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Twenty years of research into medicinal plants: Results and perspectives

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Abstract

Over the years 1981 to 2001 there has been a rapid evolution of research into medicinal plants. The major improvement has been the introduction of simple and predictive bioassays for bioactivity-guided isolation. Radical developments in separation methods have also taken place. Another important addition has been the development of hyphenated techniques involving HPLC: LC/UV, LC/MS, LC/MS^n and LC/NMR. These are indispensable nowadays for the early detection and identification of new compounds in crude plant extracts. Hyphenated techniques allow an efficient targeted isolation approach for the discovery of new lead compounds. Other areas of increasing importance include the investigation of toxic constituents of plants and phytomedicines, and the effects of genetic modifications on plant secondary metabolites.

Introduction

Medicinal plants have always had a important place in the therapeutic armoury of mankind. Up to 80% of populations in developing countries are totally dependent on plants for their primary health care. And despite the remarkable progress in synthetic organic chemistry of the twentieth century, over 25% of prescribed medicines in industrialised countries derive directly or indirectly from plants (Newman et al., 2000). This percentage can reach 50% when the over-the-counter (OTC) market is taken into consideration.

In this context, and in view of the rapid disappearance of tropical forests and other important areas of vegetation, there is good reason to pursue research in the domain of plants. Urgent attention has to be paid to as many as possible of the estimated 350,000 plant species on earth, a large percentage of which still have not been investigated for their phytochemical and pharmacological potential. This green inheritance thus represents an enormous reservoir of putative lead compounds awaiting to be discovered.

The Institute of Pharmacognosy and Phytochemistry (IPP) of the University of Lausanne has been active in this area for 20 years now, in an effort to provide new and useful bioactive compounds and to increase awareness of the possibilities available through medicinal plant research.

During this time, there has been a considerable evolution of methodology and it is the aim of this review to give an insight into the techniques introduced and some future challenges.

Evolution of separation methods

Phytochemistry has traditionally involved the isolation and structure determination of plant constituents. While, with the introduction of a whole array of spectroscopic techniques (mainly 1D- and 2D-NMR) structure elucidation has become mostly a routine affair, separation often remains a complex process, which can last from days to months, depending on the problem being tackled. The importance of proper isolation and purification cannot be over-emphasized. Chromatographic techniques which are rapid and do not lead to decomposition, material loss, or artefact formation are needed. For these reasons, and also with the aim of finding simpler solutions to complex separation problems, the last few years have seen the development of a remarkable array of new chromatographic methods, both analytical and prepar-

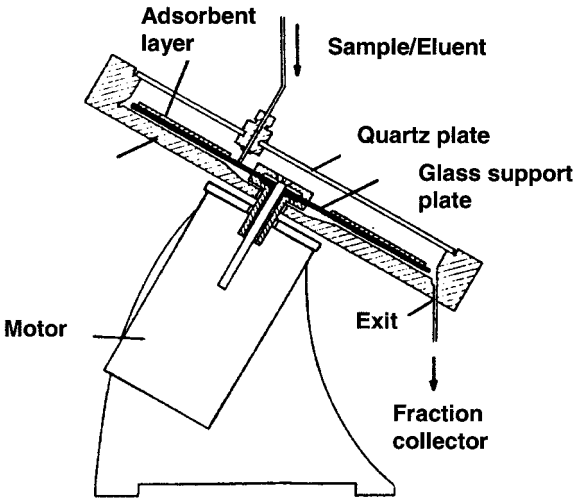

Figure 1. The Chromatotron CTLC instrument.

ative. Some of these modern preparative techniques (Hostettmann et al., 1998), used in the separation of natural products, are shown in Table 1.

For example, preparative TLC, which involves mobile phase migration through a stationary phase by capillary action, has been modified to include accelerated eluent flow by the action of a centrifugal force with rotating circular plates (centrifugal TLC, CTLC). This method avoids the need to scrape bands off the plates and has been used for the separation of numerous classes of natural products (Hostettmann et al., 1998). One instrument for CTLC applications, the Chromatotron, is shown in Figure 1.

Conventional open-column chromatography is simple, cheap and universally practised. However, it is a slow method and often produces irreversible adsorption of samples. For these reasons, various alternatives have been introduced, ranging from flash chromatography to preparative HPLC (Table 1). These techniques have in common the application of pressure to a chromatography column, or, in the case of vacuum liquid chromatography (VLC), the use of suction to force eluent through a sorbent. Applications are vast and can be found in practically every current phytochemistry journal (Hostettmann et al., 1998; Kaufman et al., 1999).

Whereas the above-mentioned methods all include solid supports, there are separation techniques which rely on liquids alone. These generally involve the partition of a sample between two immiscible solvents, the relative proportions of solute passing into each of the two phases being determined by the respective partition coefficients. The classical example is countercurrent distribution (CCD), developed in the 1940s and 1950s. More recently, pioneering work by Ito in the USA has led to the introduction of several instruments capable of performing countercurrent chromatography (CCC). Droplet countercurrent chromatography (DCCC) involves the passage of droplets of a mobile phase through a stationary liquid phase, effectively producing a constantly changing interface and leading to repeated solute partition between the two phases (Figure 2, Tanimura et al., 1970). Numerous solvent systems have been used for the separation of natural products by DCCC (Hostettmann, 1980; Hostettmann et al., 1984).

