Chapter 11

Isolation & Identification of active component from leaf extract of *Barleria lupulina*
11.1 INTRODUCTION

In Chapter 8, exact chemical nature of extract of *Barleria lupulina* had been identified. Next its ameliorating effect on mice and fish was studied in Chapter 9 and 10. In this chapter, isolation of biological active component from the leaf extract is done by column chromatography and they were analyzed by TLC, phytochemical analysis and NMR studies.

11.2 COLUMN CHROMATOGRAPHY

11.2.1 Materials And Methods

11.2.1.1 Preparation of Sample

Leaf extract (BLEE) obtained after Soxhlation (1.75 gm) (Chapter 8) was dissolved in adequate amount of 99.9% ethanol. Then it was mixed with silica gel C (mesh size 100-200, EMerck), and dried to remove the solvent. This was further used for column packing.

11.2.1.2 Column packing

Glass column, 1 meter in length and 10 cm diameter, was plugged with cotton at the bottom and packed with silica gel C mixed in 100% petroleum ether by following wet packing method (Fig 11(a)). After complete packing of column and ensuring that no air bubble has accumulated within the column, sample was poured on top following the same procedure, till complete sample was packed. Care was taken that much of solvent remains above the column layer so the column doesn’t dry up and crack due to absence of solvent.

11.2.1.3 Elution

Two types of solvent system were employed, throughout the elution process.

1. Isocratic Solvent System i.e. single solvent system
2. Gradient Solvent System i.e. mixture of solvents

Elution was done using varying concentrations of non-polar, medium polar and polar solvents as petroleum ether, chloroform, ethyl acetate, methanol and ethanol according to the increasing order of polarity (Table 11(a), Fig 11(b), Fig 11(c), Fig 11(d)).

11.2.1.4 Phytoconstituents Present (Qualitative)

After elution, each fraction was heated on water bath to evaporate the solvents and to concentrate their constituents (Fig 11(e)). Qualitative analysis of the fractions was performed by standard phytochemical tests described in Chapter 8 (Fig 11(f)).
Fig 11(a): Column packed before sample

Fig 11(b): Elution of Fraction 2 charging (terpenoid)

Fig 11(c): Elution of Fraction 8 (steroids)

Fig 11(d): Elution of Fraction 61 (towards end)
11.2.2 RESULTS

A total of 67 fractions were obtained from column chromatography, out of which, 52 yielded Phytoconstituents [Table 11(a)]. Chromatography was initiated with petroleum ether (isocratic non polar solvent) then gradually shifting to medium polar solvents (chloroform, ethyl acetate) and finally with methanol and ethanol (highly polar solvent). Compounds in pure form as terpenoids were isolated in fractions 2-5 (eluted with petroleum ether and chloroform), and in fractions 12 and 13 (eluted with ethyl acetate and chloroform); steroids were isolated in fractions 6-11 (eluted with petroleum ether and chloroform) and also in fractions 24-26 (eluted with ethyl acetate & chloroform). Further eluting with ethyl acetate and methanol in varying concentrations, the mixture yielded flavonoid in fractions 31-35, glycoside in fractions 28-30, 40-44, and finally steroid again in fractions 45-52. Each fraction was tested for their nature by performing qualitative phyto chemical tests, simultaneously. The fractions 14-23, 27 and 36-39 were mixtures of compounds and in fraction 53 to 67, phyto-constituents were absent. Tannins were present in very trace amount & in mixture in fraction number 36-39. The results for each fraction obtained in column chromatography have been summed up in Table 11(a).
Table 11(a) Solvent Systems used for elution in column chromatography and Phytoconstituents identified for each fractions

