Environmentalist (2007) 27:165–174
DOI 10.1007/s10669-007-9024-9

Review of the methods used for isolating pharmaceutical lead compounds from traditional medicinal plants

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Published online: 23 February 2007

Abstract The possibility of finding new medicines from natural sources is one of the more commonly cited reasons for preserving biodiversity, and employing indigenous knowledge of traditional healing remedies greatly increases the likelihood of discovering these hidden medicinal compounds. The main difficulties in using natural products as a source for pharmaceutical lead compounds involve separating the plethora of compounds from the original extract, as well as the gamble of time and money invested in an activity that may not yield a novel compound. However, while these difficulties exist, the potential of natural products still far outweighs the limitations of the simple structures and known modes of action of synthetic lead compounds. As such, the production of novel medicines, particularly for cancer and human immunodeficiency virus (HIV) treatments as well as the inhibition of antibiotic-resistant bacteria, now requires 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 samples in particular.

Keywords 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

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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 (Fellows and Scofield, 1995). It is the secondary metabolites of plants in biodiverse regions that invite the interest of pharmacologists seeking new lead compounds for medicines. Regions of high biodiversity contain an even greater chemodiversity, and so harbour great potential for finding new compounds (McChesney, 1996).

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. A common 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 (Fig. 1) (Black, 1997). Other well-known plant-derived pharmaceuticals include anti-malarial quinine from *Cinchona officinalis* and the painkiller, morphine, from *Papaver sonniferum* (Urban and Separovic, 2005).

Comparatively recently, the ability of chemists to synthesize purely artificial medicinal compounds lead to the production of many 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 of lead compounds, in which exists a wealth of more complex compound structures and novel modes of action (Lesney, 2004).

The emergence of antibiotic-resistant bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) (Palombo and Semple, 2002) and multi-drug-resistant tuberculosis (Cantrell et al., 2001), has lead to renewed interest in developing novel antibiotics. The continued devastation caused by the spread of human immunodeficiency virus (HIV) and various forms of cancer has also lead to intensive investigation into novel compounds from natural sources (Seidel and Taylor, 2004). This investigation has afforded the anti-leukemia agents vinblastine and vincristine from the rosy periwinkle (*Catharanthus roseus*), the powerful anticancer agent paclitaxel from the pacific yew (*Taxus brevifolia*) (Lesney, 2004), and promising results against HIV from the extracts of many plants

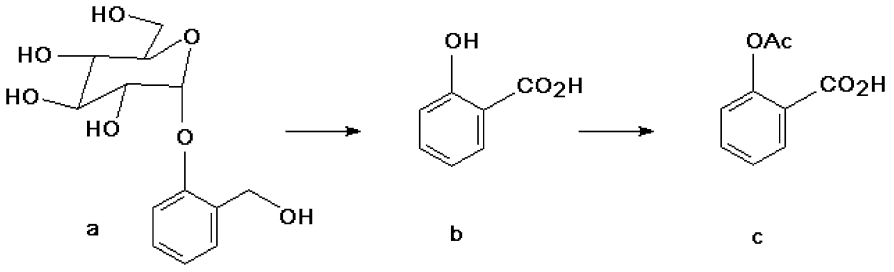

Fig. 1 The conversion of salicin (a) to salicylic acid (b) and finally to aspirin (c) (Black, 1997)

including turmeric (*Curcuma longa*) and St. John's wort (*Hypericum perforatum*) (Cowan, 1999).

It is the potential discovery of cures for illnesses such as cancers and HIV that is often given as the main reason for protecting biodiverse regions from destruction (Tyler, 1996). While this may well be the case, few companies are able to invest the money and time required in screening and isolating the active compounds from plants, and this is mainly due to the difficulty in finding compounds that have potential as pharmaceuticals. Secondly, once a species with the desired activity has been located, the process of separating the different compounds within a plant extract can be difficult and time-consuming and may or may not produce a novel compound, let alone one that can be used as 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 (Kalemba and Kunicka, 2003).

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 drugs are derived from natural products, and there are around 99% of plant species in tropical rainforests yet to be phytochemically investigated (Groombridge and Jenkins, 2002). 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 two main strategies used for the selection of plant species: random screening and ethnobotany. With over 500 000 plant species on Earth, and each of these having leaves, bark and roots with different phytochemical composition, as well as seasonal and geographical variations in the phytochemistry of many plant species, the likelihood of finding an active plant sample from a random screening survey is fairly small (Hostettman and Wolfender, 1997; Setzer, 2001; Lesney, 2004). It is even less likely that the active compound isolated from the plant sample is novel or has a novel mode of action that is more efficient than any product currently on the market.

