Chapter 8
Isolation, Purification and Characterization of Bioactive Factor from H. candolleanum
8.1 Introduction

Modern research of drug discovery from medicinal plants involves a resourceful move toward interlinking botanical, phytochemical, pharmacological, biological and molecular systems. The phytochemical based research on ethnopharmacology is considered an effective approach in the discovery of novel chemicals entities with potential as drug leads. The traditional knowledge of plants, plant extracts, decoctions, for treating several diseases, represents a source of chemical entities but no information is available on their nature. Extensive predominance of diseases and scarcity of drugs with lesser side effects has necessitated to developing satisfactory drugs from indigenous plants. Such a profile helps the plants to gain more attention from conservative organizations.

Nowadays some highly rated commercially available drugs for various diseases are natural products derived from folk medicine (Lahlou, 2013). The curative medicines available in current medicine system for various diseases are steroids, immunosuppressant’s and non steroidal anti-inflammatory drugs and most of them provide only symptomatic relief mostly without influencing the disease process and their use is coupled with the risk of relapse and danger of side effects. The fast growing scenario in the current status of medicinal plants warrants an impending need of purification and characterization of the active principles offering
therapeutic potential. Preferably compound isolation method is the best choice of methodologies, to develop novel drugs from medicinal plants, which include the combination of various analytical techniques. The initial phase of work was associated with different ethnopharmacological methods, which include extraction, sample preparation and various biological screening processes. Final phase of work include different investigative techniques which are essential for the isolation and identification of compounds liable for the biological activity claimed in the traditional use (Brusotti et al., 2014). Drug discovery from medicinal plants has traditionally been lengthier and more complicated than other drug discovery methods.

In recent records, the increased influence of plants as therapeutic agents evoke the system of active fraction guided isolation. A best known case is isolation of morphine from opium in the beginning of 19th century; cocaine, digitoxin, quinine, and codeine are some of the best known examples of similar drugs. Among them some are still used in pharma industry (Kinghorn, 2001; Newman et al., 2000; Butler, 2004; Samuelsson, 2004).

Our previous study of all the three plant parts of *H. candolleanum* revealed that the crude methanol extract of root posses more antioxidant, anti-inflammatory and anticancer activity against different *in vitro* models.
The better activity and novelty of root extract guided us to isolate, purify and characterize the bioactive factor from the root of this plant in a venture to formulate some effective drugs from it.

8.2 Materials and methods

8.2.1 Isolation, purification and characterization

Root extract of *Heracleum candolleanum* was prepared as per the procedure mentioned in chapter 3 para 3.2.2. The extract was then passed through sodium sulphate for the removal of aqueous matter. The material was then subjected to thin layer chromatography and column chromatography. About 500gm of silica gel the adsorbent (60-120 mesh size) was weighed using a ratio of 30 gm of the adsorbent to 1gm of the crude and is kept in oven for 1hr at 105°C for activation. The column was filled with a small amount of toluene. Slurry of weighted adsorbent was prepared using 100% toluene and then carefully poured into the column without any air bubbles formation. A solution of the extract was then introduced on top of the stationary phase. This layer was topped with a small layer of sand or with cotton or glass wool to protect the shape of the organic layer from the flow of the eluting solvents. The column was then eluted with the mixture of Toluene: Ethyl acetate in a ratio of 9:1, 7:3, 4:6 and 6:4. Each mixture were added around 150ml each. A total of 600ml were collected in 30 tubes. Based on TLC similar fractions were clubbed
together. About 25ml of eluting mixture was collected until there appeared to be no more solute in the column. The fractions collected were monitored by TLC and similar fractions were combined together and grouped as fraction-A (F-A), fraction-B (F-B) and fraction-C (F-C). To ensure the purity of fraction TLC analysis was carried out with the semi dry fraction using various polar solvents. The method is schematically represented in Fig 8.1.

**Fig.8-1** Schematic representation of isolation and purification of active fraction through column chromatography

Then all the three fractions were subjected to *in-vitro* DPPH analysis for verifying its activity difference, Fig.8-2. Among the three
groups, fraction-C showed good performance with a value of 59.50% and was selected for further characterization studies.

Fig.8-2 DPPH radical scavenging activity of fraction-A, fraction-B and fraction-C

**DPPH radical scavenging activity of fraction-A, fraction-B and fraction-C against reference standard BHA, values are Mean ± SD (n=6).**

Fraction-C on further TLC analysis using solvent system n-hexane-dichloromethane-ethyl acetate in a ratio of 4:4:2 showed single compound spot; indicating it as a pure compound (Compound-1).
8.2.2 Characterization of bioactive fraction

Mass spectrum was recorded in Shimadzu FSMS-1010A. $^1$H-NMR and $^{13}$C-NMR spectra were recorded with a Bruker AVANCEDPX-400 MHz in DMSO.

