Chapter-6

In Vitro Anti-inflammatory Activity of H. candolleanum
6.1 Introduction

Inflammation arises in the body as a part of bodies self repairing mechanism for patch up any injury happened by trauma, infections caused by microbial attack or with any chemical agents. The characteristic features of inflammation are pain, swelling, redness, unusual heat and uneasiness for physical functions. Inflammation helps the body to destroy the invading microorganisms and irritating agents introduced in to it. Inflammation motivates the damaged cells to release chemical mediators to patch up the destructed tissues. Non steroidal anti-inflammatory drugs are the commonly used medicine for inflammation but side effects like gastric irritation mediated ulcers were reported in several cases (Tripathi, 2008). One of the inflammatory studies solemnly suggests that, regular-dose aspirin, diclofenac, ketorolac, naproxen or nimesulide (all are NSAIDs) increased the risk of gastrointestinal bleeding (Anglin et al., 2014). Isolation and development of anti-inflammatory drugs from indigenous medicinal plants are in progress all over the world. Polyphenols isolated from the leaves of mistletoe (Loranthus micranthus Linn.) showed significant anti-inflammatory activity (Agbo et al., 2014). During inflammation, lysosomal enzymes are released into the blood. The acute or chronic inflammation is relatively associated with extracellular activity of these
enzymes. Strengthening of lysosomal membrane prevents the exposure of tissues from lysosomal enzymes and its constituents (Vadivu and Lakshmi, 2008). Human red blood corpuscle (HRBC) or erythrocyte membranes are comparable to lysosomal membrane components. Stabilization of HRBC membrane is a suitable model for studying lysosomal membrane stabilization potential of natural drugs (Gandhisan et al., 1991). The present study was conducted to evaluate the in vitro anti-inflammatory activity of *H. candolleanum*.

### 6.2 Materials and Methods

**6.2.1 Sample preparation**

Methanol extracts from the root, leaf and seed of *H. candolleanum* were prepared as per the method described in chapter 3 para 3.2.1.

**6.2.2 Inhibition of albumin denaturation**

Technique of Mizushima and Kobayashi (1968) was used for this study with minor modifications. The test mixture consisted of methanol extract of root, seed and leaf at different concentrations ie; 50µg/ml, 100 µg/ml, 150 µg/ml, 250 µg/ml, 500 µg/ml and 1% aqueous bovine albumin solution. Exactly pH of the reaction mixture was adjusted to 6.4 using 1NHCl. The samples were allowed to incubate at 37°C for 20min and then heated at 57°C for 20min. The turbidity developed was read
spectrophotometrically at 660 nm merely the samples were cooled. The inhibition percentage of protein denaturation was calculated as follows:

\[
\% \text{ inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample}) \times 100}{\text{Absorbance of control}}
\]

6.2.3 HRBC Membrane stabilization test

a) Setting up of Human Red Blood cells (HRBC’s) suspension

From a healthy person, roughly 10ml of fresh blood was collected and transferred to heparinized centrifuged tubes. The tubes were centrifuged at 3000rpm for 10min and were washed three times with equal volume of normal saline and reconstituted as 10% v/v suspension with normal saline (Sadique et al., 1989).

b) Heat-induced hemolysis

The reaction mixture (2 ml) consisted of 1 ml methanol extract of root, seed and leaf along with 1 ml of 10% HRBC’s suspension. As an alternative of the extract, saline was added to the control. Aspirin was chosen as the standard drug. The reaction mixture was incubated in a water bath at 56°C for 30 min. After incubation, the tubes were placed under running tap water to cool the solution. The testing solution was then centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was read at 560 nm (Shinde et al., 1999). Percent membrane stabilization activity was calculated by the formula:
Percentage Protection = 100 – Optical Density of Sample × 100
Optical Density of Control

