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Strategy for the Biological and Chemical Evaluation of Plant Extracts

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Abstract: When investigating large numbers of plants, it is important to have the means available to perform a dereplication of the samples in order to provide a means of selecting species for study. This can be achieved by combining simple biological assays with hyphenated HPLC analyses, such as LC/UV, LC/MS and LC/NMR. Once a candidate plant has been chosen, a suitable isolation procedure can be employed for the isolation of the active principles.

Plants are a valuable source of new natural products. Despite the availability of different approaches for the discovery of therapeuticals, natural products still remain as one of the best reservoirs of new structural types. Figures show that about 25% of all prescriptions sold in the United States are for natural products, while another 25% are for structural modifications of a natural product (ref. 1). Furthermore, Farnsworth (ref. 1) claims that 119 characterized drugs are still obtained commercially from higher plants and that 74% were found from ethnobotanical information.

Of the several hundred thousand plant species around the globe, only a small proportion has been investigated both phytochemically and pharmacologically. When one considers that a single plant may contain up to thousands of constituents, the possibilities of making new discoveries become evident. The crucial factor for the ultimate success of an investigation into bioactive plant constituents is thus the selection of plant material (ref. 2).

In view of the large number of plant species potentially available for study, it is essential to have efficient systems available for the rapid chemical and biological screening of the plant extracts selected for investigation.

CHEMICAL SCREENING

Isolation of pure, pharmacologically active constituents from plants remains a long and tedious process. For this reason, it is necessary to have methods available which eliminate unneccesary separation procedures. Chemical screening is thus performed to allow localization and targeted isolation of new or useful types of constituents with potential activities. This procedure enables

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recognition of known metabolites in extracts or at the earliest stages of separation and is thus economically very important.

Thin-layer chromatography (TLC) is the simplest and cheapest method of detecting plant constituents because the method is easy to run, reproducible and requires little equipment (ref. 3). However, for efficient separation of metabolites, good selectivity and sensitivity of detection, together with the capability of providing on-line structural information, hyphenated high performance liquid chromatographic (HPLC) techniques are preferred (ref. 4). They play an important role as an analytical support in the work of phytochemists for the efficient localization and rapid characterization of natural products.

HPLC coupled to a UV photodiode array detector (LC/UV) has been widely used for the analysis of crude plant extracts. The UV spectra of natural products obtained on-line by LC/UV give useful information on the type of constituents and in the case of certain classes of compound, such as the polyphenols, indications of oxidation patterns (ref. 5).

HPLC coupled to mass spectrometry (LC/MS) is a newer hyphenated technique. Mass spectrometry is one of the most sensitive methods of molecular analysis and yields information on the molecular weight as well as on the structure of the analytes. However, it has been difficult to achieve on-line coupling of HPLC and MS. These problems have now been overcome with the introduction of different LC/MS interfaces. For the HPLC screening of crude plant extracts (ref. 6), three interfaces have been used in our laboratory: thermospray (TSP), continuous flow fast atom bombardment (CF-FAB) and electrospray (ES). They cover the ionization of relatively small non-polar products (aglycones, MW ca. 200) to highly polar molecules (glycosides, MW ca. 2000). LC/TSP-MS allows satisfactory ionization of moderately polar constituents such as polyphenols or terpenoids in the mass range 200-800 amu. For larger, polar molecules such as saponins (MW > 800), CF-FAB or ES are the methods of choice (ref. 6).

HPLC coupled to a NMR spectrometer (LC/NMR) has, until recently, been little used mainly because of its lack of sensitivity. However, progress in pulse field gradients and solvent suppression, together with improvements in probe technology and the construction of high field magnets now allow many applications of the technique. LC/NMR has important potential for on-line structure identification of natural products. Indeed, NMR spectroscopy is by far the most powerful spectroscopic technique for obtaining detailed structural information about organic compounds in solution (ref. 7). Coupling to a HPLC instrument is straightforward and solvent suppression techniques enable the use of non-deuterated solvents (methanol or acetonitrile) under reversed-phase conditions. Water is replaced by D_2O.

