CHAPTER – IV

PURIFICATION AND ACTIVE PRINCIPLE DETERMINATION FROM THE POTENTIALLY ACTIVE HERBAL EXTRACT
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INTRODUCTION

The herbal products had been priced for their medicinal, flavouring and aromatic qualities for centuries, the synthetic products of the modern age surpassed their importance, for a while. However, the blind dependence on synthetics is over and people are returning to the naturals with hope of safety and security. Over three-quarters of the world population relies mainly on plants and plant extracts for health care. More than 30% of the entire plant species, at one time or other, were used for medicinal purposes. It is estimated that world market for plant derived drugs may account for about Rs.2,00,000 crores. Presently, Indian contribution is less than Rs.2000 crores. Indian export of raw drugs has steadily grown at 26% to Rs.165 crores in 1994-'95 from Rs.130 crores in 1991-'92. The annual production of medicinal and aromatic plant’s raw material is worth about Rs.200 crores. This is likely to touch US $1150 by the year 2000 and US $5 trillion by 2050.

It has been estimated that in developed countries such as United States, plant drugs constitute as much as 25% of the total drugs, while in fast developing countries such as China and India, the contribution is as much as 80%. Thus, the economic importance of medicinal plants is much more to countries such as India than to rest of the world. These countries provide two third of the plants used in modern system of medicine and the health care system of rural population depend on indigenous systems of medicine.
Of the 2,50,000 higher plant species on earth, more than 80,000 are medicinal. India is one of the world’s 12 biodiversity centres with the presence of over 45000 different plant species. India’s diversity is unmatched due to the presence of 16 different agro-climatic zones, 10 vegetation zones, 25 biotic provinces and 426 biomes (habitats of specific species). Of these, about 15000-20000 plants have good medicinal value. However, only 7000-7500 species are used for their medicinal values by traditional communities. In India, drugs of herbal origin have been used in traditional systems of medicines such as Unani and Ayurveda since ancient times. The Ayurveda system of medicine uses about 700 species, Unani 700, Siddha 600, Amchi 600 and modern medicine around 30 species. The drugs are derived either from the whole plant or from different organs, like leaves, stem, bark, root, flower, seed, etc. Some drugs are prepared from excretory plant product such as gum, resins and latex. Even the Allopathic system of medicine has adopted a number of plant-derived drugs, which form an important segment of the modern pharmacopoeia. Some important chemical intermediates needed for manufacturing the modern drugs are also obtained from plants (Eg. Digoxygenin, Solasodine, β-ionone). Not only plant-derived drug offers a stable market world wide, but also plants continue to be an important source for new drugs.

Today plant based products, plant extracts, natural resins and their preparations have a wide range of applications mainly in perfume and cosmetic industry, in food technology, in aroma industry and in pharmaceutical industry. This large spectrum of uses stimulated studies on natural products. The methods
used in the analysis of plants that started at the end of the 19th century, only allowed investigations on crystalline constituents isolated from these extracts. Subsequent developments on vacuum distillation techniques provided the possibility to determine the volatile components of these extracts. Along with the developments in extraction techniques, the development of chromatographic techniques primarily with planar chromatography (thin layer chromatography (TLC)) and other novel analytical methods were introduced to the benefit of scientists. Gas chromatography (GC) in the 1950’s had opened a new dimension in the analysis of volatile compounds. In the meantime high performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC) was introduced for the fractionation and isolation of more polar and non-volatile compounds.

The combination of gas chromatography and mass spectrometry (GC-MS) allows rapid identification of not only volatile components but also plant extracts, by comparing their mass spectra with available libraries which build up with reference substances recorded under the same experimental parameters. The same principle has been applied in the last decade for liquid chromatography and mass spectrometry (LC-MS) for non-volatile plant constituents. Moreover, the invention of chiral stationary phases for gas chromatography, mostly based on cyclodextrins, has facilitated the identification of the enantiomeric composition of the isolated substances, especially in essential oils. Simultaneously, the advances in spectroscopic methods such as mass spectrometry (MS) and nuclear magnetic
resonance (NMR) spectroscopy have increased the speed of the identification and structure elucidation of natural products.

