Chapter II

Isolation & Characterization of active principle from Hybanthus enneaspermus leaves
Introduction

For decades, natural products have been a well spring of drugs and drug leads. According to a survey by Newman and co-workers at National cancer institute New Delhi, 61% of the 877 small chemical entities introduced as drugs worldwide during 1981-2002 can be traced to or were inspired by natural products (Newman et al., 2003). These include natural products (6%), natural product derivatives (27%), synthetic compounds with natural product-derived pharmacophores (5%) and synthetic compounds designated on the basis of knowledge gained from a natural products i.e., a natural product mimic, 23%.

Natural product chemistry stems from the use of nature for medical purpose. The World Health Organization (WHO) estimates that approximately 80% of the world relies on natural sources for primary medical treatment and that the healthcare systems for the remaining 20% of the population also incorporate natural sources in their medical treatment (Cragg, 2002). Natural product chemistry can be defined as the exploration of nature in search of novel drugs or drug leads. Of these approximately 250,000 higher species of plants it is estimated that only 5-155 have been investigated for natural products (Cragg and Newman, 2001). Therefore, it is important that natural product chemistry continues to explore natural resources in search of new products.

In a drug discovery process, pure active compounds obtained by bioassay-guided isolation from extracts of medicinal plants, are subjected to structure-activity relationship (SAR). Toxicity and safety studies as well as clinical tests are carried out, active compounds have to be prepared on an industrial scale and an appropriate pharmaceutical formulation has to be developed before the compound can be approved as a drug. In a traditional medicine system, however, pharmacological evaluation of extracts from medicinal plants may lead to the establishment of standardized extracts. In this case, the industrial production of
these standardized extracts can start immediately after toxicity and safety studies. After formulation of the standardized extracts, clinical tests are carried out, which may lead to approval as drugs (Vlietinck et al., 1998).

**HYBANTHUS ENNEASPERMUS (L) F.MUELL.**

**Family** : Violaceae

**Common names**: Madanamast, Ratnapurus (Hindi), Orithalthamarai (Tamil), Ratna Purusha (Telugu).

**Distribution** : Warmer parts of India

**Parts used** : Leaves

**Description:**

*Hybanthus enneaspermus* is a perennial herb. 10cm high, globorous or hairy often with woody torches found in warmer parts of India. Stems are slender, creeping, and wiry. Leaves are alternate, simple, clustered, rarely opposite and linear to lanceolate, 1-5cm long. Margins are recurved to revolute, occasionally flat, sessile and shortly petiolate. Stipules present or absent, 1-4mm long. Inflorescence usually axillary cymes or racemes. Flowers are solitary, zygomorphic and mostly strong and bisexual. Sepals are unequal, linear to ovate, 3-4mm long in size, Petals are unequal, lower petals are linear-broad-spatulate (Fig 13). Upper petals are linear-oblong, 3-4mm long. Stamens are short filaments. Anthers are free. But adhering by narrow wings, often with appendages. Anterior anthers with basal nectarines. Stigma is expanded and terminal. Style is S-shaped and simple. Fruit is capsule, 4-9mm long. Ovary is globose, superior, 1-locular, usually with 3 parietal placentas. Ovules are 3-15mm.
The plant extract of *Hybanthus enneaspermus* has been reported to have anti-inflammatory, antitussive, antiplasmodial, anticonvulsant, aphrodisiac and free radical scavenging activity. It has diuretic and demulcent properties. Now it is being used in number of herbal formulations prescribed for gonorrhoea and urinary infections (Wealth of India, 1959). An infusion of the plant extract can also be given in case of cholera.

*Hybanthus enneaspermus* was investigated to evaluate *in vitro* antibacterial activity of aqueous, ethanolic, petroleum ether and chloroform extracts against *E.coli, Pseudomonas aeruginosa, Klebsiella Pneumoniae, Proteus mirabilis, Enterococcus faecalis and staphylococcus aureus* (Collins, C.H, 1970). *Hybanthus enneaspermus* was traditionally used to treat malaria and was screened for *invitro* antiplasmodial activity towards *Plasmodium falciparum* K1 chloroquine resistant and 3D7 chloroquine sensitive strains. The best inhibition of
the growth of *Plasmodium falciparum*Rk1 strain was observed with the methyl chloride extract of *Hybanthus enneaspermus* (Bernard Weniger 2003). The paste of whole plant shall be used topically to treat cough.

