REVIEW OF THE METHODS USED FOR ISOLATING PHARMACEUTICAL LEAD COMPOUNDS FROM TRADITIONAL MEDICINAL PLANTS

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ABSTRACT
The possibility of finding new medicines from natural sources is one of the most commonly cited reasons for preserving biodiversity. Further, the utilization of indigenous knowledge of medicinal plants increases the likelihood of discovering these hidden medicines. The main difficulty in using natural products as a source for pharmaceutical lead compounds lies in separating the plethora of compounds from the original extract. There is also a gamble with natural products in that the time and money invested in collecting the material and elucidating the active compounds may not yield any novel structures or compounds that can be used as pharmaceutical leads. While synthetic compounds have proven to be beneficial in the past, they are limited by structural simplicity and known modes of action. The production of novel medicines, particularly for cancer treatment and inhibition of antibiotic-resistant bacteria, now relies on the utilization of natural products. This paper provides a review of the current methods used in elucidating pharmaceutical lead compounds from natural sources, focusing on plant material in particular.

KEY WORDS
Bioactivity, lead compounds, medicinal plants, natural products chemistry, phytochemistry

1. Introduction

Areas of high biodiversity including tropical rainforests are domains of chemical warfare. In the battle for survival, plants have developed many chemical defences to ward off attackers such as bacteria, insects, fungi, and in some cases mammals that may threaten the survival of a species. While not essential for growth, these secondary metabolites do promote the spread and dominance of a species in an ecological setting, and are therefore worth the energy expended by the plant to produce them [1]. It is the secondary metabolites of plants in biodiverse regions that invite the interest of pharmacologists seeking new lead compounds for medicines. Regions of biodiversity contain an even greater chemodiversity, and so harbour great potential for finding new compounds [2,3].

Plants have been used for medicinal purposes throughout human history, and the first pharmaceuticals (that is quantified doses of medicinal compounds as opposed to crude extracts of plant material) were derived from medicinal plants. An example of this is the isolation of salicin from the bark of the willow, *Salix alba*, which was used traditionally for pain and fever. Salicin was converted into the structurally simpler salicylic acid, and was later modified into aspirin to reduce side effects (Figure 1) [4]. Comparatively recently, the ability of chemists to synthesize purely artificial medicinal compounds lead to the production of an abundance of new, effective medicines. However, as new diseases and drug-resistant strains of existing pathogens continue to emerge, the potential of wholly synthesized compounds with simple structures and known modes of action is starting to diminish. As such, attention is again being focused on natural sources for lead compounds, in which exists a wealth of more complex compound structures and novel modes of action [5].

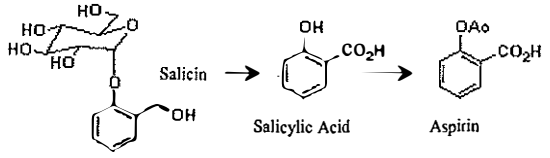


Figure 1: The conversion of salicin to salicylic acid and finally to aspirin [3].

It is the potential discovery of cures for illnesses such as cancer and human immunodeficiency virus (HIV) that is often touted as the main reason for protecting biodiverse regions from destruction [6]. While this may well be the case, large pharmaceutical companies are reluctant to invest the money and time required in screening and isolating the active compounds from plants. The main reason for this is the difficulty in finding compounds that have potential as pharmaceuticals. With over 500 000 plant species on Earth, and each of these having different parts – for example flowers, leaves, bark and roots – consisting of different phytochemical composition, as well as seasonal and geographical variations in the phytochemical composition of members of each species, the likelihood of finding the active plant part of a particular plant species from a random screening survey is fairly small [2,5,7]. The chances of this active compound being novel, and having a novel mode of action that is better than any product currently on the market, is even more remote.

Secondly, once a species with activity has been located, the process of separating out the different compounds within a plant extract can be difficult and time-consuming and may or may not produce a novel compound, let alone a novel compound that meets the criteria for a pharmaceutical lead compound. Further, as is sometimes the case, the compounds within a plant extract may only be active when working in synergy with each other, and therefore will lose that activity when separated [8].

