CHAPTER VII

PURIFICATION AND CHARACTERIZATION OF HEPATOPROTECTIVE PRINCIPLE FROM ELEPHANTOPUS SCABER LINN METHANOLIC EXTRACT

A: Purification of active principle from *Elephantopus scaber*.

7.1 Introduction

Many drugs that are on the market have come to us from folk use and use of plants by indigenous cultures. The use of natural remedies for the treatment of liver diseases has a long history and medicinal plants and their derivatives are still used all over the world in one form or the other for this purpose. Scientific evaluation of plants has often shown that active principles in these are responsible for therapeutic success. A large number of medicinal plants have been tested and found to contain active principles with curative properties against a variety of diseases. Liver protective plants contain a variety of chemical constituents like phenols, coumarins, lignans, essential oil, monoterpenes, carotenoids, glycosides, flavonoids, organic acids, lipids, alkaloids and xanthenes. Recent experience has shown that plant drugs are relatively non-toxic, safe and even free from serious side effects.

Several plant derived secondary metabolites are used in the treatment of liver diseases. Nearly 160 phytoconstituents from 101 plants have been claimed to possess liver protecting activity. In India, more than 87 plants are used in 33 patented and proprietary multi-ingredient plant formulations (Handa et al., 1986). Our studies revealed the hepatoprotective, antioxidant, antiinflammatory, antifibrotic effects of *E. scaber*. So this study was undertaken to isolate, purify
and characterize the active hepatoprotective principle of this plant in an
endeavour to formulate some effective drugs from it.

7.2 Materials and methods

7.2.1 Preparation of plant extract.

The whole plant of *E. scaber* formed the plant material. The collected
plant material was chopped, air dried at 35-40°C for a week and pulverized
in electric grinder. The powder obtained was successively extracted in
petroleum ether (60-80°C), chloroform, methanol and ethanol by using soxhlet
extractor. The extracts were then made to powder with the help of rotary
evaporator under reduced pressure. Of these different extracts collected
methanolic extract was found to be the effective fraction. So methanolic
extract was prepared in sufficient quantities and it was used for further
purification. The methanolic extract was subjected to LCMS analysis.

7.2.2 Purification protocol

Methanolic extract of the plant was prepared in sufficient quantity
using soxhlet extraction. The extract obtained was evaporated to dryness
using rotary evaporator. The extract was partitioned between petroleum ether
(60-80°C) and 10% aqueous methanol in a separating funnel. The 10%
aqueous methanolic layer was removed. The methanolic layer was again
partitioned with petroleum ether and the methanolic layer was evaporated to
dryness and subjected to column chromatography on a silica gel column. The
column was eluted successively with hexane: ethyl acetate (9:1), hexane:
ethyl acetate (3:1), hexane: ethyl acetate (1:4). The hexane: ethyl acetate
(1:4) fraction was dried and on recrystallisation from chloroform gave
yellow needle shaped crystals. The crystal obtained was subjected to LCMS
analysis.
Dried powdered plant (1000g) extracted with methanol for 72 hours using soxhlet extraction

Extract evaporated to dryness under reduced pressure (12gm obtained)

Partitioned between petroleum ether (60-80) and 10% aqueous methanol

Petroleum ether extract

10% aqueous methanolic extract

Silicagel column chromatography

Eluted with hexane: ethyl acetate (9:1)

Eluted with hexane: ethyl acetate (3:1)

Eluted with hexane:ethyl acetate (1:4)

Evaporated to dryness

recrystallised from chloroform to give yellow crystals (30mg)
The crystal obtained was subjected to LCMS analysis and also used to study the \textit{in vivo} hepatoprotective effect.

