Chapter 4

Emilia sonchifolia (L.) DC: Evaluation of the anti-inflammatory effects
4.1. Introduction

About 150 years ago the relationship between inflammation and cancer was first hypothesized by Rudolph Virchow. Inflammation in the tumour microenvironment has many cancer-promoting effects and aids in the proliferation and survival of malignant cells and further promotes angiogenesis and metastasis. Epidemiologic evidence suggests that approximately 25% of all human cancers worldwide are associated with chronic inflammation, chronic infection, or both (Morrison, 2012). Inflammation is the localized protective response of the tissue to irritation, injury, or infection, characterized by pain, redness, swelling, and sometimes loss of function. Now it was realized that inflammation has a profound role in a wide variety of diseases, including cancer. While acute inflammation is a part of the defence response, chronic inflammation can lead to cancer, diabetes, cardiovascular, pulmonary and neurological diseases (Aggarwal et al., 2006). Macrophages in particular are important tumour infiltrating cells that affect tumour growth and metastasis (Morrison, 2012). Inflammatory cells and mediators are an essential component of the tumour microenvironment. Inflammatory circuits can differ considerably in different tumours in terms of cellular and cytokine networks and molecular drivers. However, macrophages are a common and fundamental component of cancer promoting inflammation. Dissection of the diversity of cancer related inflammation is instrumental to the design of therapeutic approaches that target cancer-related inflammation (Balkwill and Mantovani, 2012). The plant *E. sonchifolia* is used in the folklore medicine for treating tumour and inflammation (Shylesh et al., 2005). Previous studies conducted on this plant revealed its anti inflammatory (Muko and Ohiri, 2000; Nworo et al., 2012) and anti tumour (Shylesh and Padikkala, 2000) properties. This study is a pioneer attempt to find out the anti-inflammatory effect of an active fraction from the medicinal plant *Emilia sonchifolia*, enriched with the major compound γ-humulene (γ-hum).
4.2. Materials and Methods

**Plant material:** - An active fraction from *E. sonchifolia*, enriched with the major sesquiterpene γ-humulene (71%) (γ-hum).

**Dosage:** - Different concentrations of γ-hum was intraperitoneally (i.p) administered for five consecutive days.

**Animals:** - Male BALB/c mice (6–8 weeks old)

4.2.1. Toxicity study

γ-hum in different concentrations (5, 10, 15, 20mg/kg b.wt.) was administered intraperitoneally to mice (n=8) for 14 days. Animals were observed for mortality, behavioral changes, and change in body weight. On 15th day, all the animals were sacrificed and selected organs such as liver, spleen, thymus, kidney, and lungs were dissected and weights were recorded. Blood was collected by heart puncture, the serum was separated and used for the analysis of hepatic and renal functions. Liver function markers, such as alkaline phosphatase (ALP) (King, 1965), glutamate pyruvate transaminase (GPT) (Bergmeyer and Bernt, 1980), and kidney function markers such as creatinine (Toro, 1975) and blood urea (Murray, 1984) were determined.

4.2.2. Carrageenan induced paw edema model

Mice were divided into five groups (n=6/group).

Group I: Carrageenan alone treated (Control)
Group II: Carrageenan+γ-hum (1mg/kg b.wt.)
Group III: Carrageenan+γ-hum (2.5mg/kg b.wt.)
Group IV: Carrageenan+γ-hum (5mg/kg b.wt.)
Group V: Carrageenan+diclofenac (10mg/kg b.wt.)
One hour, after the last dose of γ-hum or diclofenac administration, paw edema was induced by carrageenan (30μg/animal) administration on sub-plantar region of the hind paw of all mice (Winter et al., 1962). The paw thickness was measured using vernier calipers before carrageenan injection and continued at 30 min intervals for 8 hours followed by 24 hour.

4.2.3. Dextran induced paw edema model

Mice were divided into five groups (n=6/group).

Group I: Dextran alone treated (Control)
Group II: Dextran+γ-hum (1mg/kg b.wt.)
Group III: Dextran+γ-hum (2.5mg/kg b.wt.)
Group IV: Dextran+γ-hum (5mg/kg b.wt.)
Group V: Dextran+diclofenac (10mg/kg b.wt.)