Application of a force field to countercurrent chromatography by rotating the coil or cartridge which contains the stationary phase provides an accelerated method for the separation of samples. This technique has been named centrifugal countercurrent chromatography (CCCC) or centrifugal partition chromatography (CPC) and is currently the most widely employed all-liquid method for the separation of natural products and lead compounds. The great advantage of CCCC is that there is no irreversible adsorption to a solid phase and also a much diminished risk of denaturing sensitive bioactive natural products. It is possible to invert mobile and stationary phases, thus catering for a wider polarity range of samples. Use is made of CPC both for the fractionation of crude plant extracts and for the purification of compounds from simpler mixtures (Marston and Hostettmann, 1994). This is of great value when searching for new lead compounds. An example of the separation of different phenolic compounds from the Brazilian plant *Kielmeyera coriacea* (Guttiferae) is shown in Figure 3 (Cortez et al., 1999).

Development of LC hyphenated techniques

When searching for active plant metabolites and new lead compounds, biological screening followed by activity-guided fractionation is the standard procedure. The drawback with this strategy is that it frequently leads to the isolation of known metabolites. Chemical screening of crude plant extracts therefore constitutes an efficient complementary approach, allowing localization and targeted isolation of new types of constituents with potential activities. This procedure also allows for recognition of known metabolites at the earliest stages (dereplication), thus avoiding

Table 1. Preparative chromatography of natural products.

Solid-liquid chromatography	Liquid-liquid chromatography
Planar chromatography (TLC, CTLC, OPLC)	Countercurrent distribution
Open column chromatography	Droplet countercurrent chromatography (DCCC)
Vacuum liquid chromatography (VLC)	Rotation locular countercurrent chromatography (RLCC)
Pressure liquid chromatography (Flash, LPLC, MPLC, HPLC, SFC)	Centrifugal partition chromatography (CPC)

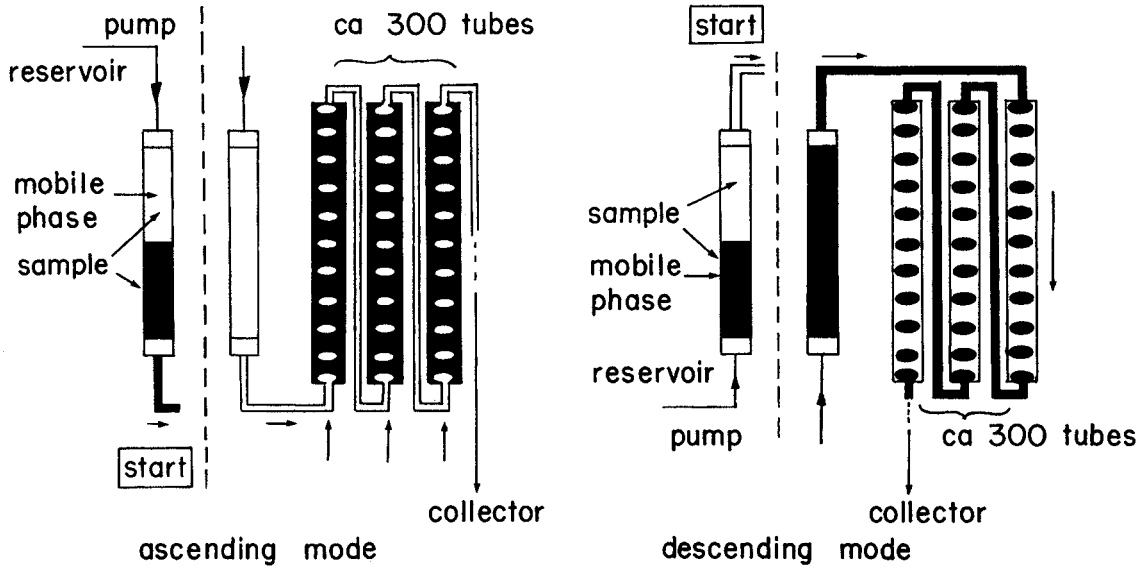

Figure 2. Droplet countercurrent chromatography.

