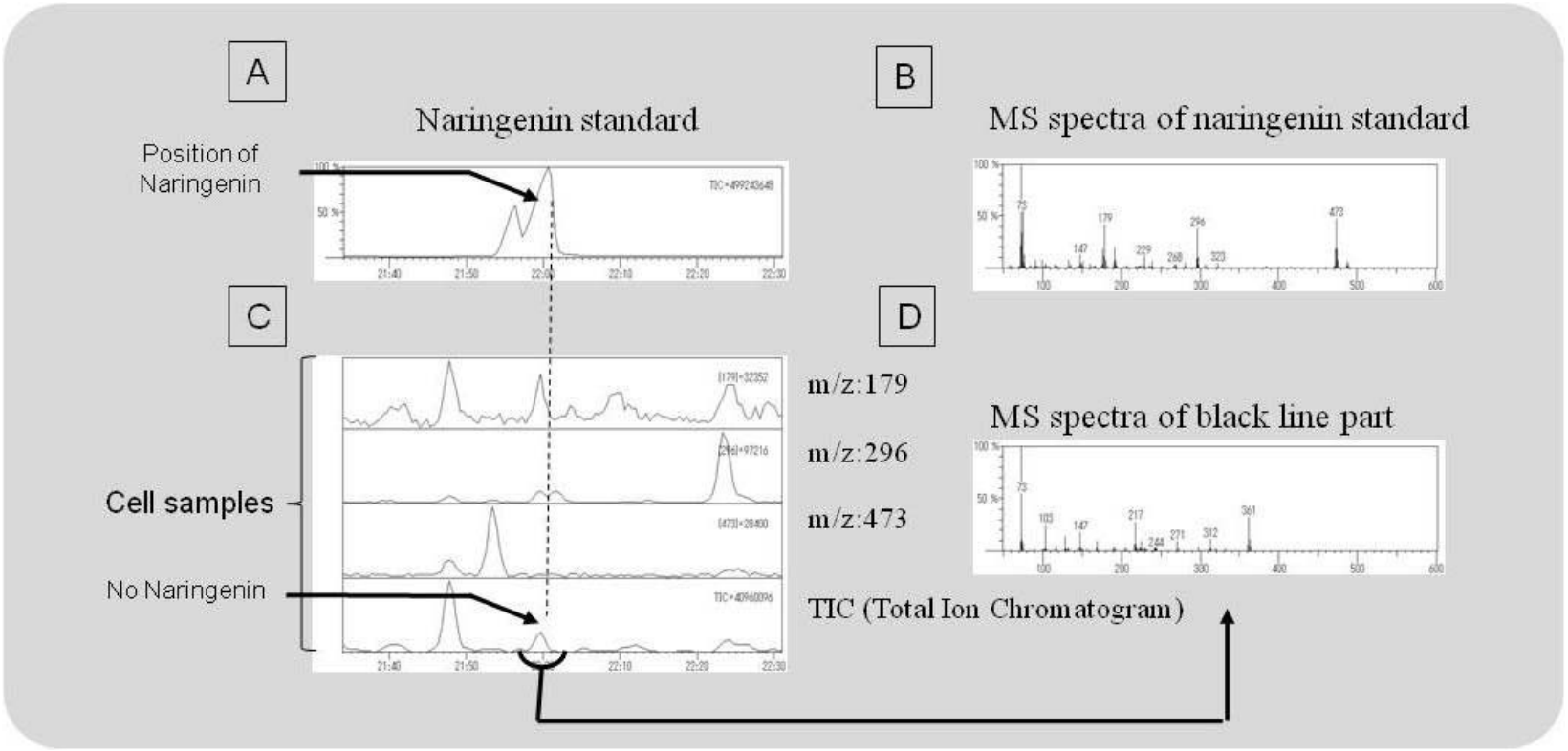

Fig. 2.5: GC-MS analysis of the cell suspension culture extracts for the detection of naringenin. **A:** naringenin standard chromatograph; **B:** MS chromatograph of the naringenin standard; **C:** Total ion chromatogram. **D:** MS chromatograph of arrowed pick in the TIC chromatogram.