BIOLOGICAL SCREENING

Screening programmes for biologically active natural products require the right bioassays. Detection of compounds with the desired activity in complex plant extracts depends on the reliability and sensitivity of the test systems used. Bioassays are also essential for monitoring the required effects throughout activity-guided fractionation: all fractions are tested and those continuing to exhibit activity are carried through further isolation and purification until the active monosubstances are obtained. The search for promising plant extracts and subsequent activity-guided isolation put specific requirements on the bioassays to be used. They must be simple, inexpensive and rapid in order to cope with the large number of samples - including extracts from

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the screening phase and all fractions obtained during the isolation procedure. They must also be sensitive enough to detect active principles which are generally present only in small concentrations in crude extracts. Their selectivity should be such that the number of false positives is reasonably small (ref. 2).

In this chapter, emphasis will be placed on TLC autographic assays, which combine TLC with a bioassay in situ and allow localization of active constituents in a complex matrix (ref. 8).

The number of available targets for biological screening is limited. Furthermore, bioassays are often not reliaby predictive for clinical efficiency. For these reasons, it is extremely helpful to have chemical screening techniques available as a complementary approach for the discovery of new molecules which might serve as lead compounds. As mentioned above, chemical screening also serves for dereplication purposes.

TLC screening for antifungal and antibacterial compounds

The use of immunosuppressive drugs and the spread of AIDS have resulted in an increasing occurrence of opportunistic systemic mycoses. The infections commonly observed in the immune-compromised host include candidiasis (*Candida albicans* and related species) of the oesophagus and mouth, cryptococcosis (*Candida neoformans*) and aspergillosis (*Aspergillus flavus, A. fumigatus, A. niger*). As there are few really effective antifungal preparations currently available for the treatment of systemic mycoses and as the efficacy of existing drugs is rather limited, it is important to find new sources of antifungal agents. Plant-derived natural products may offer potential leads for novel agents which act against these mycoses.

There is also a need to screen plants for constituents which have activity against plant pathogenic fungi: fungal attack can be economically devastating in agriculture.

Bioautography is a very convenient and simple way of testing plant extracts and pure substances for their effects on both human pathogenic and plant pathogenic microorganisms. It can be employed in the target-directed isolation of active constituents. Three bioautographic methods have been described (ref. 9): agar diffusion, direct TLC bioautographic detection and agar-overlay.

Direct bioautography is applicable to microorganisms that can grow directly on the TLC plate. The agar-overlay technique is a hybrid of the two other methods and is applicable to a broad spectrum of microorganisms. It produces well defined zones of inhibition and is not sensitive to contamination. Active compounds are transferred from the stationary phase to the agar layer (which contains the microorganism) by a diffusion process. After incubation, the plate is sprayed with a tetrazolium salt (e.g. MTT) which is converted to a formazan dye by the microorganism. Inhibition zones are observed as clear spots against a purple background.

Direct bioautographic procedures have been described for spore producing fungi such as *Aspergillus*, *Penicillium* and *Cladosporium* (ref. 10) and also for bacteria (ref. 11). Numerous antifungal compounds have been characterized using *Cladosporium cucumerinum* in a routine assay (ref. 12).

The agar-overlay assay has been used for yeasts such as *Candida albicans* and can also be applied to bacteria such as *Bacillus subtilis* (ref. 13). If phenol red is incorporated into media containing 0.6% agar and the plates are sprayed with MTT, clearer results are obtained, with dark red coloured inhibition zones appearing against a blue background (ref. 14). This method works successfully with a range of microorganisms, including *Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (ref. 14).

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TLC screening for radical scavengers and antioxidants

Another use of TLC for biological testing is as a means for discovering new antioxidants in higher plants (ref. 15). These can be detected on a TLC plate by spraying with 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. Antioxidants reduce the radical, producing white spots on a purple background. Alternatively, the bleaching of crocin (which normally gives a yellow colour on the plate) can be used to distinguish components of plant extracts with potential antioxidant or radical-scavenging properties.