\textbf{7.2.3 Liquid Chromatography Mass Spectrum analysis (LCMS) of crude methanolic extract}

\textbf{Specifications}

\begin{itemize}
  \item LC COLUMN: Reverse Phase C-18
  \item PUMP: SPD 10 AVP
  \item AUTOSAMPLER TEMP: 293K
  \item MOBILE PHASE: 0.1\% Formic acid in water: 0.1\% Formic acid in acetonitrile
  \item ELUTION MODE: Gradient [as per the customer description]
  \item IONISATION MODE: Electronic Spray Ionization/APCI
  \item MODE: Both Positive and Negative
  \item INJECTION VOLUME: 5 µl
  \item FLOW RATE: 2 ml/min
  \item COLUMN TEMPERATURE: 25°C
  \item COLUMN: PHENOMENEX RP 18
  \item COLUMN DIMENSION: 25 cm x 2.5 mm
  \item LC DETECTION: 254 nm
  \item M/Z RANGE: 40-1000 for POS and 50-1500 for NEG
  \item SOFT WARE: CLASS V P INTEGRATED.
  \item LIBRARY: METWIN 2.0
\end{itemize}
The methanolic extract of *E. scaber* was analyzed using LC-MS 2010A instrument (Shimadzu, Japan). The column and pump used were reverse phase C-18 (PHENOMENEX RP 18 with size 25 cm x 2.5 mm) and SPD 10 AVP respectively. 5 µl of sample was injected to the column using a micro syringe (1-50 µl, Shimadzu). The Mobile Phase used was 0.1% Formic acid in water : 0.1% formic acid in acetonitrile in a gradient mode. The flow rate was 2 ml/min and the column temperature was 25°C. The separated compounds were then ionized using electron spray ionization method (ESI). The spectral data were collected at 254 nm. Mass analysis was performed in the range 40-1000 and 50-1500 m/z for positive and negative ion mode. The Class VP Integrated software was used for the data analysis. The constituents of the extracts were identified by referring the LC-MS library, METWIN 2.0

### 7.2.4 LCMS analysis of purified fraction

**Specifications**

<table>
<thead>
<tr>
<th>Specification</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>WATERS</td>
</tr>
<tr>
<td>LC</td>
<td>WATERS e2695</td>
</tr>
<tr>
<td>UV</td>
<td>WATERS 2489</td>
</tr>
<tr>
<td>MS</td>
<td>WATERS 3100</td>
</tr>
</tbody>
</table>

LC COLUMN: WATERS e2695

PUMP: SPD 10 AVP

AUTOSAMPLER TEMP: 293K

MOBILE PHASE: 0.1% METHANOL LCMS grade: 0.1% Formic acid in acetonitrile

ELUTION MODE: Gradient
IONISATION MODE: Electronic Spray Ionization

MODE: Both Positive and Negative

INJECTION VOLUME: 10 µl

FLOW RATE: 2 ml/min

COLUMN TEMPERATURE: 25°C

COLUMN: PHENOMENEX RP 18

COLUMN DIMENSION: 25 cm x 2.5 mm

LC DETECTION: 220 nm

M/Z RANGE: 40-1000 for POS and 50-1500 for NEG

SOFTWARE: EMPOWER 2

LIBRARY: METWIN 2.0

Methodology

The crystal obtained from the purification of methanolic extract of *E.scaber* was analyzed using LC-MS WATERS instrument. The column and MS used were WATERS e2695 and WATERS 3100 respectively. 10 µl of sample was injected to the column using a micro syringe (1-50µl, Shimadzu). The Mobile Phase used was 0.1% methanol in a gradient mode. The flow rate was 2ml/min and the column temperature was 25°C. The separated compounds were then ionized using electron spray ionization method (ESI). The spectral data were collected at 220nm. Mass analysis was performed in the range 40-1000 and 50-1500 m/z for positive and negative ion mode. Empower 2 software was used for the data analysis. The constituents of the extracts were identified by referring the LC-MS library, METWIN 2.0.
7.3 Results and Discussion

7.3.1 Liquid Chromatography Mass Spectrum Analysis (LCMS) of crude methanolic extract

Fig 7.1 and Table 7.1 depicts the LCMS analysis data of the methanolic extract of *E. scaber*.

