Evaluation of \textit{In vitro} and \textit{In vivo} Anti-Inflammatory Activity of Aqueous Extract of \textit{Gliricidia sepium} Flowers in Rats

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Available Online: 1\textsuperscript{st} September 2014

\textbf{ABSTRACT}

The present study was aimed for scientific evaluation of the anti-inflammatory activity of aqueous extract of \textit{Gliricidia sepium} linn. (fabaceae) flowers by \textit{in-vitro} and \textit{in vivo} models. The anti-inflammatory activity of aqueous extract obtained by decoction was evaluated by \textit{in vitro} HRBC membrane stabilization assay and in \textit{vivo} carrageenan induced paw edema model in albino wistar rats. Aqueous extract showed dose dependent anti-inflammatory activity in human red blood cell membrane stabilization method at different concentrations (100-500 \textmu g/kg) with a percentage protection of 7.15, 11.25, 22.71, 24.83 and 26.95 compared to standard diclofenac 32.09\% at 10 \textmu g/kg. Diclofenac sodium at 10 mg/kg, aqueous extract administered at a dose of 250 and 500 mg/kg p.o. at 1, 3, 6 and 8 hours significantly (p<0.05) decreased and increased the volume of paw edema & \% protection compared to carrageenan group and diclofenac, respectively. The aqueous extract has shown a significant (p<0.05, p<0.01, p<0.001) percentage inhibition of paw edema 69.81±2.93 and 78.07±3.19 on 8\textsuperscript{th} hour at 250 and 500mg/kg, respectively. These results provide a scientific basis for the use of the flowers of \textit{Gliricidia sepium} as an anti-inflammatory agent.

\textbf{Keywords:} \textit{Gliricidia sepium}, Carrageenan Anti-inflammatory, Diclofenac.

\textbf{INTRODUCTION}

Inflammation is a fundamental pathological process of an immune system towards tissue damage, tissue malfunction and infection\textsuperscript{1}. This complex reaction results from the release of local hormones like Prostaglandins, histamine, serotonin and cytokines. These biochemical molecules regulate both homeostatic and pathological reactions such as inflammation, fever, pain \textsuperscript{2}. Inflammation is associated with various diseases. To treat this many of anti-inflammatory agents like corticosteroids and NSAIDS are available but these synthetic agents produce adverse effects such as damage to GIT, gastric erosions and in extreme case severe hemorrhage and death \textsuperscript{3, 4}. Consequently there is a need for development of new anti-inflammatory agents with minimum side effects. In this context the value of Herbal medicinal plants, herbs and spices is priceless as the treatment is 100\% natural with no side effects.

\textit{Gliricidia sepium} is a medium size, semi deciduous trees, native to central America which grows about 10m [33ft] height and belonging to the family fabaceae \textsuperscript{5}. It is a fast-growing, nitrogen-fixing tree used throughout the tropics for the many environmental services. Gliricidia is widely used to provide crop shade for cacao, coffee, and other shade loving crops \textsuperscript{6, 7}. The tree is also an important source of green manure, fodder, and fuel wood. Several phytochemicals like flavonoids \textsuperscript{8}, triterpenoid saponins, stigmastanol glucoside, rhamnogalactoside of kaempferol, coumarin, coumaric acid and melilotic acid and 12a-hydroxy retenoids were reported in various parts of the plant and bark, respectively \textsuperscript{9}. Furthermore, different parts of the plants are reported to have viz. antimicrobial \textsuperscript{10}, nematicidal \textsuperscript{11}, larvicidal \textsuperscript{12} antioxidant \textsuperscript{4} and it’s a folk remedy for rheumatism, headache, fever, wounds, fractures and urticaria etc \textsuperscript{13}. \textit{Gliricidia sepium} is a folk remedy for some of inflammation related diseases fractures, gangrene, head-ache, itch, prickly heat, rheumatism, urticaria, and wounds \textsuperscript{14}, till today there is no scientific report on this plant. Hence, the present study was aimed to investigate the anti-inflammatory activity of \textit{Gliricidia sepium} aqueous extract of flowers.