Despite these hindrances, the chemodiversity amongst species in biodiverse regions is certainly worth the effort of investigation. In the United States alone more than half of the top 150 prescription medicines – with an economic value of around $US80 billion – are derived from natural products, and there are still around 99% of plant species in tropical rainforests yet to be phytochemically investigated [9]. From a humanitarian perspective, 30 million people have died from infectious diseases in the past decade and a further 2 million people will die each year from tuberculosis alone [10]. Clearly a resource with many potential medicines for these types of illnesses is one that we cannot afford to lose. Efforts are being made to automate the process of isolating pharmaceutical compounds from natural sources, making them more accessible for investigation [11]. Since these methods are not yet widely used, this paper details the current methods employed to separate active compounds from the non-active compounds of plant material, following activity-guided fractionation.

2. Plant selection and screening

There are many problems produced by random screening of plants, including the selection of appropriate plant parts or sampling at the appropriate time of year for particular species. Incorrect sampling can result in many plant species being erroneously reported as not having activity against a particular illness [12]. Since each new pharmaceutical lead compound that can be developed into a new medicine has been valued at approximately $US449 million [21], this oversight can undervalue the potential of biodiverse regions.

These problems can often be overcome by investigating those species that have been traditionally used as medicines for many generations [2]. Traditional medicines, including folklore remedies, have already undergone many rigorous tests to determine their safety and medicinal suitability, and as such have proven to be a reliable source of active compounds. Large-scale studies investigating the potential of medicinal plants have reported a high correlation between the traditional use of the plants and the presence of active compounds within the plant extract [13,14]. Plants used traditionally as antiseptics, for example, have been found to inhibit the growth of many different species of bacteria [15,16].

Traditional knowledge includes details such as the season during which a particular plant species produces biologically active compounds, which part of the plant contains this biological activity, and the particular region in which a species is more active [12,17,18]. For example, it has been found that certain plant species growing at different altitudes develop differing phytochemistry, which can cause variations in biological activity [19]. Other invaluable information obtained from ethnobotanical leads involves the 'activation' of certain plant species when mixed together. Curare, for example, is a poison used for hunting by indigenous people of the Amazon basin in South America, and is obtained from mixing different plant materials together. Each separate component is inactive on its own [20].

Screening the crude extract of medicinal plants determines the presence of biologically active compounds, and therefore confirms the use of such plants in traditional medicines [16]. Initial screening of plant extracts often involves antimicrobial testing - regardless of the use of the medicinal plant - since this testing is fast, ethical, and relatively inexpensive [22]. Antimicrobial screening involves simply exposing the target organism (bacteria, fungi, viruses, or cancerous cells) to the extracted plant material, and observing the presence or absence of growth after exposure. This is a useful technique that quickly assesses if the plant extract should undergo further investigation [22].

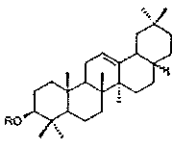
The emergence of antibiotic-resistant bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) [23], and multi-drug-resistant tuberculosis in recent years, has lead to renewed interest in developing novel antibiotics. The continued devastation cause by the spread of HIV and various forms of cancer has also lead to intensive investigation into novel compounds from natural sources [24]. This investigation has afforded the anti-leukemia agents vinblastine and vincristine from the rosy periwinkle (*Catharanthus roseus*), the powerful anticancer agent taxol from the pacific yew (*Taxus brevifolia*) [5], and promising results against HIV from many plant extracts including turmeric (*Curcuma longa*) and St. John's wort (*Hypericum perforatum*) [7]. While antimicrobial testing is the most commonly used methods to assess the medicinal potential of plants, there are many other medicinal properties that are sought after. These include anti-malarial and anti-parasitic activity [25], anti-diarrhoeal activity [26], and anti-inflammatory activity [27].