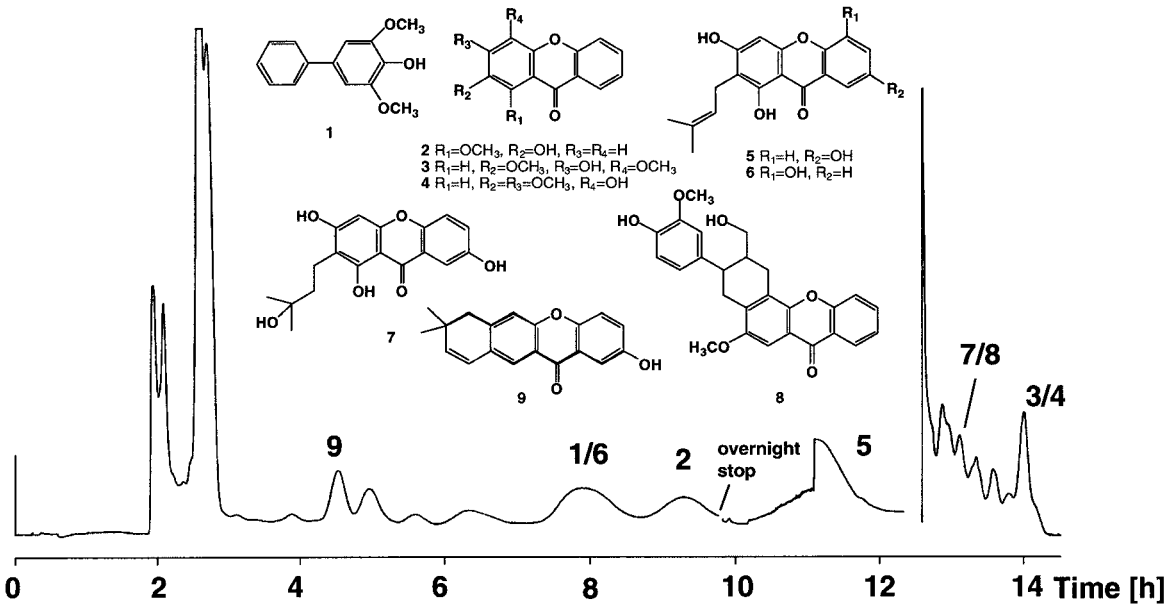

Figure 3. Separation of xanthones and a biphenyl from twigs of *Kielmeyera coriacea* (Guttiferae) by CPC.

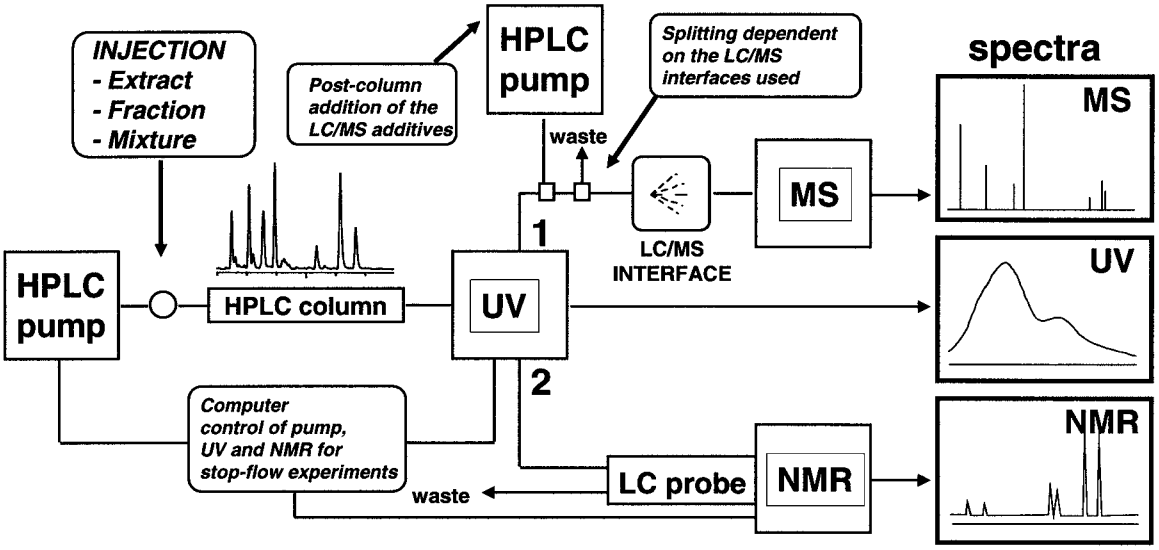

Figure 4. Equipment for LC/UV/MS and LC/UV/NMR analyses of plant extracts.

costly and time-consuming isolation of common constituents. Chemical screening can involve simple TLC analysis but a much more selective and predictive method is HPLC coupled to different detectors (hyphenated techniques). This provides efficient separation of metabolites and valuable structure information at the same time. Coupling HPLC with mass spectrometry (LC/MS) and UV diode array (LC/UV) detection has now been complemented by the connection of HPLC to nuclear magnetic resonance (LC/NMR). The latter allows the complete on-line structure determination of numerous plant metabolites (Wolfender et al., 2001). The equipment installed at the IPP allows acquisition of LC/UV/MS and LC/UV/NMR data (Figure 4). To illustrate the applications of chemical screening, the on-flow LC/^{1}H NMR analysis of a fraction from the Indonesian plant *Orophea enneandra* (Annonaceae) is shown in Figure 5. In order to determine the nature of the constituents, LC/UV/MS and LC/NMR were performed. The LC/UV spectra of **1**–**3** were typical of lignans and analysis of the on-line MS and NMR data allowed their identification as phylligenin (**1**), eudesmin (**2**) and epieudesmin (**3**). Compound **4** was shown to be polycerasoidol, a tocopherol derivative, while **5** could be assigned to the polyacetylenes after evaluation of on-line data. However, final identification of **5** as octadeca-17-en-9,11,13-triynoic acid (a new compound) was only possible after targeted isolation (Cavin et al., 1998).

Dereplication: micro-fractionation

While performing HPLC hyphenated techniques for chemical screening purposes, it is possible to combine these with on-line bioassays for the direct collection of results concerning activities of the substances under the peaks of a HPLC chromatogram (Dapkevicius et al., 2001). The number of available on-line bioassays, however, is limited and another approach is to run a micro-fractionation of the eluate from the HPLC column.