<table>
<thead>
<tr>
<th>Sl No</th>
<th>Fraction No</th>
<th>Eluent</th>
<th>Phytoco Present (Qualitative)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>100% petroleum ether</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>90% petroleum ether  + 10% chloroform</td>
<td>terpenoids</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>75% petroleum ether  + 25% chloroform</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>50% petroleum ether  + 50% chloroform</td>
<td></td>
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<tr>
<td>5</td>
<td>5</td>
<td>50% petroleum ether  + 50% chloroform</td>
<td></td>
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<tr>
<td>6</td>
<td>6</td>
<td>50% petroleum ether  + 50% chloroform</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>25% petroleum ether  + 75% chloroform</td>
<td>sterols</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>90% petroleum ether  + 10% chloroform</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>9</td>
<td>100% chloroform (isocratic)</td>
<td></td>
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<tr>
<td>10</td>
<td>10</td>
<td>100% chloroform (isocratic)</td>
<td></td>
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<tr>
<td>11</td>
<td>11</td>
<td>100% chloroform (isocratic)</td>
<td></td>
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<tr>
<td>12</td>
<td>12</td>
<td>10% ethyl acetate    + 90% chloroform</td>
<td>terpenoids</td>
</tr>
<tr>
<td>13</td>
<td>13</td>
<td>25% ethyl acetate    + 75% chloroform</td>
<td>terpenoids</td>
</tr>
<tr>
<td>14</td>
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<td>25% ethyl acetate    + 75% chloroform</td>
<td></td>
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<tr>
<td>15</td>
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<td>25% ethyl acetate    + 75% chloroform</td>
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<tr>
<td>16</td>
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<td>25% ethyl acetate    + 75% chloroform</td>
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<tr>
<td>17</td>
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<td>25% ethyl acetate    + 75% chloroform</td>
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<tr>
<td>18</td>
<td>18</td>
<td>25% ethyl acetate    + 75% chloroform</td>
<td>steroids + terpenoids</td>
</tr>
<tr>
<td>19</td>
<td>19</td>
<td>30% ethyl acetate    + 70% chloroform</td>
<td></td>
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<tr>
<td>20</td>
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<td>30% ethyl acetate    + 70% chloroform</td>
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<tr>
<td>21</td>
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<tr>
<td>25</td>
<td>25</td>
<td>90% ethyl acetate    + 10% chloroform</td>
<td>steroids</td>
</tr>
<tr>
<td>26</td>
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<td>100% ethyl acetate (isocratic)</td>
<td></td>
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<tr>
<td>27</td>
<td>27</td>
<td>98% ethyl acetate    + 2% methanol</td>
<td>steroids + glycosides</td>
</tr>
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<td>flavonoids</td>
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<tr>
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<tr>
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<td>ethanol</td>
</tr>
<tr>
<td>67</td>
<td>67</td>
<td>50%</td>
<td>ethanol</td>
</tr>
</tbody>
</table>

**Steroids + flavonoids + tannin (traces)**

**Glycosides**

**Steroids**

**Phyto-constituents absent**
11.2.3 DISCUSSION

Work on isolation of constituents from leaves of *Barleria lupulina* is very less. In previous study, Kanchanpoom *et al.* (2001) reported the presence of iridoid glucosides, phenylpropanoid glycosides, lignan glucoside, aliphatic glycoside and benzyl alcohol glycoside from the aerial part of *Barleria lupulina*. Here the author reports other class of compounds, besides glycosides as terpenoids, steroids, tannins (in traces), and flavonoids. The exact identification of the compound and molecular formula elucidation had been done by TLC and NMR analysis, which is discussed below.

11.3 PREPARATIVE TLC and 1H NMR STUDIES

11.3.1 Materials and Methods

11.3.1.1 Preparative TLC

Preparative TLC was performed for exact identification of active component, obtained in column chromatography. TLC of the fractions was run along with commercially available standards. Components of fractions were identified by comparison with Rf values of the standards.

Standard 60F$_{254}$ TLC plates (pore size; 60 Å, uv fluorescence at 254 nm, Merck, Germany) were used. The silica gel acted as the stationary phase and mobile phase was chosen by trial and error method. Very small amount of the fractions from column chromatography were put on the plates with the help of a capillary tube along with commercially available standards and TLC was run using the mobile phase (Table 11(b)). Components of the fractions were identified by comparing with $R_f$ value of the standards. Standards and mobile phase used in the experiment are given in Table 11(b).
Table 11(b): Standards and mobile phase used in TLC to identify active component of fractions

<table>
<thead>
<tr>
<th>Sl No</th>
<th>Phytochemical result</th>
<th>Standards used</th>
<th>Mobile phase used</th>
<th>Ratio of mobile phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Terpenoid</td>
<td>Ursolic acid</td>
<td>methanol : chloroform</td>
<td>7:43</td>
</tr>
<tr>
<td>2</td>
<td>Steroid</td>
<td>β Sitosterol</td>
<td>benzene : petroleum ether: chloroform : ethyl acetate</td>
<td>4:2:3:1</td>
</tr>
</tbody>
</table>

Standards for flavonoid and tannin were not available and thus they could not be identified by this method.

11.3.1.2 1 H NMR study of the fractions

1 H NMR of the fractions was performed by dissolving 15 mg of each fraction in 1 ml (CDCl₃). The NMR machine (Bruker Corp.) was operated at 400 MHz.