6.2.4 Proteinase inhibitory action

The experiment was conducted by following the method of Oyedepo and Femurewa (1995) with a slight modification. The reaction mixture contained approximately 2 ml of 0.06 mg trypsin, 1 ml of 20 mM Tris HCl buffer with a pH of 7.4 and 1 ml methanol extract of root, leaf, seed at dissimilar concentrations ie; 50 µg/ml, 100 µg/ml, 150 µg/ml, 250 µg/ml, 500 µg/ml respectively. The reaction mixture was incubated at 37°C for 5 min and then 1 ml of 0.8% (w/v) casein was added as a substrate. The mixture was incubated for an additional 20 min and to terminate the reaction nearly 2 ml of 70% perchloric acid was added. Hazy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank. The percent inhibition of proteinase was calculated using above mentioned equation.

6.3 Statistical analysis

The results are expressed as the mean ± SD for six replicates. Comparison between groups was done with one way ANOVA.

6.4 Results

Methanol extract of roots, leaves and seeds of *Heracleum candolleanum* showed a concentration dependent anti-inflammatory

6.4.1 Protein denaturation

Its effect in inhibiting heat-induced protein denaturation at different concentrations was shown in Fig 6-1. IC$_{50}$ of root, seed and leaf were found to be 55.76µg/ml, 61.44µg/ml and 552.85µg/ml respectively (Table 6-1).

![Protein denaturation activity of methanol extract of root, leaf and seed](image)

Values are expressed as mean ± SD, from a trial no of six
In Vitro Anti-inflammatory Activity of *H. candolleanum*

Table 6-1 IC\textsubscript{50} values of methanol extract of root, leaf and seed on heat induced protein denaturation, hypotonic solution induced hemolysis and proteinase inhibition.

<table>
<thead>
<tr>
<th>Plant parts And Reference standard</th>
<th>IC\textsubscript{50} values</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein denaturation activity</td>
<td>HRBC membrane stabilization activity</td>
</tr>
<tr>
<td>Root</td>
<td>55.76µg/ml</td>
<td>224.31µg/ml</td>
</tr>
<tr>
<td>Leaf</td>
<td>552.85µg/ml</td>
<td>368.73µg/ml</td>
</tr>
<tr>
<td>Seed</td>
<td>61.44µg/ml</td>
<td>200µg/ml</td>
</tr>
<tr>
<td>Aspirin</td>
<td>494.95µg/ml</td>
<td>102.37µg/ml</td>
</tr>
</tbody>
</table>

### 6.4.2 HRBC Membrane stabilization activity

The extracts were found to be effectual in diverse concentrations in inhibiting the heat-induced hemolysis. Extract (50-1000µg/ml) inhibited the heat-induced hemolysis of HRBCs to varying degrees as shown in Fig 6-2. IC\textsubscript{50} values of roots, leaves and seeds were found to be 224.31µg/ml, 368.73µg/ml and 200µg/ml respectively (Table 6-1).
Fig 6-2 HRBC membrane stabilization activity of methanol extract of root, leaf and seed

Values are expressed as mean ± SD, from a trial no of six

6.4.3 Proteinase Inhibition

Methanol extracts of HC exhibited significant anti-proteinase activity at different concentrations as shown in Fig 6-3. IC$_{50}$ values of leaves, roots and seeds were found to be 439.07µg/ml, 805.47µg/ml and 399.36µg/ml respectively (Table 6-1).
Fig 6-3 Proteinase inhibitory activity of methanol extract of root, leaf and seed