Direct fractionation can be done into microwell plates. Solvent is then evaporated and the biological test is run. Wells containing active constituents are then correlated with peaks in the HPLC chromatogram.

Introduction of simple benchtop bioassays

A combination of chemical screening with biological screening is the fastest way to arrive at new lead compounds from plants. For this purpose, it is necessary to have relatively simple biological or pharmacological tests available in order to localise the chosen activity in the plant extracts or in the numerous fractions resulting from the different purification steps which lead from the plant to the pure active constituents. These tests have to be very sensitive because the active substances may be present in the plant at very low concentrations. They are also required to be specific for

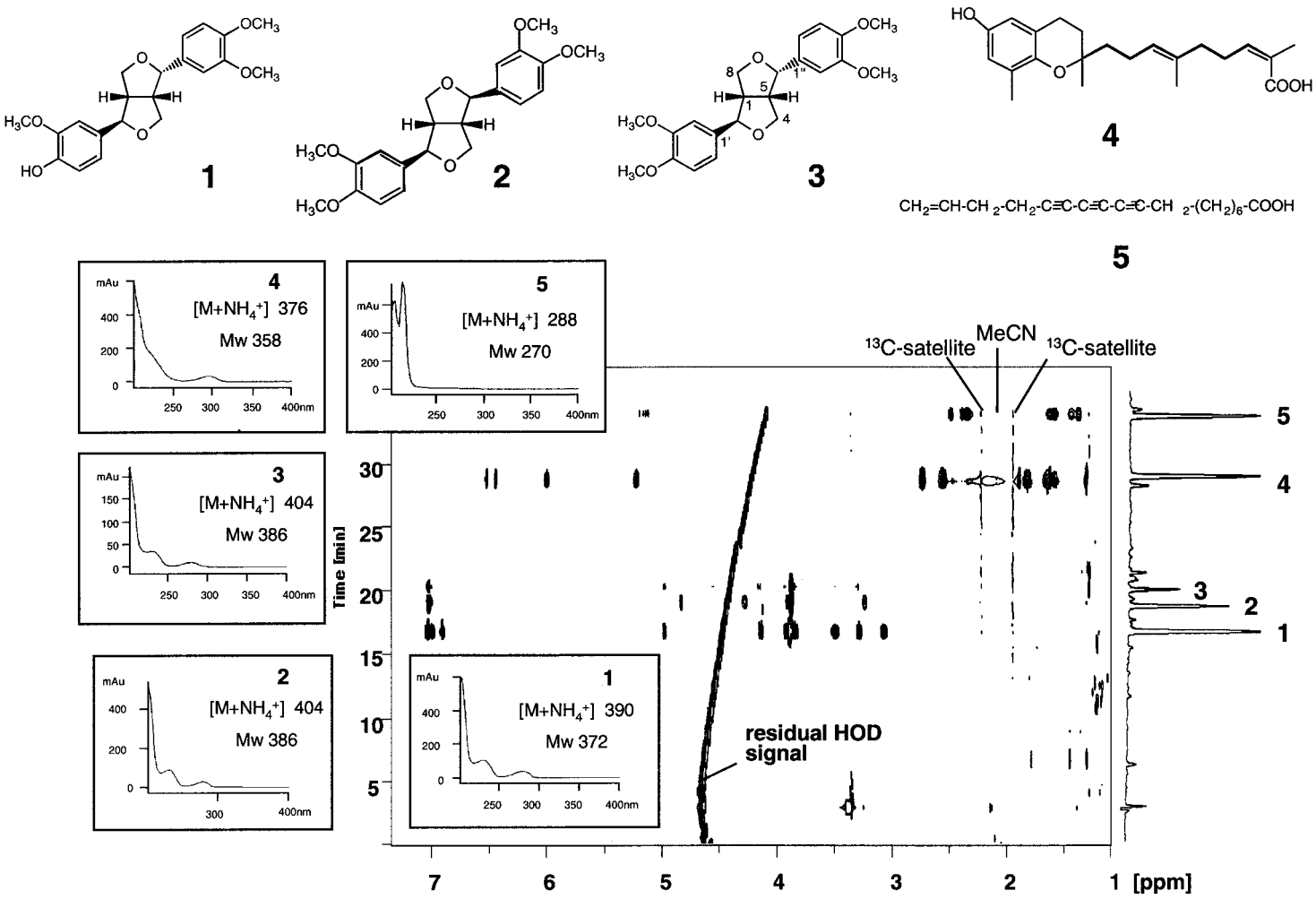

Figure 5. Bidimensional on-flow LC/^{1}H NMR chromatogram of a fraction of *Orophea enneandra* (Annonaceae) with UV spectra and molecular ions detected by LC/TSP-MS. HPLC: Nova-Pak C_{18} column (4 μm, 150 × 3.9 mm i.d.); gradient MeCN-$D_2$0 (0.05% TFA) 20:80 to 95:5 in 50 min (1 ml/min). LC/NMR: 24 scans/increment; flow cell 60 μl, 3 mm i.d.; 500 MHz.