3. Phytochemical investigation

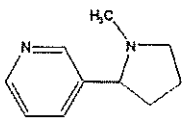
3.1 Compound classes

While there are many millions of different compounds that can be found within the thousands of plant species worldwide, they can each be classified into a distinct class of compounds based on similar characteristics. The main classes of bioactive compounds from plants include flavonoids, terpenes, alkaloids, saponins, coumarins, and tannins [7]. Flavonoids are polyphenolic compounds that produce the flavour of fruits and vegetables and the pigment of flowers. The core structure consists of two benzene rings joined by a 3-carbon chain (Figure 2), which tends to be non-polar and therefore extracted with solvents such as chloroform [28]. Terpenes are also generally non-polar, though some members of this group can be extracted with solvents as polar as methanol and water. Their structure consists of repeating isoprene units with the smallest terpene having two isoprene units ($C_{10}H_{16}$). The most recognisable member of this group is the tetraterpene β-carotene (Figure 2), which produces the orange colour of carrots. It is the terpenes that are responsible for the fragrance of essential oils [7].

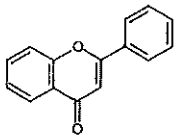
Alkaloids are the most commonly investigated group of plant compounds because they are highly active and are easily extracted from the plant material. Generally speaking, alkaloids are those compounds that consist of a heterocyclic ring with a nitrogen atom. They include caffeine, morphine, and nicotine (Figure 2) [29]. Conversely, saponins have not been widely investigated because many have haemolytic properties. Recently however, it has been shown that these effects are reduced at smaller concentrations. Triterpenoid saponins, found in dicotyledonous angiosperms, consist of an aglycone skeleton attached to a sugar moiety (Figure 2), and steroidal saponins, more common amongst monocotyledons, has a spirostane skeleton [30,31].



Saponin (aglycone skeleton)
(R=sugar moiety)

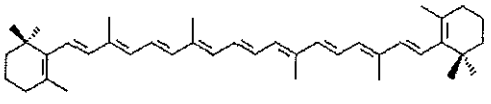


Alkaloid (Nicotine)



Flavonoid (Flavone)

Coumarin (core structure)



Terpene (β-carotene)

Figure 2: The basic structures of some bioactive compound classes.

Coumarins are most abundant in grasses and have been found to have wide-ranging activity including antimicrobial, antiviral, antithrombotic and anti-inflammatory activity. As such, they have been used as the basis for many pharmaceuticals including the oral anticoagulant warfarin. The relatively simple core structure of coumarins consists of fused benzene and α-pyrone rings (Figure 2), in contrast to the comparatively complex tannins. These compounds are generally polymeric phenolic compounds with astringent properties [7]. Tannins are commonly present in the bark of higher plant species and can also be present in the leaves and fruit but in smaller quantities. They are very useful in wound healing since they are known for their antimicrobial properties

3.2 Activity-guided fractionation of plant compounds

The plethora of different compounds and different compound classes make separation and isolation of unknown active compounds from plant material a difficult task. Activity-guided fractionation is the most frequently cited technique for separating plant compounds and isolating only those that exhibit the desired activity [30,32,33]. It uses various techniques of high-performance liquid chromatography (HPLC)-piloted column chromatography for separating plant components (Table 1), as well as biological testing to detect the desired activity within each separated fraction.

The first stage of separating plant compounds comes with the selection of solvent used for the extraction. Plant material extracted with water will only extract highly polar compounds such as tannins and saponins, while a less polar solvent, for instance chloroform, will extract more non-polar compounds such as flavonoids [7]. The selected solvent can therefore act as an initial stage of separation. Extraction of plant material with different solvents covering a range of polarities is a more thorough method of examining active compounds from traditional medicinal plants [34].

After extraction, column separation of compounds usually begins with silica gel (230-400 mesh ASTM) [35,36]. This is because this media is relatively inexpensive and effective for crude separation of extracts. Silica is a polar stationary phase that will allow the ready elution of non-polar compound classes, particularly the highly coloured terpenes. Strong polar compounds, those with hydroxy groups and those with amine groups will adhere to the silica and must be eluted with methanol. Fractionation is often visual and fractions are collected based on differing coloured bands that move through the column. As such, there is often much overlap of activity between fractions and the extent of this phytochemical overlap can be determined by HPLC analysis. Similarities of activity and HPLC profile can mean that two or more fractions can be recombined for the next stage of fractionation.