Values are expressed as mean ± SD, from a trial no of six

6.5 Discussion

For the first time the in vitro properties of methanol extract of root, leaves and seeds of *H. candolleanum* with methods of heat-induced protein denaturation, HRBC membrane stabilization and proteinase inhibition activity. In inflammatory studies, denaturation of protein is an important factor to check the cause of inflammation. Denaturation of proteins is a well documented cause of inflammation.
related with rheumatoid arthritis (Opie, 1962). Synthetic drugs such as phenylbutazone, salicylic acid, flufenamic acid etc, have dose-dependent ability to prevent heat-induced protein denaturation, but as a drug they possess certain side effects (Mizushima and Kobayashi, 1968). Without any adverse effect plant derived products have the ability to prevent protein denaturation, so scientific community sensibly select plants for research and development of anti-inflammatory drug. One of the recent study said that fruit of Zizyphus Spina-Christi successfully prevented heat induced protein denaturation (Alhakmani et al., 2014). A concentration dependent prevention of protein (albumin) denaturation by Coffea arabica (Coffee) showed its potential in-vitro anti-inflammatory activity (Chandra et al., 2012). The study of effect of H. candolleanum showed a maximum inhibition of 92.42% was observed with the methanol extract of root, followed by seed 79.32% and leaf 58.09%. Aspirin showed the maximum activity of 50.51% at the concentration of 500µg/ml with an IC50 value of 494.95µg/ml. The observed results showed that root, leaf and seed extracts of HC possess significant ability to prevent protein denaturation.

Stabilization of the HRBC’s membrane was analyzed to establish the mechanism of anti-inflammatory action of Heracleum candolleanum. Release of lysosomal enzymes during an inflammatory
condition generates a number of disorders. The extracellular functions of these enzymes are related to acute or chronic inflammation. HRBC membrane is analogous to lysosomal membrane (Chou, 1997). So it is considered to be an experimental model for anti-inflammatory studies.

Aqueous solution of *Lycopus europaeus* effectively inhibits the heat induced hemolysis of erythrocyte membrane (Aziz *et al.*, 2014). The leaf and stem of *Pergularia daemia* and *Solanum xanthocarpum* successfully prevent hypotonicity induced HRBC membrane lysis (Vijaya *et al.*, 2013). Methanol extracts of *H. candolleanum* were found to be effective in diverse concentrations in inhibiting the heat-induced hemolysis. Methanol extract of leaf showed the maximum inhibition of 68.61% at 800µg/ml, root provide a maximum protection of 89.54% at 800µg/ml and seed extract exhibited a maximum inhibition of 61.09% at 800µg/ml. Aspirin showed the maximum stabilization of 76.90% at 800µg/ml with an IC\(_{50}\) value of 102.37µg/ml. The observed *in vitro* activity suggests that the methanol extract of HC root possesses higher protein denaturation prevention and membrane stabilization activity among leaf and seed extracts.

Involvement of proteinases has been caught up in arthritic reactions regularly. Neutrophils are found to be an affluent source of proteinase, serine proteinases are associated with lysosomal granules of
them. It was previously reported that leukocyte proteinase play an important part in the initiation of tissue damage during inflammatory reactions and considerable level of protection was assured by proteinase inhibitors (Das and Chatterjee, 1995). Methanol extracts of HC exhibited significant anti-proteinase activity at different concentrations. Leaf extract showed maximum inhibition of 50.04% at 800µg/ml, roots showed maximum inhibition of 49.66% at 800µg/ml and seeds showed the maximum inhibition 57.72% at 800µg/ml. Aspirin showed the maximum inhibition of 47.37% at 800µg/ml with an IC_{50} value of 844.41µg/ml. The obtained data revealed that all the three plant parts possess potential proteinase inhibitory activity.

Traditionally *H. candolleanum* has been used by tribes as a natural medication to treat inflammatory illnesses related with arthritis (John et al., 2007). However, no studies have elucidated the mechanisms of action behind these reported uses. The root and seed of *Heracleumrigens* found in Western Ghats of India exhibited anti-inflammatory activity (Jagannath et al., 2012). The results obtained from the methanol extracts of root, leaf and seed of *H. candolleanum* supports its traditional use as an anti-inflammatory herb, yet further studies are needed to fully validate this plant’s medicinal usage. In this study we identified that the methanol fractions of *H. candolleanum* is significantly
prevent protein denaturation, HRBC membrane lysis and proteinase inhibition. This is the first study to provide positive *in vitro* results of anti-inflammatory activity of this plant.