SCREENING FOR BIOACTIVE COMPOUNDS AND THEIR SUBSEQUENT ISOLATION

An antifungal naphthoquinone from *Swertia calycina* (Gentianaceae)

Among the examples of natural products isolated in our laboratory using the TLC bioautographic approach is a 2-methoxynaphthoquinone from *Swertia calycina* (Gentianaceae), a small plant found in Rwanda. This example illustrates well the combined use of TLC and HPLC in the search for new antifungal metabolites (Fig. 1). TLC bioautography of the dichloromethane extract of *S. calycina* showed a compound which strongly inhibited the growth of *C. cucumerinum*. HPLC-UV and HPLC-MS analyses of the extract revealed the presence of three main compounds: a bitter principle, a xanthone and a naphthoquinone derivative with a MW of 188. Comparison of on-line UV and MS data with a data bank allowed identification of the bitter principle as sweroside and the xanthone as decussatin. As these have no antifungal properties, the strong activity of the dichloromethane extract was attributed to the naphthoquinone, a class of compounds which is known to have strong antimicrobial properties. Targetted isolation afforded the active compound, identified as 2-methoxy-1,4-naphthoquinone (**1**). Interestingly, quinones were previously not known to occur in the Gentianaceae. The minimum quantities of **1** required to inhibit the growth of *C. cucumerinum* and *C. albicans* on TLC plates were 0.1 and 0.4 µg, respectively (ref. 16). By comparison, the reference substance propiconazole was active at 0.1 and 0.001 µg, respectively.

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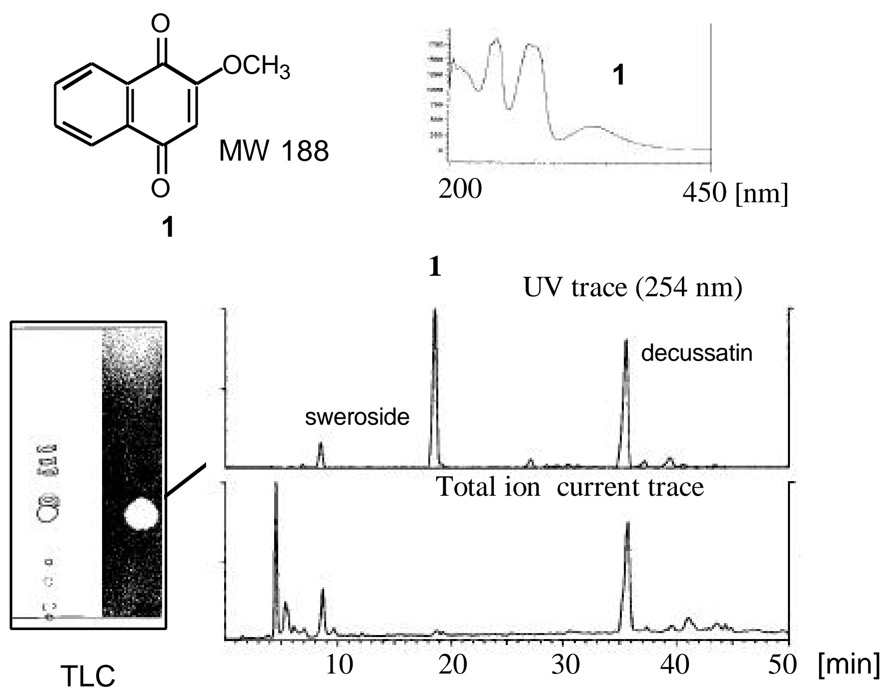

Fig. 1 TLC bioautography (*C. cucumerinum*) and LC/UV/MS analysis of *Swertia calycina* (Gentianaceae) whole plant dichloromethane extract.

Antifungal constituents of *Parinari capensis* (Chrysobalanaceae)

During the screening of Zimbabwean plants for biological activities, a dichloromethane extract of *Parinari capensis* (Chrysobalanaceae) whole plant gave a positive response in the *C. cucumerinum* TLC bioassay. Analysis of this extract by LC/UV/MS showed the presence of 3 major peaks (**2**-**4**) in the HPLC chromatogram when detecting at 210 nm (Fig. 2). LC/MS was performed with a thermospray interface, which is well adapted for the ionization of moderately polar molecules. The LC/TSP-MS of the peak **2** gave a protonated molecular ion $[M+H]^+$ at m/z 345.