The active fractions from this preliminary column are then separated further using column chromatography. For non-polar compounds, this often involves repeating the silica column [37,38], each run removing more and more of the non-active material. Polar compounds will adhere to the silica and therefore are separated more effectively with a reverse-phase C18 column [39]. This media is considerably more expensive than silica gel and therefore is not as frequently used for the crude separation of the plant extract. After initial separation by silica gel however, the fraction is much cleaner and consists of fewer non-active compounds that could potentially clog the column and reduce its potential for re-use. C18 media is made from silica gel and has long carbon chains (usually of 18 carbons in length for separation of plant material, hence the name) attached to the active sites. This reverses the polarity of the stationary phase and allows polar compounds to elute first. Detection of fractions is often achieved using a combination of visual cues and UV detection. Since polar compounds are often not coloured it is more difficult to determine visually when the first fractions begin to elute off the C18 column.

Table 1: Summary of the characteristics of stationary phases for separating plant compounds

Stationary phase	Separation stage	Separation mechanism	Solvents used	Detection of fractions
Silica	Initial	Polarity (polar)	Wide range of polarities[b]	Vis al
XAD-16	Initial[a]	Hydrophobicity	Methanol /water	Visual
C18	Secondary	Polarity (non-polar)	Methanol/water gradient	Visual/ UV
Sephadex LH-20	Tertiary	Molecular size	Methanol	UV
Preparative C18	Final	Polarity (non-polar)	Methanol/water gradient	UV

[a]Best for aqueous extracts, [b]Excluding acetone

An alternative for separating polar active compounds is the XAD-16 column [40]. This media is useful as an initial stage of separation for water extracts, particularly as a means for removing the extract from the water and dissolving it instead in a more stable methanol solution. This makes rotary evaporation of the extract much easier, allowing faster analysis of the extract. This is possible with the XAD media because the comparatively non-polar compounds of the water extract adhere to the column, while the water-soluble compounds, such as starch, elute off. The column can then be washed with methanol, which brings off compounds in a methanol solution. XAD resin is a non-ionic, hydrophobic, cross-linked polymer, which absorbs compounds into particular-sized pores. It is therefore useful for absorbing compounds with low to medium molecular weights.

Another method of separation involves the non-ionic Sephadex LH-20 gel [32,38,39,40], which - unlike silica gel or C18 - separates compounds based on molecular size rather than polarity. The porosity of this media allows large compounds to be eluted first, while smaller compounds wind their way through the pores and elute later. Medium sized compounds will fit into some pores of the polymer, and not others, so they take longer to elute than larger compounds. Separation of this type can be useful for removing any residual chlorophyll from the plant extract, which is otherwise often difficult to separate from the active compounds. Sephadex LH-20 gel is not normally utilized for crude extraction because it is much more expensive than silica gel and also because its porosity allows it to become clogged when separating anything other than more refined fractions. There may be some issues of fractions having overlapping HPLC profiles, and as such, analysis of biological activity and HPLC characteristics are required before assessing which fractions must be combined before the next stage of separation. Detection of fractions is achieved by UV detection, since there is little colour distinction from this method of separation.

As the fractionation process continues, the number of compounds in each fraction decreases. The initial extract contains thousands of compounds yet only a few of these may be active, so after many columns and activity screens, the final active fraction may consist of less than ten compounds. As the HPLC profile becomes cleaner, with fewer, more defined peaks, it becomes desirable to capture each peak from the HPLC chromatogram. This is possible using a preparative C18 column, which consists of a uniform 5μ particle size that is the same as that of the analytical HPLC column, only it allows for an injection volume approximately fifty times greater than the analytical scale [40]. Conditions such as the appropriate solvent system can be optimised on the HPLC, and these conditions can be used to run the preparative column. This will produce a very similar chromatogram, which means that single peaks can be captured as fractions. The single peaks can relate to individual compounds - providing that the solvent system has been optimised - and can therefore purify the active compounds as well as isolating them.