8.2.3 Bioactive assays of Compound-1

In vitro antioxidant and anti-inflammatory assays were conducted to evaluate the bioactivity of compound-1. Antioxidant studies were done with DPPH radical scavenging, Nitric oxide radical scavenging, Hydroxyl radical scavenging and superoxide scavenging methods (Chapter-3, para 3.2.4.2, 3.2.4.3, 3.2.4.4 and 2.3.4.5). The in vitro models used for anti-inflammatory studies were protein denaturation method, HRBC membrane stabilization method and proteinase inhibitory method (Chapter 6, para 6.2.2, 6.2.3 and 6.2.4).

8.3 Results

Different phytochemical class tests were performed as per standard protocols mentioned in Harbone. Compound-1 gave positive test for coumarins, the yellow colour developed by treatment with 10% alcoholic KOH disappears by the addition of HCl.

8.3.1 Structural elucidation

The chemical structure of Compound-1 was elucidated by spectroscopic methods. $^1$H-NMR spectrum (Fig 8-3) showed two aromatic
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proton peak at $\delta$ 6.25 and $\delta$ 8.25 which corresponds to C-3 and C-4 protons. The sharp peak at $\delta$ 4.25 reflects the presence of methoxy group in the molecule. At $\delta$ 6.2, a proton peak indicates the possibility of oxygen containing five member cyclic rings with double bond (Attia *et al*., 2015).

Fig 8.3 $^1$HNMR spectrum of Compound-1

The $^{13}$C-NMR study (Fig 8-4) established the presence of carbonyl groups by signals at 166ppm and 175ppm. Carbon resonance from carboxylic acid derivates generally appears in the 160-180ppm region. The C-6 proton appeared as a single peak at $\delta$112.25 and C-7 proton at
δ156.25. A strong peak at δ 145.32 indicates the presence of C-5 (Abyshev et al., 1988).

![CNMR spectrum of Compound-1](image)

**Fig 8.4** $^{13}$CNMR spectrum of Compound-1

The ESI-MS spectrum of compound (Fig: 8-5) with a molecular ion peak (M+H)$^+$ at 217m/z confirms the possibility of molecular formula C$_{12}$H$_8$O$_4$ (Wangchuck *et al.*, 2012). All the above findings are in agreement with data reported for bergapten in literatures (O’Neill *et al*., 2013; Dincel *et al*., 2013; Fujioka *et al*., 1999).
Based on $^1$H-NMR, $^{13}$C-NMR and ESI-MS spectra Compound-1 was assertively characterized as bergapten and its chemical structure is illustrated in Fig 8-6.

![Chemical structure of Bergapten](image_url)
8.4 Bergapten

Among several phenolic groups coumarins are considered as an effective secondary metabolite found in plant kingdom, famous for its diverse bioactivities for instance anti-inflammatory, anticancer, antifungal, antibacterial, anticoagulant, antiviral, antihypertensive, antitubercular, anticonvulsant, antioxidant, antihyperglycemic and neuroprotective properties (Venugopala et al., 2013). Furanocoumarins are derivatives of coumarin with pharmacologically important group phytoalexins (Wong and Kitts, 2006; Smith et al., 2004; Ozcelik et al., 2004) and a set of allelochemical compounds (Stevenson et al., 2003). Bergapten (5-methoxypsoralen) is a linear furanocoumarin predominantly distributed in four angiosperm families: Apiaceae, Moraceae, Rutaceae and Leguminose (Sidwa-Gorycka et al., 2003; Franke et al., 2001; Milesiet al., 2001). The isolated bergapten from hydroalcoholic extract of *Heracleum nepalense* root exhibited significant inhibition of release of tumor necrotic factor-α (TNF-α) and interleukin-6 (IL-6) (Bose et al., 2011). The coumarin component bergapten present in *Ficus hirta* possess moderate antioxidant activity (Yi et al., 2013) and bergapten from *H. crenatifolium* showed significant anticonvulsant activity (Tosun et al., 2008). Anti-viral and anti-inflammatory activity of bergapten isolated from *Ammimajus L* has been reported (Saleim and Ouf, 2012) and bergapten from *Fatoua pilosa* proved
antitubercular activity against *Mycobacterium tuberculosis* (Chiang *et al*., 2010).

(Spectroscopic study: Uwin Life Sciences, Bangalore. Technical advice: National Institute for Interdisciplinary Science and Technology (NIIST) and TBGRI, Thiruvananthapuram)

**8.4.1 In vitro antioxidant activity of Compound-1**

The free radical scavenging activity of C-1 was studied using different *in vitro* analysis models like DPPH, Superoxide, Nitric oxide and Hydroxyl radical methods. The results acquired from antioxidant studies indicated that Compound-1 exhibited significant free radical quenching activities and the IC\textsubscript{50} values of compound-1 on different free radical models were presented in Table 8-1.