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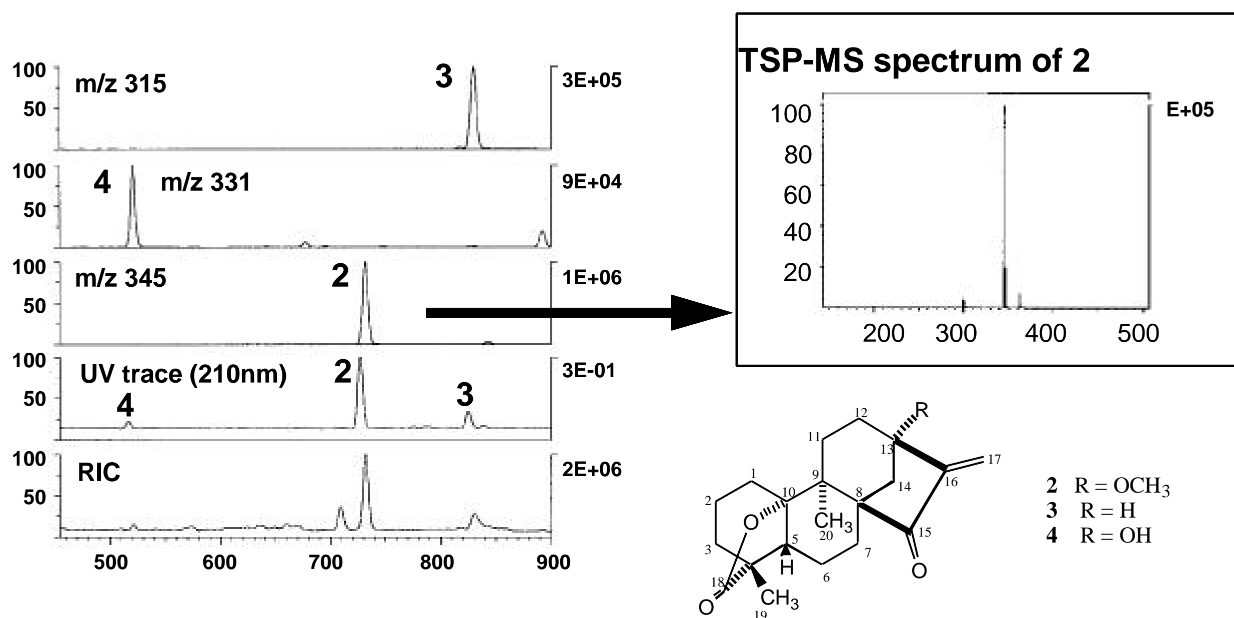

Fig. 2 LC/UV/MS analysis of *Parinari capensis* (Chrysobalanaceae) whole plant dichloromethane extract. HPLC: column Novapak RP-18; CH_3CN-H_2O 5:95 ∅ 70:30 in 30 min. TSP-MS: source temp. 280ºC; vaporizer temp. 100ºC; ammonium acetate buffer.

Fractionation of the extract was performed by a combination of medium-pressure LC, silica gel CC and Sephadex LH-20 gel filtration to give three major constituents **2**-**4**. Strucure elucidation by a combination of 1D- and 2D-NMR analysis showed the three compounds to be kaurenoid diterpenes, two of which, **2** and **4**, were new natural products.

The absolute configuration of **2** was determined from the crystal structure of the brominated derivative (crystallized from hexane-ethyl acetate). Completion of bromination was assured by DCI-MS. Thus **2** was identified as (4*R*,9*R*)-10-hydroxy-13-methoxy-9-methyl-15-oxo-20-norkaur-16-en-18-oic acid γ-lactone.

Antifungal activities were determined by a dilution assay. Compounds **2** and **3** inhibited growth of *C. cucumerinum* with MIC values of 20 µg/ml. Amphotericin B, used as positive control, gave a MIC value of 1 µg/ml (ref. 17). An independent investigation of *P. curatellifolia* gave the same three compounds and described their cytotoxic activities (ref. 18).

Iridoid glucosides with radical scavenging properties from *Fagraea blumei* (Loganiaceae)

Fagraea blumei (Loganiaceae) is a tree growing in southeast Asia. As part of our search for new antioxidants in higher plants, we detected a series of radical scavengers in the methanolic stem bark extract of *F. blumei* . These compounds exhibited on TLC plates a strong yellow or blue fluorescence which prompted us to undertake their isolation. The stem bark was successively extracted with dichloromethane and methanol. The methanolic extract was then fractionated by Sephadex LH-20 followed by centrifugal partition chromatography, medium pressure liquid chromatography and high performance liquid chromatography on RP-18 to afford blumeosides A-D (**5**-**8**) (ref. 15). Blumeosides are new iridoid glucosides containing either a hydroxy or a