One hour, after the last dose of γ-hum or diclofenac administration, paw edema was induced by dextran (30μg/animal) administration on sub-plantar region of the hind paw of all mice (Maity et al., 1998). The paw thickness was measured using vernier calipers before dextran injection and continued at 1 hour intervals for 6 hours followed by 24 and 48 hour.

4.2.4. Formalin induced paw edema model

Mice were divided into five groups (n=6/group).

Group I: Formalin alone treated (Control)
Group II: Formalin+γ-hum (1mg/kg b.wt.)
Group III: Formalin+γ-hum (2.5mg/kg b.wt.)
Group IV: Formalin+γ-hum (5mg/kg b.wt.)
Group V: Formalin+diclofenac (10mg/kg b.wt.)

One hour, after the last dose of γ-hum or diclofenac administration, chronic inflammation was induced by sub-plantar injection of freshly prepared 2%
formalin in sterile water on the hind paw of all mice (Chang and Lewis, 1989). The paw thickness was measured using vernier calipers before and after formalin injection and was continued up to fifteen days.

In all the above three models the percentage inhibition of paw thickness was calculated using the formula:

\[
\% \text{ inhibition of paw thickness} = \frac{(tCn - tC0) - (tTn - tT0)}{(tCn - tC0)}
\]

Where, \( tCn = \) paw thickness at particular time point of control animal; \( tC0 = \) paw thickness before induction of control animal; \( tTn = \) paw thickness at particular time point of treated animal; and \( tT0 = \) paw thickness before induction of treated animal.

4.2.5. Determination of the effect of \( \gamma \)-hum on proinflammatory cytokines, CRP and NO

Mice were divided into three groups (n= 6/group). To elicit macrophages 0.1 ml of 5% sodium caesinate was injected intraperitoneally to all the animals. Group I was kept as normal control; Group II were treated with single dose of Lipopolysaccharide (LPS) (250\( \mu \)g/animal) intraperitoneally and group III animals received single dose of LPS two hours after the last dose of \( \gamma \)-hum (5mg/kg b.wt) treatment. After 6 hours, all the animals were sacrificed. Blood was collected by heart puncture, serum was separated and used for the estimation of inflammatory mediators like interleukin-1 beta (IL-1\( \beta \)), interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-\( \alpha \)) and C-reactive protein (CRP) using ELISA kits according to manufacturer’s instructions. The estimation of nitric oxide was done by Griess assay (Green et al., 1982). For this an equal volume of griess reagent was added to the serum collected, incubated at room temperature for 10 minutes and optical density was measured at 540nm.
4.2.6. Determination of the effect of γ-hum on TNF-α Production by macrophages

Macrophages were collected from the peritoneal cavity of all the above three (section 4.2.5.) groups of animals after injecting 5 ml of phosphate buffered saline (PBS) followed by aspiration. Macrophages \((2\times10^5)\) were plated on to 96-well titer plates and incubated for 2h at 37\(^0\)C in RPMI medium. Non-adherent macrophages were removed after incubation and fresh medium was added and again incubated for 24h at 37\(^0\)C in CO\(_2\) atmosphere. The plates were centrifuged after incubation and the medium (100μl) from each well was added to L929 cells \((5\times10^3\text{cells/well})\). Plates were incubated for 48 hours and the cells were evaluated morphologically by fixing and staining with crystal violet and the cell density was assessed by MTT assay.

4.2.7. Determination of the effect of γ-hum on iNOS, COX-2 gene Expression in LPS-activated Macrophages

Macrophages were elicited in mice by injecting 5% sodium caesinate intraperitoneally. Two hours after the last dose of γ-hum administration mice were treated with single dose of LPS (250μg/animal). After six hour all the animals were sacrificed macrophages were collected and cDNA was synthesized. cDNA was then amplified using specific mouse primers of inducible nitric oxide synthase (iNOS); Cyclooxygenase-2 (COX-2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH was selected as a stable reference gene under the experimental conditions in this study.