Figure 7.1 Liquid Chromatography Mass Spectrum Analysis (LCMS) of Crude Methanolic Extract of *E. scaber*
Table 7.1  Compounds present in the methanolic extract of *E.scaber*

<table>
<thead>
<tr>
<th>SL NO</th>
<th>COMPOUND NAME</th>
<th>MOLECULAR MASS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SCABERTOPIN</td>
<td>358.39</td>
</tr>
<tr>
<td>2</td>
<td>α SANTALOL</td>
<td>220.35</td>
</tr>
<tr>
<td>3</td>
<td>COPAENE</td>
<td>204.35</td>
</tr>
<tr>
<td>4</td>
<td>HOMOCYSTEINE</td>
<td>135.19</td>
</tr>
<tr>
<td>5</td>
<td>HYDROXYMETHYL BENZOIC ACID</td>
<td>152.15</td>
</tr>
<tr>
<td>6</td>
<td>METHOXY CINNAMALDEHYDE</td>
<td>162.19</td>
</tr>
<tr>
<td>7</td>
<td>VASICINONE</td>
<td>202.22</td>
</tr>
<tr>
<td>8</td>
<td>HYDROXY-L-TRYPTOPHAN</td>
<td>220.23</td>
</tr>
<tr>
<td>9</td>
<td>DIHYDROXY FLAVAN</td>
<td>242.28</td>
</tr>
<tr>
<td>10</td>
<td>α LICANIC ACID</td>
<td>292.42</td>
</tr>
<tr>
<td>11</td>
<td>GLUTATHIONE</td>
<td>302.33</td>
</tr>
<tr>
<td>12</td>
<td>ARACHIDIC ACID</td>
<td>312.54</td>
</tr>
<tr>
<td>13</td>
<td>MALVIDIN</td>
<td>331.31</td>
</tr>
<tr>
<td>14</td>
<td>MALTOSE</td>
<td>342.31</td>
</tr>
<tr>
<td>15</td>
<td>AJACONINE</td>
<td>359.51</td>
</tr>
<tr>
<td>16</td>
<td>APODINE</td>
<td>366.42</td>
</tr>
<tr>
<td>17</td>
<td>VERNODALOL</td>
<td>392.41</td>
</tr>
<tr>
<td>18</td>
<td>RUTARIN</td>
<td>424.41</td>
</tr>
<tr>
<td>19</td>
<td>FOLIC ACID</td>
<td>441.41</td>
</tr>
</tbody>
</table>
The methanolic extract of *E. scaber* was subjected to LC-MS analysis and was found to contain 30 compounds including terpenoids, aminoacids, flavonoids, proteins, carbohydrates, phenols, fixed oils, vitamins etc. The sesquiterpene lactones identified include scabertopin, dihydroelephantopin, elascaberin and vernadalol. The essential oils include α-santalol and copaene. Flavonoids include luteolin and dihydroxyflavan, alkaloids like vasicinone and ajaconine, phenols like rutarin and coumaric acid, polyphenols like anthocyanin and malvidin were also identified. Certain carbohydrates and oligosaccharides such as maltose and stachyose were also present. Amino acids such as homocysteine and hydroxy tryptophan were also found to be present.
Researchers have proved that vasicinone possess antioxidant properties (Nilani et al., 2009). Certain studies also proved the antimicrobial and anti-inflammatory activities of this alkaloid (Singh and Sharma, 2013). Oxidative stress and inflammation induced by ischemia-reperfusion was reduced by malvidin, the anthocyanin polyphenol (Jakesevic et al., 2013). This study thus proved the antioxidant efficacy of this compound. p-Coumaric acid (3-[4-hydroxyphenyl]-2-propenoic acid) is a ubiquitous plant metabolite with antioxidant, anti-inflammatory and anticancer properties (Yoon et al., 2013). Ferulic acid, a natural antioxidant act against oxidative stress induced by oligomeric amyloid β-peptide on sea urchin embryo (Picone et al., 2013).