11.3.2 RESULTS

11.3.2.1 Preparative TLC

The fractions after column chromatography (test) along with the identified compounds in TLC and corresponding \( R_f \) values of standard and test are shown in Table 11(c) and Fig 11(g). In fraction No. 13, terpenoid was identified as ursolic acid (\( R_f \) value = 1), in fraction No. 24-26, steroid as β sitosterol (\( R_f \) value = 0.59) and in Fraction No. 28-30, glycoside as Sitosterol-3-O-glucoside (\( R_f \) value = 0.35)
Table 11(c): Preparative TLC: Identification of terpenoid, steroid and glycoside by comparison with commercially available standards

<table>
<thead>
<tr>
<th>Sl No</th>
<th>Fraction No</th>
<th>Phytochemical Result (Test)</th>
<th>Standard used</th>
<th>Mobile phase used</th>
<th>Rf value of standard</th>
<th>Rf value of test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13</td>
<td>Terpenoid</td>
<td>Ursolic acid</td>
<td>Methanol: chloroform (7:43)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>24-26</td>
<td>Steroid</td>
<td>β sitosterol</td>
<td>Benzene : petroleum ether: chloroform: ethyl acetate (4:2:3:1)</td>
<td>0.59</td>
<td>0.59</td>
</tr>
<tr>
<td>3</td>
<td>28-30</td>
<td>Glycoside</td>
<td>Sitosterol-3-O-glucoside</td>
<td>Benzene : chloroform: ethyl acetate: methanol (18:2:1:4)</td>
<td>0.35</td>
<td>0.35</td>
</tr>
</tbody>
</table>

{methanol : chloroform (7:43) didn’t give fruitful results}

Fig 11(g): Identification of terpenoid, steroid and glycoside by comparison with standards by TLC

Ursolic acid (terpenoid)  β sitosterol (steroid)  Sitosterol-3-O-glucoside (glycoside)
11.3.2.2  **1 H NMR study of the fractions**

The 1H NMR results of ursolic acid, β sitosterol and sitosterol-3-O-glucoside are shown in Fig 11(h), Fig 11(i) and Fig 11(j) respectively.

Five main signal peaks are observed in case of ursolic acid. The highest signal peak (doublet) is observed at 1.293 and 1.254 intensities, and chemical shift between 1.5 to 1.1 ppm. In case of β sitosterol, five doublets, one doublet-of-doublets and one triplet are observed, representing that the protons are arranged in three different coupling patterns. Some peaks in this NMR are at 5.823, 4.941, 4.307 etc signal intensities. Thirdly, the NMR data of Sitosterol-3-O-glucoside reveals three peaks, among which, the highest signal intensity is 1.254 between 71.30 ppm chemical shifts.
Fig 11(h): 1H NMR study of ursolic acid
Fig 11(i): 1H NMR study of β sitosterol
Fig 11(i): 1H NMR study of Sitosterol-3-O-glucoside
Thus the terpenoid was identified as Ursolic acid, steroid as β sito sterol and glycoside as Sitosterol-3-O-glucoside.

11.3.2.3 **Structures**

The structures of these compounds along with molecular formula and IUPAC names are given as under

1. **Ursolic acid**
   a) Molecular formula : C\textsubscript{30}H\textsubscript{48}O\textsubscript{3}
   b) IUPAC name : 3β-hydroxy-urs-12-en-28-oic-acid (Furtado et al., 2008)
   c) Structure

![Ursolic acid structure]

2. **β sito sterol**
   a) Molecular formula : C\textsubscript{29}H\textsubscript{5}O
   b) IUPAC name : 17-(5-Ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol
      (Ref: http://en.wikipedia.org/wiki/Beta-Sitosterol)
   c) Structure

![β sito sterol structure]
3. **Sitosterol-3-O-glucoside**
   a) Molecular formula : $C_{35}H_{60}O_6$
   b) IUPAC Name : (3-beta)-Stigmast-5-en-3-yl-beta-D-glucopyranoside
   c) Structure

![Structure of Sitosterol-3-O-glucoside]