4.3. Results

4.3.1. Toxicological Evaluation

The toxicity study (table 4.1) revealed the no observed-adverse-effect level of γ-hum as 15mg/kg body weight. The doses of 5, 10, and 15mg/kg body weight, administered for 14 days, did not produce any mortality, change in behavior,
### Table 4.1. Toxicity profile

| Abbreviations: D/T, dead/treated mice; ALP, alkaline phosphatase; GPT, glutamate pyruvate transaminase. |
|---|---|---|---|---|---|
| Control | Concentrations of γ-hum (mg/kg body weight) | 5 | 10 | 15 | 20 |
| Mortality (D/T) | None | None | None | None | None | None |
| Behavioural change | None | None | None | None | None | None |
| Change in body weight (g) | +2.13±1.3 | +2.12±0.6 | +2.06±0.4 | +2.01±0.2 | +1.92±1.5 |

Relative organ weights (g/100g body weight)

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<tbody>
<tr>
<td>5.32±0.32</td>
<td>0.38±0.03</td>
<td>0.11±0.01</td>
<td>1.36±0.24</td>
<td>0.60±0.03</td>
</tr>
<tr>
<td>5.38±0.16</td>
<td>0.41±0.02</td>
<td>0.11±0.01</td>
<td>1.34±0.13</td>
<td>0.61±0.02</td>
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<tr>
<td>5.23±0.18</td>
<td>0.36±0.02</td>
<td>0.10±0.01</td>
<td>1.34±0.16</td>
<td>0.60±0.03</td>
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<tr>
<td>5.19±0.18</td>
<td>0.39±0.02</td>
<td>0.12±0.01</td>
<td>1.34±0.15</td>
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<td>4.97±0.15</td>
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<td>1.31±0.12</td>
<td>0.57±0.03</td>
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<table>
<thead>
<tr>
<th>1. Serum ALP (U/ml)</th>
<th>2. Serum GPT (U/ml)</th>
<th>3. Blood urea (mg/dl)</th>
<th>4. Serum creatinine (mg/dl)</th>
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<tr>
<td>13.5±0.40</td>
<td>58.35±3.7</td>
<td>43.38±1.5</td>
<td>0.91±0.01</td>
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<td>13.4±0.42</td>
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<td>14.21±0.8</td>
<td>59.97±4.6</td>
<td>44.31±0.8</td>
<td>0.93±0.02</td>
</tr>
<tr>
<td>15.8±1.08</td>
<td>64.17±7.0</td>
<td>43.55±2.7</td>
<td>0.99±0.05</td>
</tr>
<tr>
<td>14.5±2.01</td>
<td>63.91±6.1</td>
<td>43.22±1.0</td>
<td>0.98±0.13</td>
</tr>
</tbody>
</table>

Values are the mean ± standard deviation. All the treated animals were carefully examined for 14 days for any signs of toxicity (behavioural changes and mortality). “None” means that no toxic symptoms were seen during the observation period.
body weight, relative organ weight, and hepatic and renal functions when compared with untreated animals. Administration of γ-hum at 20mg/kg body weight produced slight weight loss. Based on these results and some preliminary screening on biological activity, we selected the nontoxic lowest dose of 5mg/kg b.wt. for further studies (Gilycь and Kuttan, 2016).

4.3.2. Effect of γ-hum on carrageenan induced paw edema

In mice carrageenan administration produced an increase in paw thickness. This increase was very high in the control group of animals compared to γ-hum and diclofenac treated group of animals. At the third hour this increase was reduced (p<0.001) by about 61% in γ-hum 5mg/kg b.wt. treated group. There was a reduction of about 47% and 37% in γ-hum 2.5mg/kg b.wt. and γ-hum 1mg/kg b.wt. treated group of animals respectively. Administration of the standard drug diclofenac produced 53% inhibition in the paw thickness at the same time point (figure 4.1).

4.3.3. Effect of γ-hum on dextran induced paw edema

Dextran administration also produced an increase in paw thickness. In the dextran alone treated control group the increase was high compared to γ-hum and diclofenac treated group of animals. At the third hour this increase was reduced (p<0.001) by about 60% in γ-hum 5mg/kg b.wt. treated group. There was a reduction of about 45% and 31% in γ-hum 2.5mg/kg b.wt. and γ-hum 1mg/kg b.wt. treated group of animals respectively. The standard drug diclofenac produced 52% inhibition in the paw thickness (figure 4.2).