These problems can often be overcome by using ethnobotany to investigate those species that have been traditionally used as medicines for many generations (Hostettman and Wolfender, 1997). 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 (such as altitude) in which a species is more active (Latz, 1995; Okundae, 2002; Chandra, 2004; Jagetia and Baliga, 2005). As a result of their long-term use, traditional medicines, including folklore remedies, are generally considered to be safe and medically suitable, 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 plants and the presence of active compounds within the plant extract (Ieven et al., 1979; Zavala et al., 1997; Murillo-Alvarez et al., 2001; Palombo and Semple, 2001; McRae et al., 2003).

Screening the crude extract of medicinal plants determines the presence of biologically active compounds, and therefore validates the use of such plants in traditional medicines (Palombo and Semple, 2001). 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 (Vlietnick and Apers, 2001). Antimicrobial screening involves simply exposing the target organism (bacteria, fungi, viruses, or parasites) to the extracted plant material, and observing the presence or absence of growth after exposure. This is a useful technique that quickly determines if the plant extract should undergo further investigation (Vlietnick and Apers, 2001; Diallo et al., 2001). Other sought-after medicinal properties from plant extracts include anti-diarrhoeal activity (Akah et al., 1999), anti-inflammatory activity (Islam et al., 1995), and anti-tumour activity (del Barrio and Parra, 2000).

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 usually 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, and coumarins (Cowan, 1999). Flavonoids are polyphenolic compounds that produce the flavour of fruits and vegetables as well as the red and blue pigments of plants, and have been used in taxonomic studies of angiosperms (Hrazdina, 1982). The core structure consists of a C_{15} skeleton with two aromatic rings joined by a chromane ring (Fig. 2) (Aruoma et al., 2003).

Terpenes are responsible for the fragrance of essential oils (Cowan, 1999), and they consist of repeating isoprene units with monoterpenes having two isoprene units ($C_{10}H_{16}$). The most recognisable member of this group is the tetraterpene β-carotene (Fig. 2), which produces the orange colour of carrots. Coumarins are most abundant in grasses and have been found to have wide-ranging activity including antimicrobial, antiviral, antithrombotic and anti-inflammatory. As such, they have been used as the basis for many pharmaceuticals including the oral anticoagulant, warfarin. The

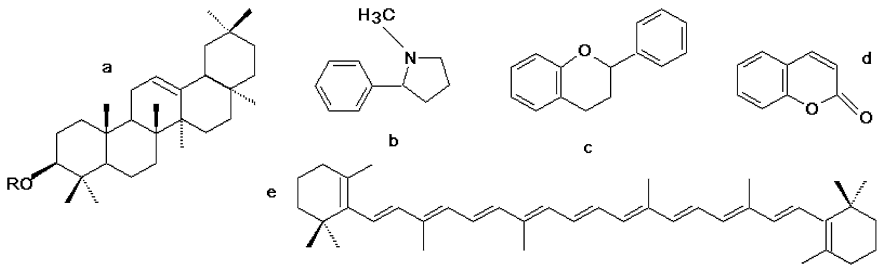

Fig. 2 The basic structures of some bioactive compound classes (a) aglycone skeleton of a triterpenoid saponin, where R refers to a sugar moiety, (b) the alkaloid nicotine, (c) general core structure of a flavonoid, (d) coumarin core structure, and (e) the tertraterpene β-carotene

relatively simple core structure of coumarins consists of fused benzene and α-pyrone rings (Fig. 2) (Cowan, 1999).

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 (Fig. 2) (Collins et al., 1990). Conversely, saponins have not been widely investigated because some were known to have haemolytic properties. Recently however, it has been shown that the medicinal properties of some plants (including ginseng) are due to saponins that can be consumed safely, and therefore they can have applications as pharmaceuticals. Triterpenoid saponins, found in dicotyledonous angiosperms, consist of an aglycone skeleton attached to a sugar moiety (Fig. 2) (Sparg et al., 2004), and steroidal saponins, more common amongst monocotyledons, have a spirostane skeleton (Massiot et al., 2002).

3.2 Activity-guided fractionation of plant compounds

The plethora of different compounds within the different compound classes makes 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 (Adesina et al., 2000; Massiot et al., 2002). It involves 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 use of many different types of separation media can help pull compounds apart more efficiently than reliance on one type of media. Some of the more commonly used separation media will be discussed here.