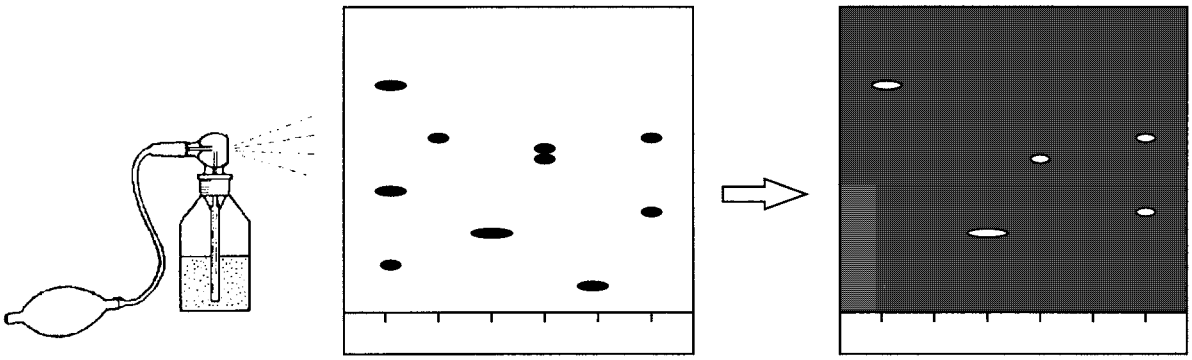

Figure 6. Bioassay for antifungal activity against the plant pathogenic fungus *Cladosporium cucumerinum* by TLC autobiography.

the target involved. The principal targets for biological tests can be divided into 6 groups:

- lower organisms: microorganisms (bacteria, fungi, viruses)
- invertebrates: insects, crustaceans, molluscs
- isolated subcellular systems: enzymes, receptors
- animal or human cell cultures
- isolated organs of vertebrates
- whole animals

Compilations of certain biological tests exist (Gebhardt, 2000; Hostettmann, 1991; Houghton, 2000) and many other assays are used by pharmaceutical companies in the high throughput screening (HTS) of samples.

For benchtop bioassays in the phytochemical laboratory, simple and rapid tests are also required but the scale of screening is obviously orders of magnitude lower than HTS in industry. Thus the targets employed at the IPP involve the first three categories in the above list: lower organisms, invertebrates and isolated subcellular systems (Table 2).

For investigating antifungal or antibacterial activity, the process is relatively simple. For example, a plant extract or an isolated substance is placed in contact with human pathogenic fungi. It is then straightforward to observe inhibition of spore growth or death of the fungus. This can be done in classical solution assays or, alternatively by thin-layer bioautography. Bioautography on TLC plates involves running samples in a suitable solvent and drying the plate. A nutrient medium is inoculated with the microorganism and then sprayed on the plate. In the case of the plant pathogenic fungus *Cladosporium cucumerinum*, white-coloured areas of inhibition are visible against a grey background of fungal spores after incubation (Figure 6). Direct bioautography is not possible with yeasts such as *Candida albicans* and a rapid agar overlay assay is used. An agar layer containing the microorganism is spread over the TLC plate and there is transfer of active compounds from the stationary phase into the agar by diffusion. After incubation, the plate is sprayed with methylthiazoyltetrazolium chloride (MTT) which is converted into a purple MTT formazan dye by the yeast.

The advantage of these TLC bioautographic methods is that bioactivities can be associated with specific spots on the plate. This simplifies the localization of active compounds and helps the design of subsequent isolation strategy.

When the causes of a disease are known, it is possible to perform direct *in vitro* tests on the receptors or enzymes (of human or animal origin) implicated in the pathology of the complaint. Enzymatic tests are generally very specific and very sensitive. They are of great value for the screening of large numbers of samples. The experiments are often relatively easy and require only small amounts of material. Furthermore, the thin-layer chromatography approach can also be extended to enzymes. The first candidate enzymes chosen were the cholinesterases because of the current interest in the search for new drugs for the management of Alzheimer's disease by a mechanism involving inhibition of acetylcholinesterase (AChE). Galanthamine, an alkaloid from the snowdrop (*Galanthus nivalis*, Amaryllidaceae), is a potent inhibitor of AChE and has recently been introduced in Europe and the USA for the treatment of Alzheimer's disease. A simple TLC bioautographic method has been developed in which AChE is sprayed onto the plate after migration of samples. Following incubation and reaction with a colour reagent, areas of enzyme inhibition are seen as white spots on a purple background (Marston et al., 2002).

Table 2. Some simple bioassays used for biological screening.