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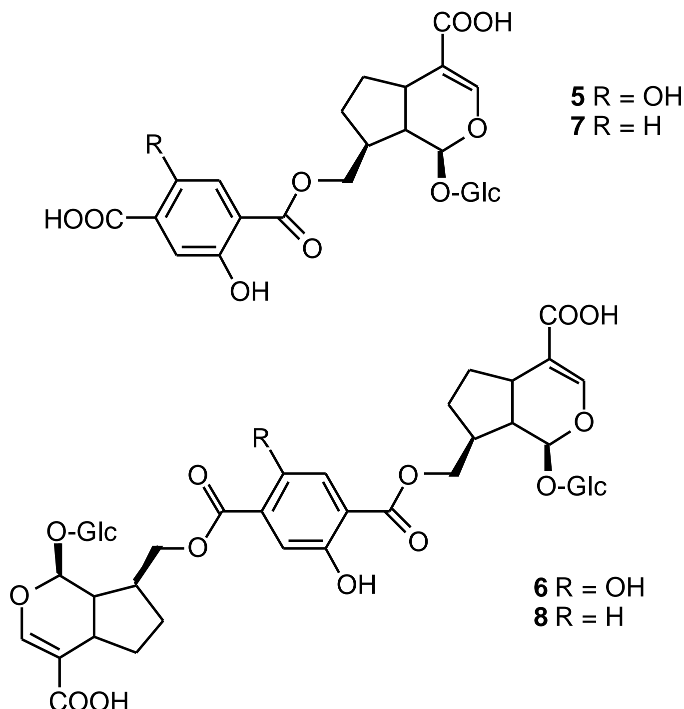
dihydroxy terephthalyl moiety. While iridoids and secoiridoids are widespread in Loganiaceae, terephthalic acid derivatives are rather uncommon in plants.



Radical scavenging properties of blumeosides were evaluated against the DPPH radical. By using DPPH as a TLC spray reagent, **5** and **6** (10 µg) appeared as yellow spots against a purple background, while the same amount of **7** and **8** did not react with the radical. Compounds **5-8** were also tested against DPPH in a spectrophotometric assay. Quercetin and BHT were used as reference compounds. The activity of blumeoside A (**5**) remained lower than that of quercetin but was higher than that of BHT. Compound **6** was less active than **5**, while **7** and **8** did not reduce significantly the free radical. Interestingly the two compounds which contain a hydroquinonic moiety exhibited the strongest radical scavenging activity in this assay.

The antioxidative activity of compounds **5-8** was also evaluated spectrophotometrically on the bleaching of the water soluble carotenoid crocin. Compounds **5**-**8** were all active in this assay but their potency remained lower than that of rutin. Among the iridoids, **5** and **8** exhibited the strongest activity, comparable to that of gallic acid.

CONCLUSIONS

Plants contain thousands of constituents and are a valuable source of new and biologically active molecules. For their investigation, it is important to have the necessary tools at hand. These include suitable biological assays and chemical screening methods.

Bioassays should be as simple as possible and attempts should be made to have access to a large number of different tests so that many biological properties can be screened. Existing assays,

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however, are often not reliably predictive for clinical efficiency and care should be taken when interpreting the results.

The bioassays summarized here involve antifungal, antibacterial and antioxidant/radical scavenging activities. They are most effective when used in conjunction with chemical screening methods so that ubiquitous and unimportant compounds can be excluded. For chemical screening, HPLC coupled with different detection methods e.g. UV, MS provides a great deal of preliminary information about the content and nature of constituents found in the active extracts. In certain cases, combination with a spectral library and pre- or post-column derivatization allows structure determination on-line. By selective ion monitoring in LC/MS or even LC/MSMS, it is possible to achieve the detection of specific target molecules - those, for example, which have already been found to exhibit a particular activity. The recent introduction of other hyphenated techniques such as LC/NMR (ref. 19) will render the on-line structure determination of metabolites even more accurate and rapid.

Once the novelty or utility of a given constituent is established, it is then necessary to process the plant extract in the usual manner, to isolate samples for full structure elucidation and biological testing.

The combination of biological and chemical screening provides important information about plant constituents but will not be a sufficient condition for the discovery of potent new drugs if suitable pharmacological models (or disease-specific assays in agrochemistry) are not available. It is thus essential to adopt a multidisciplinary approach when working in this field. Efficient collaborations with pharmacologists and medical doctors, plant pathologists and biologists is crucial to see the complete development of an interesting lead compound into a exploitable product.

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