Terpenes, or terpenoids, are represented with more than 80000 compounds in this group of secondary metabolites, comprising more than 80000 structures. They show extraordinarily diverse structures and exhibit a large variety of physical and physiological properties. Their number increases every year with the addition of new structures, most of which have different biological effects. In plants, the production of terpenoids is much more common than in animals or microorganisms. The production of large quantities of these natural products, their accumulation in cells and other storage compartments, their secretion in the plant body or emission from the plant to its surrounding is a challenging subject for biochemists, phytochemists and biologists in terms of finding out not only the biosynthetic pathway of these compounds in such specialized organisms, but also identifying their physiological properties in association with other living organisms.

In the present study, the effective herbal anti-Alzheimer’s drug was purified and the active principles were analyzed using TLC and HPTLC. Apart from this the photochemical components of the effective herb *S. album* and its sections were taken and microscopically analyzed
MATERIALS AND METHODS

PURIFICATION OF EFFECTIVE HERBAL ANTI-ALZHEIMER’S METABOLITES

TLC AND HPTLC instrumentation

Quantitative and qualitative analysis were performed with the help of HPTLC instrument. The HPTLC system (Camag, Muttenz, Switzerland) consists of (1) TLC scanner connected with a PC running WinCATS software under MS Windows (2) NT; Linomat V Sample applicator, (3) Photo documentation system Camag, Reprostar III.

Spotting of samples

The chromatographic estimation was performed by streaking the extracts in the form of narrow bands of 6 mm length on the precoated silica gel 60 F254 aluminum TLC plate (5 cm ×10 cm), at a constant application rate of 150 µl/s and gas flow of 10 s/µL was employed with help of Camag 100 µl syringe connected to a Nitrogen tank using a Camag Linomat V (Camag, Muttenz, Switzerland). The space between these bands was kept 15 mm. 5µl of 1% concentration solution from each three extracts (Methanol, Chloroform and Petroleum ether) was placed as a spot.

Plate development and chromatographic conditions

After spotting the plate, it is subjected to linear ascending development up to a distance of about 90 mm in a solvent system of Toluene: Ethyl acetate:
Diethylamine: Methanol: Chloroform in the ratio of 10:6:2:2:1 v/v., at Camag Twin Trough glass chamber, which was saturated with the same solvent system at room temperature just 10 minutes prior to development.

**Scanning of plate**

TLC plate was dried in flowing air at room temperature. Densitometric scanning was carried out using Camag TLC Scanner III (Camag, Muttenz, Switzerland) between wavelength of 200-450 nm with a slit dimension of 6.00 × 0.30 mm, with scanning speed of 20 mm/s, and data resolution was at 100 µm/step. The source lamps for radiation were deuterium and tungsten lamps. All remaining measurement parameters were left at default settings. The chromatograms were integrated and regression analysis and statistical data were generated using WinCATS evaluation software (Version 1.4.2.8121).

**Photo documentation of plate**

After the scanning, images of the plate were taken by using three different wavelength of lights (254 nm by UV lamp, 366 nm by mercuric lamp and 400-800 nm by white lamp) with the help of Photo documentation system of Camag, Reprostar III.

**PHYTOCHEMICAL ANALYSIS**

**Identification Tests**

The tests were done to find the presence of the active chemical constituents such as alkaloids, glycosides, terpenoids and steroids, flavonoids, reducing sugar and tannin by the following procedure:
Alkaloid

Alkaloids are basic nitrogenous compounds with definite physiological and pharmacological activity. Alkaloid solution produces white yellowish precipitate when a few drops of Mayer’s reagents are added (Siddiqui and Ali, 1997). Most alkaloids are precipitated from neutral or slightly acidic solution by Mayer’s reagent (Evans, 2002). The alcoholic extract was evaporated to dryness and the residue was heated on a boiling water bath with 2% hydrochloric acid. After cooling, the mixture was filtered and treated with a few drops of Mayer's reagent. The samples were then observed for the presence of turbidity or yellow precipitation.

Glycoside

Glycosides are compounds which upon hydrolysis give rise to one or more sugars (glycones) and a compound which is not a sugar (aglycone or genine). To the solution of the extract in glacial acetic acid, few drops of ferric chloride and concentrated sulphuric acid were added, and observed for a reddish brown coloration at the junction of two layers and the bluish green color in the upper layer (Siddiqui and Ali, 1997).