Activity	Test type
Antibacterial	*Escherichia coli*, *Bacillus subtilis*
Antifungal	*Candida albicans* (Human pathogenic)
	Cladosporium cucumerinum (Plant pathogenic)
Antimitotic	*Micrasterias denticulata*
Larvicidal	*Aedes aegypti* (yellow fever)
Molluscicidal	*Biomphalaria glabrata* (schistosomiasis)
Spermicidal	Human spermatozoids
Acetylcholinesterase inhibition	Enyme TLC assay

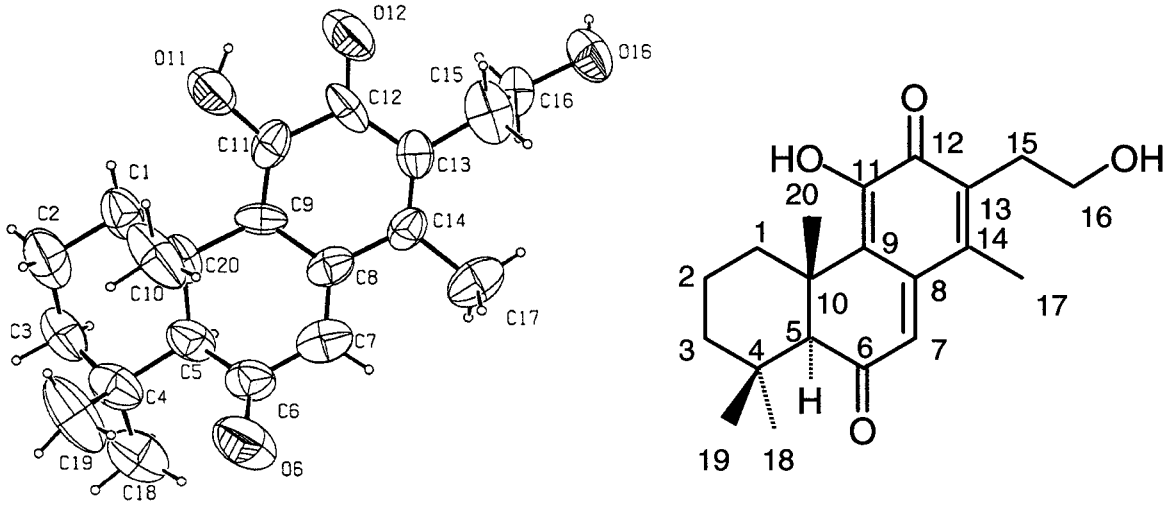

Figure 7. Structure of the major antifungal quinone from *Bobgunnia madagascariensis.*

Mention should also be made of a TLC assay using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical which is used to screen for radical scavenging activity (Cuendet et al., 1997). Radical scavengers reduce the radical, producing white spots on a purple background. Antioxidants can be detected by the bleaching of β-carotene (or crocin) on TLC plates (Pratt and Miller, 1984).

Plants studied and new lead compounds isolated

Over the years, a number of different research topics have been undertaken at the IPP. Most of these are listed in Table 3. A small selection of the topics will be discussed here.

One of the most intensely studied areas of research has been the investigation of plants with antifungal action. The increasing incidence of mycoses associated with AIDS and also those arising after treatment by immunosuppressive drugs has given fresh impetus to the search for novel antifungal agents. There are few really effective antifungal preparations currently indicated for the treatment of systemic mycoses and their efficacy is rather limited. New lead compounds are also needed in the field of agrochemistry.

More than 2000 plant extracts have been screened for their antifungal properties by the IPP using TLC bioautographic techniques. A dichloromethane extract of the root bark of the pan-African tree *Bobgunnia madagascariensis* (Leguminosae, formerly known as *Swartzia madagascariensis*) was found to give extremely good results in these tests. Chemical screening data suggested the presence of compounds with interesting chemical structures in this extract. Phytochemical investigation of the extract led to the isolation of 14 new diterpene quinones. The major constituent, a phenanthrene-3,9-dione (Figure 7), was also the strongest inhibitor of fungal growth (Schaller et al.,

Table 3. Areas of research at the IPP 1981-2001.

Schistosomiasis/molluscicides	Antimalarials
Antifungals	Antibacterials
Antioxidants	Radical scavengers
Spermicidals	
Gentians and IMAO	Epilobium and BPH
Yellow star thistle and neurological disorders of horses	Floppy trunk disease of elephants
Aphrodisiac plants	Genetically-modified organisms
Toxic plants	

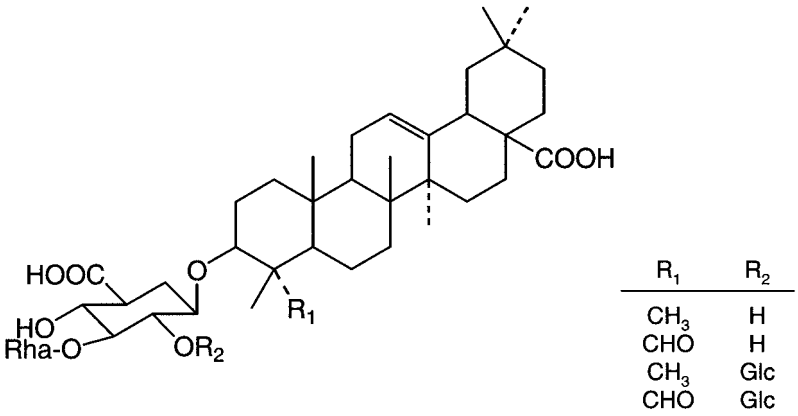

Figure 8. Structures of saponins isolated from fruits of *Bobgunnia madagascariensis*.