**11.3.3 DISCUSSION**

Ursolic acid is a pentacyclic triterpenoid compound (Lai et al., 2007) and is a major component of traditional medicinal herb as *Hedyotis diffusa*, *Eribotrya japonica* and *Ligustrum lucidum* (Lai et al., 2007). The five signal peaks obtained in our NMR result of ursolic acid (Fig 11(h) represents its pentacyclic nature. The highest peak which is a doublet represents the single proton of the OH group which is near the $=O$ group at the right hand side of its molecular structure. The other weak signal peaks are for the protons at the side chains. This triterpenoids have shown hepatoprotective (Liu 1995), antiallergic (Banno et al., 2004), anti-ulcer (Ovesná et al., 2004), cardioprotective (Senthil et al., 2007), antimicrobial (Ngouela et al., 2005), antiinflammatory, analgesic (Vasconcelos et al., 2006) and antioxidant (Huang et al., 1994) activities. It has potent anti tumor activity also (Liu 2005; Ovesná et al., 2006). Ursolic acid acts on different stages of tumor development as tumor initiation and promotion and also plays important role in tumor cell differentiation apoptosis (Liu 2005; Ovesná et al., 2006) and inhibit tumor angiogenesis (Sohn et al., 1995). Pathak et al., (2007) had shown that this terpenoid inhibits STAT 3 activation pathway, leading to suppression of Human Multiple Myeloma Cells. Even, ursolic acid inhibits PMA-induced inflammation and tumor promotion in mouse skin (Huang et al., 1994). Subbaramaiah et al., (2000) proved that ursolic acid suppresses the activation of COX-2 gene expression by inhibiting the PKC signal transduction.
pathway. Even ursolic acid attenuates D-galactose-induced inflammatory response in mouse prefrontal cortex through inhibiting AGEs/RAGE/NF-κB pathway activation (Jun et al., 2010). The presence of ursolic acid in our extract of Barleria lupulina has shown anti-elastogenic, anti-tumor, anti-cancer and radio-protective activities on fish and mice (Das and Sur 2012).

β sitosterol is one of the phytosterols that has structure similar to cholesterol. It is a white waxy powder with a characteristic odor. It is widely available in varieties of plants and plant parts as Nigella sativa, Serenoa repens (saw palmetto), avocados, Pygeum africanum, cashew fruit, rice bran, Cucurbita pepo (pumpkin seed), wheat germ, corn oils, soybeans, sea-buckthorn and Wrightia tinctoria etc. (http://en.wikipedia.org/wiki/Beta-Sitosterol). The five doublets, one doublet of doublets and one triplet is observed in our NMR study of this steroid (Fig 11(i) represent the hexacyclic and pentacyclic groups; the connecting chain and the methyl groups respectively. β sitosterol reduces cholesterol level in blood (therefore sometimes used in treating hypercholesterolemia), inhibits absorption of cholesterol in intestine (Matsuoka et al., 2008) and thus causes less cholesterol absorption in the body. Even, this sterol is used in the treatment of benign prostatic hyperplasia (BPH) (Berges et al., 1995) and prostatic carcinoma (Stephen et al., 2005). Jourdain et al., (2006) proved that beta-sitosterol in combination with polyphenols from cocoa inhibits proliferation of prostatic cancer cell growth. β sitosterol is also found to be present in leaves of Barleria lupulina of the present work (Das and Sur, 2012).

Glycosides are molecules that contain a sugar (glycone) and a non-sugar (aglycone) moiety. When the sugar moiety is a glucose derivative, it is called a glucoside and sitosterol-3-O-glucoside falls under the class β D glycoside (http://chemxo.com/EN/products/J82ASB-00019291-005.html). The three peaks of Sitosterol-3-O-glucoside our NMR study (Fig 11(j) represent the gluco-pyranose sugar, cyclo-hexagon and cyclo-pentagon groups and the free side chains. Jares et al., (1990) reported the presence of sitosterol-3-O-β-d-glucopyranoside in extract of roots of Senecio bonariensis. Gohar et al., (2009) had stated that methanolic extracts of seeds of Ceratonia siliqua L. has a rich source of natural anti-oxidants, which contains β sitosterol-3-O-glucoside along with other flavonol glycosides. Hence, the presence of β sitosterol-3-O-glucoside in the present work also
accounts for the anti-clastogenic, anti-tumor, anti-cancer and radio-protective activities of *Barleria lupulina*.

In the present work, we are the first to report the rich source of ursolic acid, β sito sterol and sitosterol-3-O-glucoside in leaves of plant *Barleria lupulina*. Leaf extract of this plant containing these components, had been found by us to have anti clastogenic, anti cancer, radio protective and anti tumor activities (in mice and fish) which had been discussed earlier. It is discussed above that ursolic acid, β sitosterol show potent anti cancer activity. Therefore, in conjugation with sitosterol-3-O-glucoside, these three components together act as a potent anti cancer agent in mice and fish models.

Presently, effect of X-rays and γ rays is carried out on house musk shrew *Suncus murinus* in our laboratory. Protection by *B. lupulina* is also studied.

### 11.4 Conclusion

Therefore we are the first to report anti-cancer, radio-protective, anti-clastogenic and anti-tumor activities in ethanolic extracts of leaves of *Barleria lupulina*. Patenting had been done in this respect, which is discussed earlier.