After extraction of plant material by exposure to a single organic solvent or, more thoroughly, a range of different solvents with different polarities, column separation of compounds usually begins with silica gel (230–400 mesh ASTM) (Blatt et al., 2002; Cottiglia et al., 2002). This is because this media is relatively inexpensive and effective for crude separation of extracts. Silica is a polar stationary phase that allows the ready elution of non-polar compounds, particularly highly coloured terpenes. Highly polar compounds with hydroxyl or amine groups can strongly adhere to the silica, so this

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

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

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

media is not ideal for such compounds. 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 in activity and HPLC profile can mean that two or more fractions can be recombined for the next stage of fractionation.

An alternative for separating polar active compounds is with Amberlite XAD-16 resin (Machado et al., 2002). This media is useful for removing organic material from aqueous solutions and therefore can be used to concentrate the small amounts of active compounds from aqueous extracts. The active compounds can then be eluted with methanol. 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.

The active fractions from the preliminary column are then separated further with repeated column chromatography. For non-polar compounds, this often involves repeating the silica column (León et al., 2004; Yang et al., 2004), each run removing more and more of the non-active material. Polar compounds are separated more effectively with a C18 column, which consists of silica gel with long carbon chains attached to the active sites (Feng et al., 2003). This reverses the polarity of the stationary phase and allows polar compounds to elute first. C18 is considerably more expensive than silica gel and therefore is not as frequently used for the crude separation of the plant extract. However, after initial separation by silica gel or XAD resin, the fraction is much cleaner and consists of fewer non-active compounds that could potentially obstruct the column and reduce its potential for re-use. Detection of fractions is often achieved using a combination of visual cues and UV detection.

Another method of separation involves non-ionic Sephadex^{TM} LH-20 gel (Adesina et al., 2000; Machado et al., 2002; Feng et al., 2003; Yang et al., 2004), which 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. Separation of this type can be useful for removing chlorophyll from the plant extract as well as separating compounds that are closely eluting on silica gel or C18. Sephadex^{TM} LH-20 gel is not normally utilized for crude extracts because it is much more expensive than silica gel, and also because its porosity allows it to become congested when separating anything other than more refined fractions. 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, making it possible to see clear, distinct peaks in the HPLC chromatogram. Of the thousands of compounds in the initial extract, only less than ten may be present in the active fraction after repeated chromatography. The optimal conditions used to separate these compounds with analytical HPLC can also be used with a preparative C18 column so each separated compound can be collected as a fraction. Preparative C18 consists of a uniform 5 μm particle size (Machado et al., 2002)—the same grade as the analytical HPLC column—and the column is much larger, enabling around 50 times more material than the analytical column to be loaded onto it. It is particularly useful for producing sufficient quantities of isolated compounds for

characterization and biological screening. Purification can then be achieved through repeated chromatography of the isolated compound.

These stages of separation can be immensely time consuming and difficult, since they deal with natural compounds of unknown structure and characteristics. It is also the high probability of elucidating a known compound despite all the work involved that acts as a deterrent for many companies working with medicinal plants, however improvements in the separation and characterization of natural products will encourage further investigation in this field.

3.3 Characterization of natural products

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 include 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 to the appropriate target site within the body, while retaining the pharmacophore of the 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.

The structure of the purified active compound can be determined using information obtained from various spectroscopic techniques. The most common technique for elucidating natural products involves nuclear magnetic resonance (NMR) spectroscopy, an instrument that has been a major advantage in determining the structure of unknown compounds. Samples can generally be recovered after analysis since NMR does not destroy the compound to analyze it. Other methods of structural elucidation involve mass spectroscopy (MS) to determine the molecular weight of compounds and infrared (IR) spectroscopy to ascertain functional groups. Both of these techniques destroy the sample, but can provide additional information to confirm the NMR-determined structure. 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.

More recently, the use of combined spectroscopic technologies has provided a more efficient method of elucidating the structure of natural products. These so-called ‘hyphenated techniques’ involve streamlined analysis of crude plant extracts separated by HPLC. The more traditional LC-MS and GC-MS instruments are gradually being replaced with the more effective LC-NMR and LC-NMR-MS and the powerful but not yet widely utilized LC-UV-NMR-MS-FTIR (Urban and Separovic, 2005). The use of such instrumentation significantly reduces the amount of materials and solvents

required for the separation and characterization process and also allows for the rapid identification of any novel compounds in a plant extract. This technology combined with high throughput screening of crude extracts and isolated compounds will allow natural products to be processed more rapidly. These advances will enable the vast wealth of natural compounds to be more fully explored.

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 still many medicinal plants yet to be properly assessed as sources of 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, and the application of new hyphenated technologies will make these compounds more accessible.

Acknowledgments The authors acknowledge, with great appreciation, the contributions of Noel Hart, senior principal research scientist and separations chemist for CSIRO Molecular and Health Technologies, to this investigation.

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