4.3.4. Effect of γ-hum on formalin induced paw edema

Formalin induced chronic inflammation produced an increase in paw thickness in control group. When γ-hum was administered at concentrations 5mg/kg b.wt., 2.5mg/kg b.wt. and 1mg/kg b.wt., there was a reduction in the paw thickness (p<0.001) in these three groups 58%, 47% and 39% respectively. These results
Figure 4.1. Effect of γ-hum on carrageenan induced paw oedema formation

Figure 4.2. Effect of γ-hum on dextran induced paw oedema formation
were comparable to the results obtained by the standard drug diclofenac (55\%) (figure 4.3).

### 4.3.5. Effect of γ-hum on proinflammatory cytokines, CRP and NO

There was a significant increase in the level of proinflammatory cytokines TNF-α (547.95±35pg/ml), IL-1β (119.51±12pg/ml), IL-6 (356.38±39pg/ml) in the sera of LPS induced animals compared to normal animals without any treatment. This increase was found to be significantly \(p<0.001\) inhibited by the administration of LPS+γ-hum (5mg/kg b.wt.) the values are TNF-α (149.18±21pg/ml), IL-1β (59.31±7.8pg/ml), IL-6 (125.41±13pg/ml) (Figure 4.4). The serum C- reactive protein (CRP) and Nitric oxide (NO) level after treatment with γ-hum (5mg/kg b.wt.) in LPS stimulated animals was significantly reduced (\(p<0.001\)) in comparison with LPS alone treated group (table 4.2).

### 4.3.6. Effect of γ-hum on TNF-α production by macrophages

Peritoneal macrophages were elicited by injecting 200μl of 5\% sodium caesinate solution. On the day of sacrifice 5ml of PBS or HBSS was injected into the peritoneal cavity of the mice. The peritoneal fluid containing macrophages was aspirated and the cells were washed and suspended in the culture medium at desired cell concentrations. Bio assay for TNF-α level was done using L929 cell line because this cell line is TNF sensitive and the inflammatory cytokine TNF-α can produce direct cytotoxicity to these cells. When normal macrophage culture supernatant without LPS induction was added to L929 cells it will not produce any cytotoxicity. But culture supernatant of macrophages with LPS induction was added to L929 cells it produced 100\% toxicity. When the culture supernatant of macrophages collected from mice with γ-hum (5mg/kg b.wt.) + LPS induction it produced only 14\% cytotoxicity with almost normal cell morphology (figure 4.5) indicating the inhibition of TNF-α production by γ-hum treatment.
Figure 4.3. Effect of \(\gamma\)-hum on formalin induced paw oedema formation

![Graph showing effect of \(\gamma\)-hum on formalin induced paw oedema formation.](image)

***p < 0.001

Figure 4.4. Effect of \(\gamma\)-hum on the proinflammatory cytokine levels in LPS induced animals

![Graph showing effect of \(\gamma\)-hum on proinflammatory cytokine levels.](image)
Table 4.2. Effect of γ-hum on LPS induced serum CRP and NO level

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CRP (µg/ml)</th>
<th>NO (µmol)</th>
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<tbody>
<tr>
<td>Normal</td>
<td>547±21</td>
<td>20.05±1.41</td>
</tr>
<tr>
<td>LPS alone</td>
<td>4388±192</td>
<td>77.46±9.98</td>
</tr>
<tr>
<td>LPS+ γ-hum (5mg/kg b.wt.)</td>
<td>1383±182***</td>
<td>32.33±4.44***</td>
</tr>
</tbody>
</table>

Values are mean ± SD. ***p<0.001 compared with LPS alone control
4.3.7. Effect of γ-hum on iNOS, COX-2 gene Expression in LPS-activated Macrophages

According to the gene expression analysis in unstimulated macrophages neither iNOS nor COX-2 expression were detectable. In response to LPS stimulation the level of iNOS and COX-2 were increased markedly. Treatment with γ-hum (5mg/kg b.wt.) reduced both iNOS and COX-2 expression in LPS stimulated macrophages. The inhibition in the expression of iNOS and COX-2 was evident from the band intensity (figure 4.6).