2000). Its structure was elucidated by different chemical and spectroscopic techniques, with final confirmation by single crystal X-ray analysis. The absolute configuration was determined by X-ray analysis of the 4-bromobenzoate derivative. The antifungal activity of the major compound was compared with that of commercial antifungal agents towards a panel of commercial and clinical fungi, such as several *Candida* species and pathogenic fungi of the type *Aspergillus*. Activities considerably superior to those of amphotericin B and fluconazole were obtained against *Candida* species. This prompted the incorporation of seven of the isolated diterpene quinones in preclinical testing.

The ultimate aim is to introduce new antifungal drugs which can be topically applied for dermatomycoses, useful for the treatment of systemic mycoses associated with HIV infections and which may be of importance in agrochemistry.

Certain plants have insect repellent or insecticidal properties, while others are active against insect larvae or molluscs (molluscicides). Screening tests for these activities on invertebrates are simple to perform. Insecticidal or larvicidal plants can play an important rôle in the prevention of tropical parasitic diseases, like malaria or yellow fever, transmitted by mosquitoes. As for molluscicidal plants, they can stop the propagation of schistosomiasis (bilharzia), a parasitic disease with a mollusc (freshwater snail) as intermediate host, which affects over 250 million people in Third World countries. *Bobgunnia madagascariensis* bears large fruits which were shown to exhibit molluscicidal activity. Phytochemical investigation showed that molluscicidal activity of the fruits was due to the saponin content of the plant. Four glucuronides of oleanolic acid and gypsogenin (Figure 8) were isolated from the aqueous extract. The highest activity of these saponins against *B. glabrata* snails was a LC_{100} of 3 mg/l for the rhamnosyl glucuronide of oleanolic acid, a value which is comparable to that of synthetic molluscicides. Two field trials were performed in Tanzania in order to test the molluscicidal activity of *B. madagascariensis* in a natural habitat. The densities of aquatic snails dropped to zero one week after a single application of *B. madagascariensis* water extract. The snails were observed only at low densities and never reached the initial density during the short-

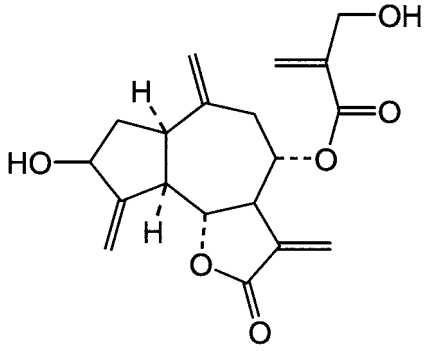

Figure 9. Structure of cynaropicrin from *Centaurea solstitialis*.

term and long-term follow-up period of five months (Suter et al., 1986).

A neurological disease of horses in California known as 'chewing disease' or 'yellow star thistle poisoning' has been linked to the ingestion of large amounts of yellow star thistle (*Centaurea solstitialis*, Asteraceae). The symptoms, abnormal movement disorders, resemble those of Parkinson's disease in humans so a phytochemical investigation of the thistle was undertaken. This work was done in collaboration with Professor Peter Jenner from King's College London because the London group was able to test samples in primary neuronal cultures of foetal rat brain. Thus a bioactivity-guided fractionation approach was possible, resulting in the isolation of four sesquiterpene lactones. Two of these, including cynaropicrin (Figure 9) were highly neurotoxic in the cell culture assay and may be responsible for the ability of the plant to cause neurodegenerative changes in the brain of horses (Wang et al., 1991).

Future directions

While much work has been done on the isolation of new lead compounds and the investigation of bioactive constituents of plants, there are still many avenues to be explored. More representative bioassays need to be introduced with suitable targets. Faster and more efficient dereplication techniques are continuously being sought for.

An aspect of plant constituents and especially phytotherapeuticals which needs considerable attention is the problem of toxicity. For substances with small therapeutic indices, such as the cardiac glycosides, there is an inherent danger of toxic responses. For certain well-known preparations such as ginkgo extracts, there is the possibility of finding traces of toxic material – in this case the ginkgolic acids.

A recent problem has been the confusion of components contained in a vegetable slimming preparation. Intoxications (mainly end stage renal failure) have resulted by the inadvertent substitution of constituent plants because of their similar Chinese names. Thus *Aristolochia fangchi* (Aristolochiaceae) has been used instead of the correct plant, *Schisandra tetrandra* (Menispermaceae). The genus *Aristolochia* contains nephrotoxic and carcinogenic aristolochic acids (Figure 10) and it is essential to have methods available for their selective and sensitive determination in plant preparations. For this reason, a LC/UV/MS analysis has been developed to provide an on-line detection of aristolochic acid I in plant preparations. Aristolochic acid I has a characteristic UV spectrum and the mass spectrum in the APCI mode shows a predominant ion at *m/z* 295 $[M\text{-}NO_2]^+$ (Figure 11). Under the conditions of analysis, a detection limit of 2 ng was determined for both UV and MS measurements (Ioset et al., 2002). Similar procedures need to be developed for other toxic principles which occur in plant preparations.