Thus the methanolic extract of *E. scaber* contains a number of secondary metabolites having antioxidant, anti-inflammatory and anticancer properties. These compounds having these reported properties may in single or in combination contribute to the hepatoprotective, antioxidant, anti-inflammatory and antifibrotic effects of methanolic extract of *E. scaber*.

### 7.3.2 LC-MS analysis of purified fraction

LCMS analysis of Purified fraction from *E. scaber* methanolic extract was done and the spectrum obtained is depicted as Fig 7.2. The integrated library search confirmed the presence of five compounds present in the purified fraction of *E. scaber* as listed in Table 7.2.
Figure 7.2 Liquid Chromatography Mass Spectrum Analysis (LCMS) of purified fraction of methanolic extract of *E.scaber*

Table 7.2 Compounds present in the purified fraction of *E.scaber*

<table>
<thead>
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<th>SL NO</th>
<th>COMPOUND NAME</th>
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</tr>
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<tbody>
<tr>
<td>1</td>
<td>SCABERTOPIN</td>
<td>358.39</td>
</tr>
<tr>
<td>2</td>
<td>DIHYDROELEPHANTOPIN</td>
<td>346</td>
</tr>
<tr>
<td>3</td>
<td>ELESCABERIN</td>
<td>376</td>
</tr>
<tr>
<td>4</td>
<td>LUTEOLIN</td>
<td>286.2</td>
</tr>
<tr>
<td>5</td>
<td>METHOXY CINNAMALDEHYDE</td>
<td>162.1</td>
</tr>
</tbody>
</table>
The methanolic extract of *E. scaber* was subjected to fractionation procedure and yellow crystals were obtained. The yellow crystals were subjected to LC-MS analysis and were found to contain sesquiterpene lactones -Scabertopin, dihydroelephantopin and elescaberin, flavanoid-luteolin and oil-cinnamaldehyde.

Scabertopin has been proved to be an antitumour agent *in vitro* in SMMC-7721, Caco-2 and HeLa cell lines in a dose dependent manner. (Xu et al., 2006).Elescaberin exhibited significant inhibitory activity against human SMMC-7721 liver cancer cells *in vitro* (Liang et al., 2008).

Luteolin is a yellow crystalline compound. It is a flavonoid. It is thought to play a role in the human body possibly as an antioxidant, a free radical scavenger and a promoter of carbohydrate metabolism (Johnson et al., 2008). Basic research results indicate luteolin as an anti-inflammatory agent. It has been suggested for multiple sclerosis on the basis of *in vitro* work (Theoharides, 2009). Luteolin displayed an anti-HepatitisC virus activity with EC$_{50}$ values of 4.3 µM in a cell-based antiviral assay (Liu et al., 2012). Luteolin exhibited a good inhibition of NS5B polymerase enzymatic function with an IC$_{50}$ of 1.12 µM according to the method used (Luo et al., 2000).

Recent research documents anticancer activity of cinnamaldehyde/cinnamic aldehyde observed in cell culture and animal models of the disease. Proliferation, invasion, and tumor growth were inhibited in a murine A375 model of human melanoma, though only at high doses not achievable through dietary intake (Cabello et al., 2009).
B. Hepatoprotective efficacy of the purified fraction of *E.scaber*

The purified fraction of *E.scaber* on LC-MS analysis contains Scabertopin, dehydroelephantopin, elescaberin, luteolin and methoxy cinnamaldehyde. After purification the fraction was tested for its efficacy as a hepatoprotectant.

7.4 Materials and methods

7.4.1 Preparation of purified plant material

For animal experiment the purified plant material was prepared as a suspension in 5% Tween 80.

7.4.2 Animals

Male albino rats of Wistar strain weighing between 120 to 150g were used for the experimental purpose. The animals were kept as explained in section 2.2

7.4.3 CCl₄ induced hepatotoxicity in rats

Rats were divided into five groups with six animals in each group.