These stages of separation can be immensely time consuming and difficult since they deal with natural compounds of unknown structure and characteristics. It is the separation processes and the only slight probability of a promising end result despite all the work involved that acts as a deterrent for many pharmaceutical companies to working with medicinal plants. Further complications arise from the characterisation of the purified active compounds.

3.3 Characterization of active compounds

Once purified, the structure of the active compound can be elucidated using various programs of the nuclear magnetic resonance spectrometer (NMR) [36,37,38,40]. The use of this instrument in structural elucidation is a major advantage since it alleviates the need to chemically degrade the active compound to determine its structure. Because of this, compound structures can be characterised using very small amounts of the compound, as little as 1 mg for large compounds with a 500 MHz NMR. One of the difficulties with elucidating the structure of an unknown natural compound is the possible complexity of that compound, which can on occasion, make the structure impossible to characterize, even with NMR technology.

It is only once the compound has been characterized that it can be assessed in terms of its potential as a lead compound, and indeed if the compound is actually novel and worthy of further investigation. To be considered as a pharmaceutical lead, the active compound must have a reasonably simple structure so it can be synthesized, or have a novel mode of action that is much more efficient than current drugs. Other criteria

includes having a high level of the desired activity (active down to low concentrations), and of course it must be a novel compound.

A pharmaceutical compound is created by altering the basic structure of the lead compound. These changes may make the compound more effective against the target organism, make the compound simpler, or reduce any side effects. The lead compound may also be fitted with hydrophobic or hydrophilic groups to enable more rapid transport around the body, but the core structure will still be that of the original compound. Since activity and mode of action are the most critical characteristics of the lead compound and because they are so intrinsically linked to the compound structure, accurate structural elucidation is a key factor in obtaining lead compounds from natural sources. Therefore, the structure as determined by the NMR spectra should be confirmed with mass spectrometry. This technique destroys the sample, but is necessary to further prove the NMR-determined structure by calculating the molecular mass of the sample.

Determination of the structure of the active compound is essential for assessing the potential as a lead compound, but involves a great deal more time and resources than the biological screening. For this reason, there are many medicinal plants yet to be properly assessed as potential sources of lead compounds. Efforts to automate the separation process will aid in the elucidation process and should lead to more interest and investment in utilizing plants as sources of pharmaceutical lead compounds.

4. Conclusions

The vast chemical diversity of plants in biodiverse regions is a promising source of novel lead compounds that is still relatively unexplored. The indigenous knowledge of traditional medicinal plants is a valuable tool for targeting potentially active species from the wealth of plants in these regions, which may be of great importance as new medicines. Though there are many difficulties involved in the current methods for isolating active compounds from plant material, the potential of this resource for pharmaceutical compounds is such that it should not be disregarded.

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REFERENCES:

[1] Fellows, L., Scofield, A.: 1995, 'Chemical diversity in plants', in: Intellectual property rights and biodiversity conservation – an interdisciplinary analysis of the values of medicinal plants, University Press, Cambridge, Great Britain, pp. 19-44.
[2] Hostettman, K., Wolfender: 1997, 'The search for biologically active secondary metabolites', Pesticide Science, 51 (4), p. 471-482.
[3] McChesney, J.: 1996, 'Biological diversity, chemical diversity, and the search for new pharmaceuticals'. in: Medicinal Resources of the tropical forest – biodiversity and its importance to human health, Balick, M.J., Elisabetsky, E., Laird, S.A. (eds), Columbia University Press, New York, pp. 11-18.
[4] Black, D.: 1997, 'One hundred years of aspirin', The Lancet, 350 (9075), pp. 437-439.
[5] Lesney, M.: 2004, 'Nature's pharmaceuticals – Natural products from plants remain at the core of modem medicinal chemistry', Today's Chemist at Work, July, 27-32.
[6] Tyler, V. E.: 1996, 'Natural products and medicine: an overview'. in: Medicinal Resources of the tropical forest – biodiversity and its importance to human health, Balick, M.J., Elisabetsky, E., Laird, S.A. (eds), Columbia University Press, New York, pp. 3-10.
[7] Cowan, M.: 1999, 'Plant products as antimicrobial agents', Clinical Microbiology Reviews, 12 (4), 564-582
[8] Kalemba, D., Kunicka, A.: 2003, 'Antibacterial and antifungal properties of essential oils', Current Medicinal Chemistry, 10 (10), 813-829.
[9] Groombridge, B., Jenkins, M.: 2002, 'World Atlas of Biodiversity: Earth's Living Resources for the 21st Century', University of California Press, USA.
[10] Cantrell, C., Franzblau, S., Fischer, N.: 2001, 'Antimycobacterial plant terpenoids', Planta Medica, 67(7), p. 685-694.
[11] Li, H., Chen, F.: 2004, 'Preparative isolation and purification of bergapten and imperatorin from the medicinal plant *Cnidium monnieri* using high-speed counter current chromatography by stepwise increasing the flow-rate of the mobile phase', Journal of Chromatography A, 1061, 51-54.