4.4. Discussion

In the present study, anti-inflammatory effect of γ-hum was assessed by two acute inflammatory models of paw edema induced by carrageenan and dextran and one chronic inflammatory model induced by formalin. The mucopolysaccharide carrageenan is used for liberating inflammatory and proinflammatory mediators like prostaglandins, leukotrienes, histamine, bradykinin and TNF-α. The biphasic carrageenan induced inflammation consists of two phases, the first phase starts with the release of histamine, serotonin, and kinins (Bhukya et al., 2009) and the second phase is related to the release of prostaglandin like substances (Brooks and Day, 1991). Histamine and serotonin released by mast cells were considered to be responsible for dextran induced inflammation. Vasodilation, increased permeability and an increase of blood flow ultimately leading to an increase in paw size is the result of the marked vascular changes induced by these inflammatory mediators (Igbe et al., 2010). From the results it was evident that γ-hum is able to reduce the acute inflammation induced by these two agents and the results were comparable to the standard drug diclofenac. The chronic inflammation induced by formalin closely resembles human arthritis. The effect of formalin is also biphasic the second phase of reactions is the result of chemical insult which results in tissue damage and the tissue mediated response is with the involvement of histamine, prostaglandins and bradykinin (Wheeler and Cowan, 1991).
Figure 4.5. Effect of γ-hum on the production of TNF-α by the macrophages in LPS induced animals

A. L929 cells treated with normal macrophage culture supernatant
B. L929 cells treated with LPS alone treated macrophage culture supernatant
C. L929 cells treated with LPS+γ-hum treated macrophage culture supernatant

Bioassay of L929 cells

Figure 4.6. Effect of γ-hum on COX-2 and iNOS gene expression in LPS stimulated macrophages
LPS is a component of the bacterial cell wall, which stimulates the inflammatory response by activating the production of inflammatory mediators such as TNF-α, IL-1β, IL-6, CRP and NO. The proinflammatory cytokines TNF-α, IL-1β and IL-6 recruit additional immune cells to sites of infection or tissue injury (Bosca et al., 2005). In the present study, administration of γ-hum could inhibit significantly the LPS induced production of proinflammatory cytokines, CRP and NO. This may be due to the regulatory effect of γ-hum on the immune cell response in a state of inflammation. Macrophages are known to play a major role in the innate immune defence system and represent one of the main cellular sources of COX-2 expression upon exposure to different stimuli. The Cycloxygenase-2 (COX-2) expression is induced by a wide range of stimuli including LPS, pro-inflammatory cytokines such as IL-1β and TNF-α and growth factors such as epidermal growth factor (Steele et al., 2003). COX-2 is responsible for the increased production of prostaglandins, the key mediators or the main culprit responsible for inflammation (Firdous et al., 2015).

The release of NO by activated macrophages is an important cytotoxic/cytostatic mechanism of nonspecific immunity. In macrophages, NO is generated from l-arginine and molecular oxygen by iNOS. Induction of iNOS activity and the subsequent NO synthesis in macrophages may be caused or modulated by activators of these cells, including such bacterial products as LPS, muramyl dipeptide and especially some cytokines such as IFN-γ, TNF-α, and IL-1β. Activation of the iNOS results in the production of reactive nitrogen species like nitric oxide that damage DNA and cell membranes (Ohshima and Bartsch, 1994). γ-hum inhibits the gene expression of iNOS and COX-2 in LPS stimulated macrophages.

For the proper functioning of the immune system there should be a balance between the proinflammatory and anti inflammatory mediators. The regulatory effect of γ-hum involves the apt moulding of the body defences with the proper level of expression of the participating genes and the resulting concentration of its products. The anti-inflammatory properties of α humulene and transcaryophyllene that share close similarity with γ humulene has already been reported (Fernandes et al., 2007). γ-humulene is a major phytochemical
ingredient of some of the non drug anti-inflammatory products available today like CannaFX®21™, MedFX®Alpha™ (Norwood, MA,USA). The results obtained in the present study strongly indicate the anti-inflammatory effect of γ-hum obtained from E. sonchifolia, the plant with proven anti-inflammatory effects.