**Phytochemical studies:**

The detection of secondary metabolites in medicinal plants play a strategic role in the phytochemical investigation of crude plant extracts and is very important in regard to their potential pharmacological effects. The ethanolic extract of selected medicinal plant was screened for secondary metabolites such as, flavonoids, phenols, triperpinoids, steroids, tannins, alkaloids, glycosides and saponins.

**Objectives:**

1. To isolate and characterize active principle from the leaves of *Hybanthus enneaspermus*.

**Materials and methods:**

Ethanolic extract of the leaves of *Hybanthus enneaspermus*:

Preparation of ethanolic extract from the leaves of *Hybanthus enneaspermus* was carried out as per the “Materials and methods”-chapter.
Isolation of active fraction from the ethanolic extract of *Hybanthus enneaspermus* leaves:

Ethanolic extract of *Hybanthus enneaspermus* leaves

<table>
<thead>
<tr>
<th>Evaluation of Hepatoprotective activity</th>
<th>Ethanol extract fractionated on silica gel column by eluting different solvent systems in their increasing order of polarity (Hexane, EA, EA:IPA 1:1)</th>
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</table>

Fraction 1  Fraction 2  Fraction 3  Fraction 4

No activity  No activity  No activity  Shown activity

Reverse phase purification by column chromatography

n-hexane  100%

Ethylacetate  100%

n-hex: EA

[8:2]

[7:3]

[6:4]

[9:1]

5%ethylacetate: methanol

Purified fraction was collected (2 compounds were unable to separate)

TLC performed

Electronic absorption spectra performed ← HPLC performed ←
The isolation of the active fraction was carried out using gravity column chromatographic method. The ethanolic extract of *Hybanthus enneaspermus* leaves was chromatographed on using reverse phase C18 Chromatography column using hexane as a solvent and eluted with 100% hexane, 100% ethyl acetate and n-hexane: ethyl acetate (9:1), methanol: ethyl acetate(9:1) solvent systems in their increasing order of polarity. After collection of fractions they have monitored by TLC on silica gel plates using suitable solvent systems.

**Antihepatotoxicity activity of Ethylacetate (100%) fractions(1,2,3) and fractions(4) ethanolic extract of *H. enneaspermus* at a dose of 50mg /kg bw. (Mean±SD)**

<table>
<thead>
<tr>
<th>Groups</th>
<th>AST</th>
<th>ALT</th>
<th>ALP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>52.63±0.42</td>
<td>81.63±0.38</td>
<td>132.53±0.42</td>
</tr>
<tr>
<td>Paracetamol(300mg)</td>
<td>91.52±0.33</td>
<td>172.42±0.39</td>
<td>316.57±0.36</td>
</tr>
<tr>
<td>Fraction1 (50mg) + P 300mg</td>
<td>50.18±0.26</td>
<td>83.03±0.31</td>
<td>130.28±0.32</td>
</tr>
<tr>
<td>Fraction2 (50mg) + P 300mg</td>
<td>49.22±0.26</td>
<td>78.96±0.30</td>
<td>120.93±0.31</td>
</tr>
<tr>
<td>Fraction3 (50mg) + P 300mg</td>
<td>35.13±0.23</td>
<td>81.20±0.41</td>
<td>134.93±0.30</td>
</tr>
<tr>
<td>Fraction4 (50mg) + P 300mg</td>
<td>56.61±0.38</td>
<td>90.50±0.33</td>
<td>198.31±0.37</td>
</tr>
</tbody>
</table>

Values are expressed as Mean±SE by Duncan’s multiple range test (DMRT). Means having same subscripts in each column don’t differ significantly at 0.01 level by Duncan’s Multiple range Test (DMRT), p<0.01.

Note: Units observed IU/L

From above table it is clear, fraction1,2,3 doesn’t showed activity related to hepatoprotectiveivity. Hence fraction 4 was selected as it showed activity. The fraction4 was analyzed for further compound determination.
TLC analysis:

Thin layer chromatography was carried out on Merck TLC plates (silica gel coated). Sample was loaded on the plate and the separation of compounds of fractionated sample was carried out in the TLC chamber after saturating the chamber with suitable solvent system i.e. ethanol: chloroform (7:3). The compounds separated were visualized under the conditions of visible, ultraviolet light and iodine vapors.