[12] Jagetia, G., Baliga, M.: 2005, 'The effect of seasonal variation on the antineoplastic activity of *Alstonia scholaris* R. Br. in HeLa cells', Journal of Ethnopharmacology, 96 (1-2), 37-42.
[13] Ieven, M., Vanden Berghe, D., Mertens, F., Vlietinck, A., Lammens, E.: 1979, 'Screening of higher plants for biological activities 1. Antimicrobial activity', Planta medica, 36, 311-321.
[14] Murillo-Alvarez, J., Encarnación, D., Franzblau, S.: 2001, 'Antimicrobial and cytotoxic activity of some medicinal plants from Baja California Sur (Mexico)', Pharmaceutical biology, 36 (6), 445-449
[15] McRae, J., Palombo, E., Harding, I., Crawford, R.: 2003, 'Antimicrobial activity of traditional medicinal plants, in M. Mowlaei, A. Rose, and J. Lamborn, (eds.), Proceedings of 7th Annual EERE Conference, December 2003, Environmental Engineering Research Event, Victoria, pp. 225-233.
[16] Palombo, E., Semple, S.: 2001, 'Antibacterial activity of traditional Australian medical plants', Journal of Ethnopharmacology, 77 (2-3), 151-157
[17] Latz, P.: 1995, Bush fires and Bushtucker – Aboriginal plant use in central Australia, IAD Press, Alice Springs, Australia.
[18] Okundae, A.L. 2002. '*Ageratum conyzoides* L. (Asteraceae)', Fitoterapia, 73 (1): 1-16.
[19] Chandra, S.: 2004, 'Effect of altitude on energy exchange characteristics of some alpine medicinal crops from central Himalayas', Journal of Agronomy and Crop Science, 190 (1), 13-20.
[20] Philippe, G., Angenot, L., Tits, M., Frédérich, M.: 2004, 'About the toxicity of some Strychnos species and their alkaloids', Toxicon, 44 (4), 405-416.
[21] Setzer, M., Setzer, W., Jackes, B., Gentry, G., Moriarity, D.: 2001, 'The medicinal value of tropical rainforest plants from Paluma, north Queensland, Australia', Pharmaceutical Biology, 39 (1): 67-78
[22] Vlietinck, A., Apers, S.: 2001, 'Biological screening methods in the search for pharmacologically active natural products', in C. Tringali (ed), Bioactive compounds from natural sources: isolation, characterization and biological properties, Taylor & Francis, London, pp. 3-24.
[23] Palombo, E., Semple, S.: 2002, 'antibacterial activity of Australian plant extracts against methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant enterococci (VRE)', Journal of Basic Microbiology, 42 (6), 444-448.
[24] Seidel, V., Taylor, P.: 2004, 'In vitro activity of extracts and constituents of *Pelagonium* against rapidly growing mycobacteria', International Journal of Antimicrobial Agents, 23 (6), 613-619.
[25] Diallo, D., Marston, A., Terreaux, C., Touré, Y., Smestad Paulsen, B., Hostettman, K.: 2001, 'Screening of Malian medicinal plants for antifungal, larvicidal, molluscicidal, antioxidant and radical scavenging activities', Phytotherapy Research, 15 (5), 401-406.