Genetically modified organisms (GMO) are another domain of high actuality. While much is known about the influence of genetic modification on primary metabolites of plants, virtually no research has been undertaken to evaluate changes in secondary metabolites. This aspect could, however, be of considerable importance – for example, in potatoes, differences in secondary metabolite composition could affect their resistance to disease. Consequently, a study has begun at the IPP to develop analytical methods for the safety assessment of GMO by looking at the secondary metabolite composition. The coupled techniques of LC/UV and LC/MS are being used to measure α-solanine and

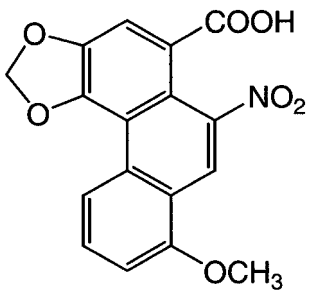

Figure 10. Structure of aristolochic acid I.

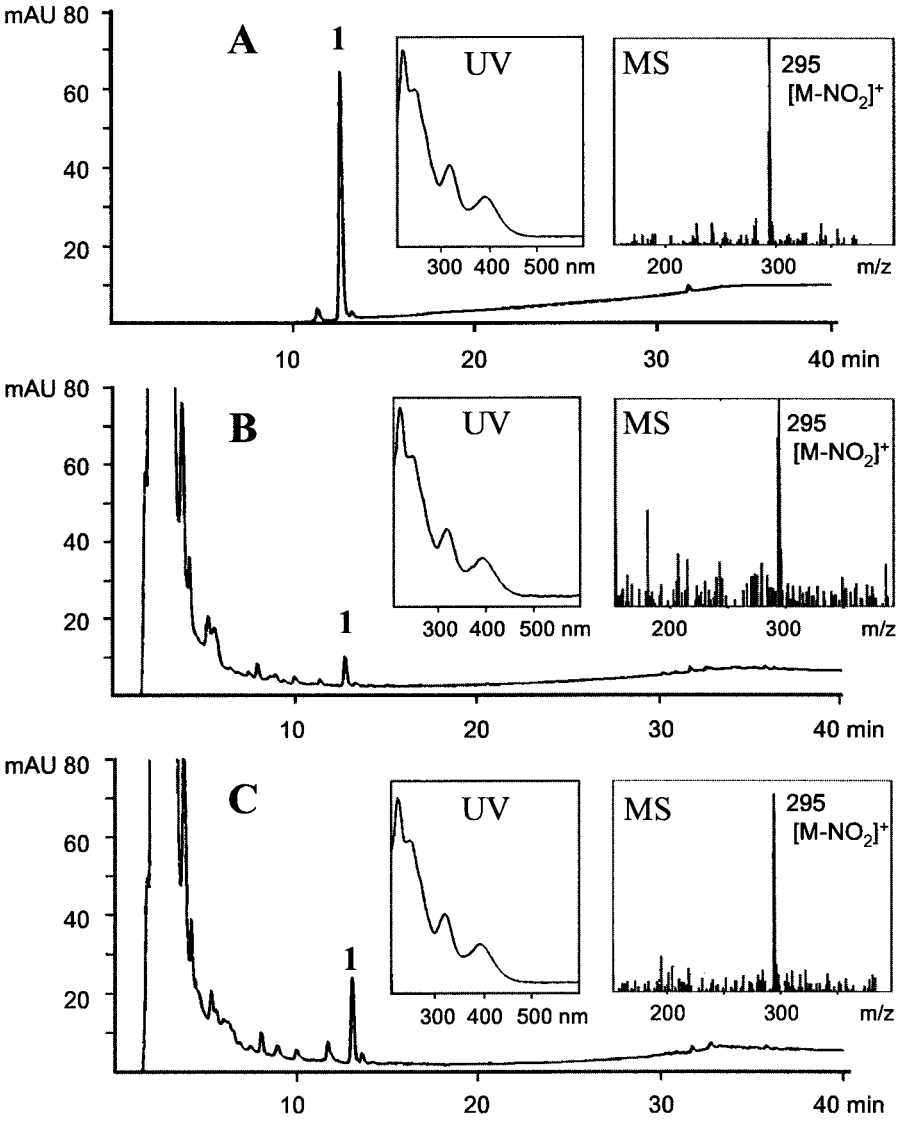

Figure 11. Detection of aristolochic acid I (**1**) by LC/UV/MS (wavelength 254 nm). A) Aristolochic acid I reference sample; B) Methanol extract of *Aristolochia acuminata* leaves; C) Mixture of A) and B).

α-chaconine levels in normal and genetically modified potatoes, since these two glycoalkaloids are known to show toxicity. These studies need to be extended to other GMO, such as soya products and maize.

Conclusions

Even though only a limited selection of the therapeutical applications and bioactivities of plant products is outlined here, a general idea is given of their extraordinary usefulness.

The value of the dual chemical-biological screening approach has been amply demonstrated for the discovery of important plant-derived bioactive compounds and work is continuing on various other leads from extracts of material collected in Africa, South America and Asia.

Not only do natural products themselves possess significant pharmacological properties but they also provide a source of very important lead compounds which can be modified for the development of new therapeutics. The introduction of new bioassays and the expansion of screening programmes will provide a valuable source of new drugs in the future, a reservoir which should be sensibly exploited before the large-scale destruction of these natural resources goes too far. The most effective strategy is to perform multidisciplinary work on the development of drugs from plants, a task that can only be effectively tackled by collaboration between botanists, ethnobotanists, pharmacognosists, phytochemists, biologists, pharmacologists and medical doctors.

Acknowledgements

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