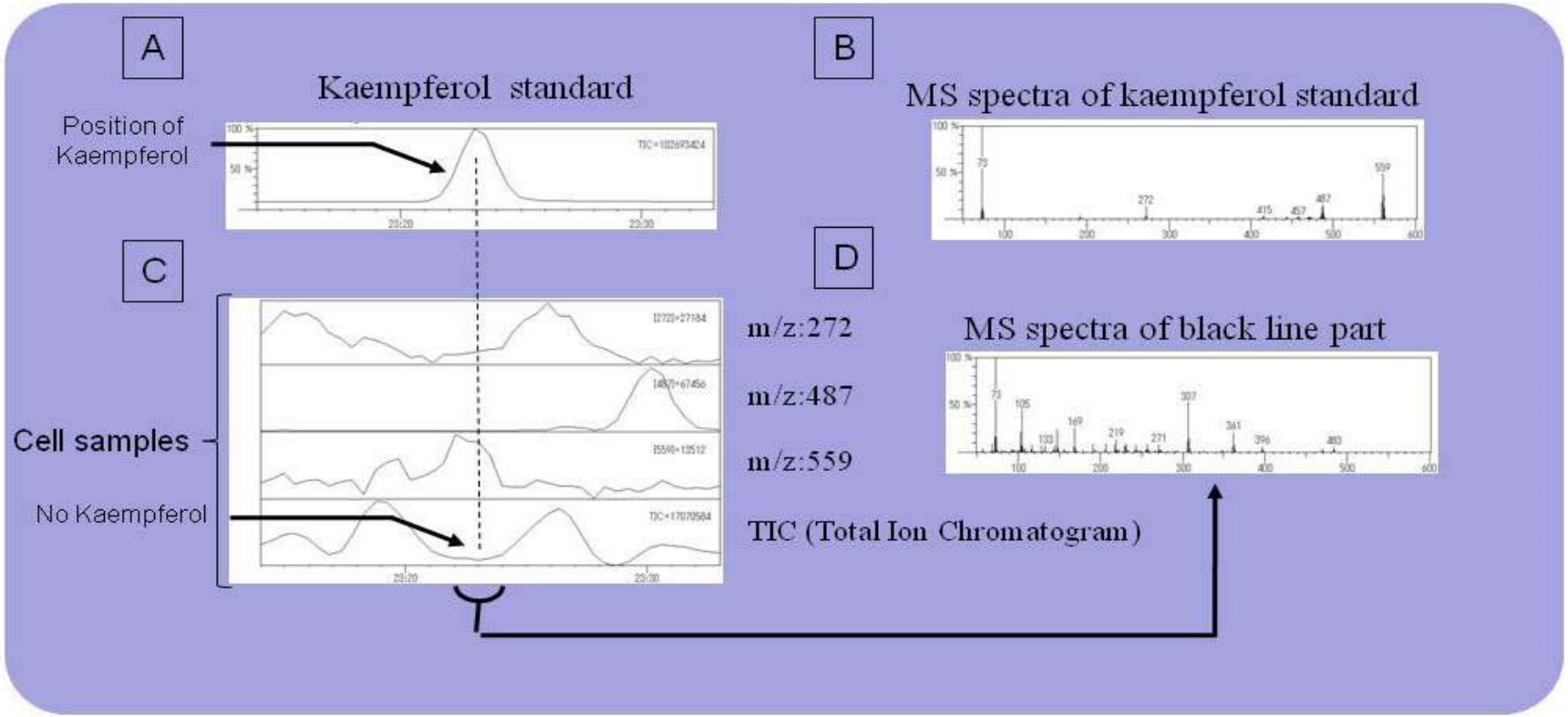

Fig. 2.6: GC-MS analysis of the cell suspension culture extracts for the detection of kaempferol. **A:** Kaempferol standard chromatograph; **B:** MS chromatograph of kaempferol standard; **C:** Total ion chromatogram. **D:** MS chromatograph of the arrowed pick in TIC chromatogram.