<table>
<thead>
<tr>
<th>Antioxidant Methods</th>
<th>Compound-1 (IC\textsubscript{50})</th>
<th>Reference compound (IC\textsubscript{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH</td>
<td>276.42 µg/ml</td>
<td>193.40 µg/ml</td>
</tr>
<tr>
<td>Superoxide</td>
<td>479.66 µg/ml</td>
<td>226.73 µg/ml</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>262.44 µg/ml</td>
<td>218.34 µg/ml</td>
</tr>
<tr>
<td>Hydroxyl radical scavenging</td>
<td>230.16 µg/ml</td>
<td>172.81 µg/ml</td>
</tr>
</tbody>
</table>

*In vitro* antioxidant activities of compound-1 isolated from *H. candolleanum*. Reference standard for DPPH-BHA, Nitric oxide-Curcumin, Hydroxyl radical-BHA and Superoxide-Ascorbic acid. Values are Mean ± SD (*n*=6).
8.4.2 In vitro antiinflammatory activity of Compound-1

Antiinflammatory activity of Bergapten has great importance because the tribal leads of *H. candolleanum* as an anti-inflammatory drug argue the scientific community to reveal the truth behind it. From the data it was very clear that Compound-1 showed strong anti-inflammatory character (Table 8.2).

Table 8.2 In vitro antiinflammatory activities of Compound-1 isolated from *Heracleum candolleanum*

<table>
<thead>
<tr>
<th>Antiinflammatory Methods</th>
<th>Compound-1 (IC$_{50}$)</th>
<th>Aspirin (IC$_{50}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein Denaturation</td>
<td>179.44µg/ml</td>
<td>247.47 µg/ml</td>
</tr>
<tr>
<td>HRBC Membrane Stabilization</td>
<td>230.45µg/ml</td>
<td>231.99µg/ml</td>
</tr>
<tr>
<td>Proteinase Inhibitory Activity</td>
<td>251.71µg/ml</td>
<td>263.88µg/ml</td>
</tr>
</tbody>
</table>

*In vitro antiinflammatory activities of Compound-1 isolated from *H. candolleanum* against standard drug Aspirin. Values are Mean ± SD (n=6).

8.5 Discussion

In relation to significant bioactive results methanol extract of *H. candolleanum* was subjected to fractionation studies. Among the three fractions obtained through chromatographic techniques fraction–C exhibited more bioactivity against DPPH scavenging test. The yellow color formation of fraction-C on addition of alcoholic KOH established the
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The presence of furanocoumarins. By TLC method the purity of fraction is checked and presence of single compound is confirmed. Then the structure was identified by $^1$H-NMR, $^{13}$C-NMR and ESI Mass spectrum. The data obtained by NMR studies were compared with literature and identified the Compound-1(C-1) as Bergapten. Further the mass spectrum of Compound -1 confirmed its identity. Then antioxidant and anti-inflammatory studies of compound-1 was carried out with different *in-vitro* models. Data obtained from antioxidant studies revealed that Compound-1 exhibited dose dependent increase in activity with concentrations of 10µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, 250 µg/ml and 500 µg/ml, in all tests of DPPH radical scavenging, superoxide scavenging, nitric oxide scavenging and hydroxyl radical scavenging. Compound-1 showed lowest IC$_{50}$ value (230.16µg/ml) for hydrogen peroxide scavenging which is comparable with that of reference compound (172.81µg/ml). At 500 µg/ml concentration C-1 showed maximum DPPH radical scavenging activity and it was comparable with that of standard curcumin. C-1 was highly efficient in nitric oxide radical scavenging (IC$_{50}$ 262.44 µg/ml) that was comparable with the reference standard BHA (IC$_{50}$ 218.34 µg/ml). A strong superoxide radical scavenging activity of C-1 was obtained at a concentration of 500µg/ml with an IC$_{50}$ value of 479.66µg/ml and the
reference compound Ascorbic acid showed a corresponding IC$_{50}$ value of 226.73µg/ml.

*In-vitro* anti-inflammatory studies proved that C-1 showed dose dependent protein denaturation, HRBC membrane stabilization and proteinase inhibitory activity against reference standard Aspirin. A concentration dependent inhibition of protein denaturation was observed at various concentrations of 25 µg/ml, 50 µg/ml, 100 µg/ml, 250 µg/ml and 500 µg/ml. At 500µg/ml C-1 performed the best with an IC$_{50}$ value of 179.44µg/ml corresponding to reference standard Aspirin (IC$_{50}$ 247.47); however, the effect of Aspirin was found to be less when compared with C-1. In HRBC membrane stabilization C-1 showed the highest inhibition at 500 µg/ml with an IC$_{50}$ value of 230.45µg/ml and it was close to the IC$_{50}$ of aspirin (231.99µg/ml). This proved that C-1 may possibly inhibit the release of lysosomal content of neutrophils at the site of inflammation. Neutrophils are considered as rich source of proteinases and in arthritic inflammation their presence is highly detected. C-1 at 500µg/ml exhibited significant anti proteinase activity with an IC$_{50}$ value of 251.71µg/ml. The standard drug aspirin expressed a corresponding IC$_{50}$ value of 263.88µg/ml.

Bergapten was already been reported as major bioactive compound from several plants including different species of *Heracleum*. This is the
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first time bergapten was reported from *H. candolleanum* root, however literature review revealed that bergapten was reported from fruits of *H. candolleanum* (Chacko *et al.*, 2000). From the observations we can conclude that the antioxidant, antiinflammatory and anticancer property of *H. candolleanum* root may be attributed to the presence of linear furanocoumarinbergapten. Further isolation and purification of other fractions of this plant is recommended which could yield some novel bioactive compounds.