[26] Akah, P., Aguwa, C., Agu, R.: 1999, 'Studies on the antidiarrhoeal properties of *Pentaclethra macrophylla* leaf extracts', Phytotherapy Research, 13 (4), 292-295.
[27] Islam, C., Bandyopadhyay, S., Banerjee, S., Dutta, M., Das, P.: 1995, 'Preliminary studies on the anti-inflammatory effects of *Swertia chirata* in albino rats', Indian Journal of Pharmacology, 27 (1), 37-39.
[28] Aruoma, O., Bahorun, T., Jen, L.: 2003, 'neuroprotection by bioactive components in medicinal and food plant extracts', Mutation Research/ Reviews in Mutation Research, 544 (2-3), 203-215.
[29] Collins, D., Culvenor, C., Lamberton, J., Loder, J., Price, J.: 1990, 'Plants for medicines – a chemical and pharmacological survey of plants in the Australian region', CSIRO Publications, Australia.
[30] Massiot, G., Chen, X., Lavaud, C., Le Men-Olivier, L., Delaude, C., Viari, A., Vigny, P., Duval, J.: 1992, 'Saponins from stem bark of *Petersianthus macrocarpus*, Phytochemistry, 31 (10), 3571-3576.
[31] Sparg, S, Light, M., van Staden, J.: 2004, 'Biological activity and distribution of plant saponins', Journal of Ethnopharamcology, 94 (2-3), 219-243.
[32] Adesina, S., Idowu, O., Ogundaini, A., Oladimeji, H., Olugbade, T.: 2000, 'Antimicrobial constituents of the leaves of *Acalypha wilkesiana* and *Acalypha hispida'*, Phytotherapy Research, 14 (5), 371-374.
[33] Shah, A., Cross, R., Palombo, E.: 2004, 'Identification of the antibacterial component of an ethanolic extract of the Australian medicinal plant, *Eremophila duttonii*', Phytotherapy Research, 18 (8), 615-618.
[34] Khan, M., and A. Omoloso. 2002. Antibacterial, antifungal activities of *Barringtonia asiatica*, Fitoterapia, 73 (3), 255-260.
[35] Blatt, C., Chávez, D., Chai, H., Graham, J., Cabieses, F., Farnsworth, N., Cordell, G. Pezzuto, J., Kinghorn A.: 2002, 'Cytotoxic flavonoids from the stem bark of *Lonchocarpus* aff. *Fluvialis'*, Phytotherapy Research, 16 (4), 320-325.
[36] Cottiglia, F., Bonsignore, L., Loy, L., Garau, D., Floris, C., Casu, M.: 2002, 'Structural elucidation and antibacterial activity of a new coumarinolignoid from *Daphne gnidium* L', Magnetic Resonance in Chemistry, 40 (8), 551-553.
[37] León, L., Maldonado, E., Cruz, A., Ortega, A.: 2004, 'Tenuflorins A-C: New 2-phenoxychromones from the leaves of *Mimosa tenuiflora'*, Planta medica, 70 (5), 536-539
[38] Yang, S., J. Huo, Y. Wang, L. Lou, and J. Yue. 2004. Cytotoxic sesquiterpenoids from *Eupatorium chinense*, Journal of Natural Products, 67 (4), 638-643
[39] Feng, X., M. Dong, Gao, Z., Xu, S.: 2003, 'Three new triterpenoid saponins from *Ixeris sonchifolia* and their cytotoxic activity', Planta Medica, 69 (11), 1036-1040.
[40] Machado, T., I. Leal, A.Amaral, K. dos Santos, M., da Silva, and R., Kuster. 2002. 'Antimicrobial ellagitannin of *Punica granatum* fruits', *Journal of the Brazilian Chemical Society*, 13 (5), 606-610