Table 6 PHYTOCHEMICAL ANALYSIS

<table>
<thead>
<tr>
<th>Secondary metabolites in EEHE</th>
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<tbody>
<tr>
<td>Alkaloids</td>
</tr>
<tr>
<td>Saponins</td>
</tr>
<tr>
<td>Steroids</td>
</tr>
<tr>
<td>Flavonoids</td>
</tr>
<tr>
<td>Tannins</td>
</tr>
<tr>
<td>Triterpinoids</td>
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</table>

+ = Present
- = Absent

Separation of active principle using HPLC:

The active fraction which was concentrated using soxhelt evaporation was dissolved approximately the 20mg/ml in methanolic medium. This sample was subjected to HPLC under the given solvent system for isolation of active fractions of Hybanthus enneaspermus.

COLUMN : Reverse phase, C18 ODS, Gemini 110A; Size - 18cm X 0.5cm
MOBILE PHASE : Methanol: water (70:30)
WAVELENGTH : 254nm
FLOW RATE : 0.5ml/min
VOLUME OF SAMPLE: 20ul (per injection)

Results:

TLC: TLC of crude resulted in streaking of compounds indicating the presence of compound.

*Fig: 16 TLC of the crude ethanolic extract  TLC of the purified extract*

The soxhelt evaporated product upon injection to HPLC the sample was fractionated into one active fraction at the retention time of 2.169 min (fig.14 & 15). This fraction on TLC showed two active compounds in the solvent system of ethanolic extract in ethanol: chloroform (7:3). These compounds were further subjected to their chemical nature. This compound was scratched from the plate and analysed for various biochemical molecules. Upon analysis it showed the presence of 1, 3 butadienes and unsaturated dicarboxylic compounds. The fraction isolated from HPLC was subjected for absorption scanning from the wavelengths range of 200 to 500nm. This compound has absorption maxima at 217nm and 408nm.
Fig: 14 HPLC analysis of crude ethanolic extract:

Fig: 15 HPLC analysis of active principle:
Fig: 17 Absorption spectra of active principle of *Hybanthus enneaspermus* after separation on HPLC

\[ \lambda_{\text{max}} \, 217 = 1.3 \text{ butadienes} \]

\[ \lambda_{\text{max}} \, 408 = \text{unsaturated dicarboxylic compounds} \]

Discussion:

Plants, especially used in Ayurveda can provide biologically active molecules and lead structures for the development of modified derivatives with enhanced activity and / or reduced toxicity. The small fraction of flowering plants that have so far been investigated have yielded about 120 therapeutic agents of
known structures from about 90 species of plants. Some of the useful plant drugs include vinblastine, vincristine, taxol, podophyllotoxin, camptothecin, digitoxigenin, gitoxigenin, digoxigenin, tubocurarine, morphine, codeine, aspirin, atropine, pilocarpine, cascicine, allicin, cucumirin, artemesinin and ephedrine. In some cases, the crude extract of medicinal plants may be used as medications. On the other hand, the isolation and identification of the active principles and elucidation of the mechanism of action of a drug is of paramount importance. Hence, works on both mixture of traditional medicine and single active compounds are very important. Modern science and technology have an essential role to play in the process. Standardisation and validation of known herbal medicines and other relative aspects of preparation need to be focussed (Joy et al., 2001).

The experimental analysis related to biochemical, HPLC and T.L.C as the active fraction separation from crude extract of *Hybanthus enneaspermus* revealed the presence of an active molecule, which had absorption maxima at 217nm and 408nm. These two molecules were separated as single sharp peak on reverse phase HPLC analysis at the retention time of 2.169 min in the solvent systems of MeOH. This molecule is apolar and able to protect the liver of mice from the damage induced by paracetamol. The biochemical analysis suggested that this molecule may be an aromatic which has no free aminoacid or peptide bond attachment. The presence of free amino group and peptide bonds has provided with the negative results of ninhydrin and biuret tests, respectively. Therefore the molecule isolated from *H.enneaspermus* leaves is aromatic with λmax of 217nm and 408nm. However this require further analysis for its specific action on various tissues of animals.
Therefore the present study based on its chemical and spectroscopic analysis conclude that it may be an aromatic molecule with attached moieties of 1,3 butadienes and dicarboxylic compounds present in the preparation of *Hybanthus enneaspermus* as active principles. This principle in the earlier studies also showed the properties of "hepatoprotectivity" in ayurveda. However it require further elucidation for the structure determination.