Terpenoid and steroid

Four milligrams of extract was treated with 0.5 ml of acetic anhydride and 0.5 ml of chloroform. Then concentrated solution of sulphuric acid was added slowly and red violet color was observed for terpenoid and green bluish color for steroids (Siddiqui and Ali, 1997).
Flavonoid

Four milliliters of extract solution was treated with 1.5 ml of 50% methanol solution. The solution was warmed and metal magnesium was added. To this solution, 5-6 drops of concentrated hydrochloric acid was added and red color was observed for flavonoids and orange color for flavones (Siddiqui and Ali, 1997).

Tannins

To 0.5 ml of extract solution 1 ml of water and 1-2 drops of ferric chloride solution was added. Blue color was observed for gallic tannins and green black for catecholic tannins (Iyengar, 1995).

Reducing Sugar

To 0.5 ml of extract solution, 1 ml of water and 5-8 drops of Fehling’s solution was added at hot and observed for brick red precipitate.
RESULTS

The results of basic constituents of *S. album* shows the presence of water soluble components at a rate of 2.21% (w/w) and alcohol soluble particles at a rate of 4.10% (w/w). It was also noted that around 12.06% (w/w) of components were last during drying at 105°C. Interestingly the ash value and acid insoluble ash were nil in the experimental plant *S. album*. The results are shown in the table 18.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ash (% w/w)</td>
<td>Nil</td>
</tr>
<tr>
<td>Acid insoluble ash (% w/w)</td>
<td>Nil</td>
</tr>
<tr>
<td>Water soluble extractive (% w/w)</td>
<td>2.21</td>
</tr>
<tr>
<td>Alcohol – soluble extractive (% w/w)</td>
<td>4.10</td>
</tr>
<tr>
<td>Loss on drying at 105°C (% w/w)</td>
<td>12.06</td>
</tr>
</tbody>
</table>

**TLC**

The *S. album*, which was used as a experimental drug was purified using thin layer chromatography. The fraction colour and their Rf values in different absorption methods are given in table: 19. Based on the results the plate visuvalized in UV 254 nm showed five fractions with Rf values 0.33, 0.44, 0.65, 0.76 and 0.92 respectively. Interestingly all the fractions found to be green in colour, but in UV 366nm visuvalization showed only one blue fraction at the Rf value of 0.69. The results of vanillin sulphuric acid dipped plates showed seven different fractions with three distinct colours. The Rf value noticed were 0.15, 0.18, 0.21, 0.36, 0.49, 0.81, and 0.92. The HPTLC results of *S. album* are shown in
Plate 5 and the data are tabulated below. It was noted that, the *S. album* extracts produced 11 peaks with different area percentage as 1.8-0.37, 1.02, 1.30, 0.15, 5.55, 15.53, 11.22, 6.84, 14.02 and 12.19% respectively. It also reduces the peak height of 15.4, 0.5, 0.2, 9.7, 4.9, 52.9, 45.1, 64.0, 35.9 and 54.2 AV respectively. It was also noted that, the starting position of the peaks were 1-0.00, 2-0.00, 3-0.11, 4-0.16, 5-0.21, 6-0.28, 7-0.38, 8-0.56, 9-0.70, 10-0.77 and 11-0.90 RF respectively.

**Organic qualitative analysis.**

The qualitative analysis of *S. album* is given in table 20. It was noted that, the alcoholic extract contains triterpenoids, sugar, phenol and tannins,. Interestingly the alcohol extracts did not have any steroid, flavonoid, quinine, alkaloid, coumarin and furanoid. The results also showed that the alcoholic extract yielded 21.98 g extract per kg.