- **Group I** - Animals served as vehicle control and received oral administration of liquid paraffin twice a week at the dose of 3ml/kg bw

- **Group II** - Animals constituted the toxic control group which received oral administration of LP+CCl₄ in the ratio 1:2 (V/V) twice a week at the dose of 3ml/ kg bw

- **Group III** - Animals were same as in GroupII but received daily silymarin at a dose of 50 mg/ kg bw orally
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Group IV - Animals were same as in Group II but received daily purified fraction obtained from *Elephantopus scaber* at a dose of 2.5 mg/kg bw orally

Group V - Animals were same as in Group II but received daily purified fraction obtained from *Elephantopus scaber* at a dose of 5 mg/kg orally

The duration of the experiment was for 14 days. Animals were kept starved overnight on the fourteenth day. On the next day they were sacrificed by decapitation, by making an incision on jugular vein to collect blood. Blood was allowed to clot and serum separated.

7.4.4 Biochemical evaluation

The effect of *E.scaber* methanolic extract and silymarin CCl₄ induced hepatic dysfunction in rats was evaluated biochemically. Biochemical parameters such as AST, ALT, ALP and LDH were estimated in serum.

7.5 Results and discussion

Table 7.3 depicts the various serum parameters. The activities of serum AST, ALT, ALP and LDH were significantly elevated in carbon tetrachloride treated rats, compared to vehicle control. Administration of *E.scaber* purified fraction at doses 2.5mg/kg bw and 5mg/kg bw and silymarin along with CCl₄ reversed the hepatotoxin induced changes in the activities of AST, ALT, ALP and LDH in serum. It was found that the purified extract exhibited a better hepatoprotection at a dose of 5mg/kg bw compared to 2.5mg/kg bw.
Table 7.3 Effect of purified fraction of *Elephantopus scaber* on AST, ALT, ALP and LDH in carbon tetrachloride intoxicated rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
<th>ALP (U/L)</th>
<th>LDH (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle control</td>
<td>157.8 ± 3.4</td>
<td>59.5 ± 4.6</td>
<td>29.61±1.98</td>
<td>155.3 ± 3.7</td>
</tr>
<tr>
<td>Group 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCl₄-treated</td>
<td>840.5 ± 17.3*</td>
<td>565.3 ± 13.5*</td>
<td>196.92±3.94*</td>
<td>768.3 ± 26.8*</td>
</tr>
<tr>
<td>Group 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCl₄+silymarin</td>
<td>185.8 ± 5.4#</td>
<td>76.3 ± 5.2#</td>
<td>91.32±1.83#</td>
<td>186.8 ± 6.0#</td>
</tr>
<tr>
<td>Group 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCl₄+2.5mg/kg bw of purified fraction</td>
<td>237.1 ± 5.0 “88%”</td>
<td>107.9 ± 4.6 “90%”</td>
<td>119.74±2.85# “46%”</td>
<td>213.9 ± 7.0# “90%”</td>
</tr>
<tr>
<td>Group 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCl₄+5mg/kgbw of purified fraction</td>
<td>184.3 ± 4.0 “96%”</td>
<td>65.1 ± 5.8 “98%”</td>
<td>93.54±0.97# “62%”</td>
<td>171.0 ± 5.5# “97%”</td>
</tr>
</tbody>
</table>

Values are mean±S.D.,n=6

*p*≤0.05 vs normal control

#p*≤0.05 vs CCl₄ control

% protection given in parenthesis

From the *in vivo* studies it has been proved that the purified fraction containing sesquiterpene lactones such as Scabertopin, dihydroelephantopin and Elescaberin, flavonoid - luteolin and oil-cinnamaldehyde has hepatoprotective effect. Detailed investigations are required to confirm the protective mechanism of *E.scaber*. It can be concluded that the hepatoprotective and antioxidant activity exhibited by *E.scaber* extract may be due to these compounds present in the purified fraction. More than that the active components may also be responsible for the antifibrotic and anti-inflammatory activities of *E.scaber*. 