2.5 Discussion

The above results demonstrate that galangin is not produced in the cell suspension culture of *H. aureonitens.* There are several other reports that show the absence of certain compounds in the cells suspension cultures while they are present in the intact plants. An example of this is the callus culture of ginseng (*Panax ginseng*), in which polyacetylene, one of the anti-cancer compounds in intact ginseng plants, was not detected in the callus cultures (Yang et al., 1999). The absence of galangin in the cell suspension cultures of *H. aureonitens* could be due to different reasons. It has been stated that many of the natural products of interest are synthesized in organized tissues including leaves, roots and flowers. It is widely accepted that some levels of cellular or tissue differentiation is necessary for the biosynthesis of many compounds (Lindsey and Yeomem, 1983). The absence of peroxidise in carrot cells suspension cultures was attributed to lack of cell or tissue differentiation (Dornenburg and Knorr, 1995). In this experiment undifferentiated cells were used in the cell suspension cultures. This could be a possible reason for the absence of galangin in the cell suspension cultures of *H. aureonitens.* The experiment also showed that the tested flavonoid intermediates as well as kaempferol were not detected in the of *H. aureonitens* cell suspension culture. Accordingly it can be concluded that the known flavonol biosynthetic pathway in the cell suspension culture of *H. aureonitens* is not functioning. Previous experiments demonstrated that in some species of cell suspension cultures of parsley (*Petroselinum crispum*) and a few related species, flavonoids were absent in dark-grown cells and accumulate rapidly upon UV irradiation (Matern et al., 1983). It has also been shown that flavonoids, CHS protein, and CHS, PAL, and 4CL transcripts all accumulate in the same epidermal cells, following UV irradiation (Schmelzer et al., 1988; Wu and Hahlbrock, 1992). When using *Arabidopsis,* UV irradiation induces flavonoids (particularly kaempferol derivatives) and sinapate esters and isoflavonoids and psoralens in other species

(Hahlbrock, 1981; Beggs et al., 1985; Li et al., 1993; Lois, 1994). This phenomenon is attributed to the UV protection role of flavonoids in plants (Jordan, 1996; Reuber, et al., 1996).

It has been also mentioned that some of the phenylpropanoid compounds are induced in response to wounding or to feeding by herbivores. A good example would be kaempferol, another member of the flavonol group of compounds. The accumulation of flavonols such as kaempferol and its glycosides are induced by both wounding and pollination in petunia stigmas and appear to be required for normal pollen development (Mo et al., 1992; van der Meer et al., 1992; Vogt et al., 1994). The absence of galangin in the cells of *H. aureonitens* suspension cultures can be attributed to the dark condition or the absence of any kind of stresses in this experiment.

2.6 Conclusions

The results in this chapter showed that galangin and all the tested intermediates from the known flavonol biosynthetic pathway were not detected in the cell suspension culture samples of *H. aureonitens*. It can thus be concluded that galangin is a flavonoid-group member which is not inducible in this system, although it is produced in the leaf samples. One of the differences between these two samples is the different growing conditions, such as the absence or presence of light. It is therefore recommended that further experiments with *H. aureonitens* suspension cultures should include factors such as light in association with some biotic elicitors.

There are some compounds present in large amounts in the cell suspension cultures of *H. aureonitens,* and these were observed in much lower quantities in the leaf samples (Fig. 2.2). This leads to some questions that will be addressed in further chapters.

1- What is the observed compound in the cell suspension cultures of *H. aureonitens*?

2- Is the galangin biosynthetic pathway blocked and is there any relationship between these compounds and the galangin biosynthetic pathway?

3- Whether the tested intermediates of the galangin biosynthetic pathway in the cell suspension cultures are detected in the intact plant samples as well.