**Table 19. Qualitative photochemical analysis of Santalum album**

<table>
<thead>
<tr>
<th>Test</th>
<th>Santalum album</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steroid</td>
<td>- ve</td>
</tr>
<tr>
<td>Triterpenoids</td>
<td>+ ve</td>
</tr>
<tr>
<td>Flavanoids</td>
<td>- ve</td>
</tr>
<tr>
<td>Sugar</td>
<td>+ ve</td>
</tr>
<tr>
<td>Phenol</td>
<td>+ ve</td>
</tr>
<tr>
<td>Quinone</td>
<td>- ve</td>
</tr>
<tr>
<td>Tannin</td>
<td>+ ve</td>
</tr>
<tr>
<td>Alkaloid</td>
<td>- ve</td>
</tr>
<tr>
<td>Coumarin</td>
<td>- ve</td>
</tr>
<tr>
<td>Furanoid</td>
<td>- ve</td>
</tr>
</tbody>
</table>
DISCUSSION

New drugs may be discovered from a variety of natural sources or even they can be synthesized. In the recent years, plant materials have served as a reservoir of potential new drugs. Though herbalism is practiced since many years, very less works are being carried out in isolation and evaluation of bioactive compounds from marine sources. Most drugs discovered recently are the result of carefully defined research programs of screening, molecular identification and mechanism based drug design. Random screening techniques may be employed initially as a means to detect an unknown activity of the test compound or substance. Prospective drug substances must undergo preclinical testing for biological activity in order to access their potential as useful therapeutic agents. It is essential to assess the pharmacological activity of a new drug in animals before human studies are being conducted.

In recent years, secondary plant metabolites (phytochemicals), previously with unknown pharmacological activities, have been extensively investigated as a source of medicinal agents. Thus, it is anticipated that phytochemical analysis with adequate efficacy will be used to determine the bioactivity of that plants because, basic phytochemical compounds have the ability to alter the activity profile of natural medicine.

Since time immemorial, man has used various parts of plants in the treatment and prevention of various ailments. During the past decade, traditional systems of medicine have become increasingly important in view of their safety.
Current estimates suggest that, in many developing countries, a large proportion of the population relies heavily on traditional practitioners and medicinal plants to meet primary health care needs. Although modern medicine may be available in these countries, herbal medicines (phytomedicines) have often maintained popularity for historical and cultural reasons. Concurrently, many people in developed countries have begun to turn to alternative or complementary therapies, including medicinal herbs (Farnsworth and Soejarto, 1991).

India possesses rich floristic wealth and diversified genetic resources of medicinal plants. It has a widely ranging tropical and the agro climatic conditions, which are conductive for introducing and domesticating new and exotic plant varieties. The use of plants, plant extracts and pure compounds isolated from natural sources provided the foundation to modern pharmaceutical compounds. The well known Indian Systems of Medicine, namely, the Ayurveda and Siddha use predominantly plant based raw materials. Most of these traditional preparations and formulations have been found to be a reservoir of pharmaceuticals (Arora et al., 2003).

The traditional use validation has focused on medicinal plants (Wallace, 2004) for the effective treatment of human diseases. Investigations were restricted to weather or not the plant could rectify the disease. Lot of bioactive and pharmacologically potent drugs have been reported from medicinal plants. Various activities as anti fungal antidiabetic, anti-inflammatory and antioxidant drugs have been isolated form medicinal plants. AD I is characterized clinically by cognitive, impairment and pathological deposition of beta amyloid plaques and neuro
fibrillary tangles in cortex and hypocampus regions of the brain. The ginkgobiloba 
(Stackman et al., 2003), Thespesia populnea (Vasudevan and Parlie 2007), Abana 
(Parlie and Vasudevan 2007) and S. album (Jackson et al., 2009) were proven as 
effective drugs for this chronic memory loss. Apart from this the reducing 
oxidating agents like antioxidants, anti-inflammatory drugs and anticholineesterase 
drugs showed some positive results for the management of AD. In the current 
study, efforts are being taken to purify the active principles and characterize the 
compound through phytochemical evaluations. It was noted that, the alcoholic 
extract contains triterpenoids, sugar, phenol and tannins. Interestingly the alcohol 
extracts did not have any steroid, flavonoid, quinine, alkaloid, coumarin and 
furanoid. The TLC and HPTLC methods were adapted to purify S. album which 
was proven as an effective substance to cure AD in single and in combined forms. 
The S. album extracts produced 11 peaks with different area percentage as 1.8 – 
0.37, 1.02, 1.30, 0.15, 5.55, 15.53, 11.22, 6.84, 14.02 and 12.19% respectively, one 
among these fractions might be responsible for this activity.