\textbf{MATERIALS AND METHODS}

Plant collection and identification: Flowers of \textit{Gliricidia sepium} was collected from Acharya Nagarjuna University campus, Guntur district of Andhra Pradesh. The plant was identified, confirmed and authenticated by comparing with voucher specimen available at survey of Medicinal plants and collection unit, Department of Botany.

Preparation of Aqueous Plant Extract: The flowers were cut into small pieces, powdered and the crude drug is boiled directly with distilled water at 60°-70°c for 3hours. Then it is cooled and filtered. The extract was concentrated under reduced pressure and stored in vacuum desiccators,

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which was used for in-vitro and in-vivo anti-inflammatory investigations.

Phytochemical Screening: Preliminary qualitative phytochemical screening of Gliricidia sepium flower extract shows a positive test for carbohydrates [Benedict’s test and Barfoed’s test], proteins [Millons test], flavonoids [Shinoda test], saponins [froth formation test] and Tannins [color reaction with ferrous chloride].

Table 1: In vitro anti-inflammatory activity of aqueous extract of Gliricidia sepium

<table>
<thead>
<tr>
<th>Groups</th>
<th>% Hemolysis Mean ± SEM</th>
<th>% protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diclofenac 10µg/ml</td>
<td>67.9±0.085</td>
<td>32.09</td>
</tr>
<tr>
<td>GSAE 100 µg/ml</td>
<td>92.8±0.0236***</td>
<td>7.15</td>
</tr>
<tr>
<td>GSAE 200µg/ml</td>
<td>88.7±0.0731***</td>
<td>11.25</td>
</tr>
<tr>
<td>GSAE 300µg/ml</td>
<td>77.3±0.0853***</td>
<td>22.71</td>
</tr>
<tr>
<td>GSAE 400 µg/ml</td>
<td>75.2±0.0873***</td>
<td>24.83</td>
</tr>
<tr>
<td>GSAE 500µg/ml</td>
<td>73.0±0.101***</td>
<td>26.95</td>
</tr>
</tbody>
</table>

Results are MEAN±SEM, ***P<0.001 [Dunnett’s post hoc test] significant when compared with standard Diclofenac.

Fig. 1: In vitro anti-inflammatory activity of aqueous extract of Gliricidia sepium and diclofenac.

Table 2: Effect of Gliricidia sepium aqueous flower extract on carrageenan induced rat paw oedema volume

<table>
<thead>
<tr>
<th>S.No.</th>
<th>1h</th>
<th>3h</th>
<th>4h</th>
<th>8h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control [Saline]</td>
<td>0.48±0.021</td>
<td>0.56±0.017</td>
<td>0.60±0.035</td>
<td>0.71±0.029</td>
</tr>
<tr>
<td>Diclofenac 10 mg/kg</td>
<td>0.31±0.017</td>
<td>0.27±0.019</td>
<td>0.22±0.014</td>
<td>0.11±0.01</td>
</tr>
<tr>
<td>Aq.extract 250 mg/kg</td>
<td>0.45±0.023***</td>
<td>0.36±0.015*</td>
<td>0.29±0.026ns</td>
<td>0.21±0.014**</td>
</tr>
<tr>
<td>Aq.extract 500 mg/kg</td>
<td>0.37±0.037*</td>
<td>0.31±0.028**</td>
<td>0.25±0.032ns</td>
<td>0.16±0.023ns</td>
</tr>
</tbody>
</table>

Results are MEAN±SEM, [n=6] *P < 0.05, **P<0.01 and ***P<0.001 [Dunnett’s post hoc test] significant when compared with standard Diclofenac.

Fig. 2: Effect of Gliricidia sepium aqueous flower extract on carrageenan induced rat paw volume

Results are MEAN±SEM, [n=6] *P < 0.05, **P<0.01 and ***P<0.001 [Dunnett’s post hoc test] significant when compared with standard Diclofenac.

Acute Toxicity [LD50] Study: Acute oral toxicity of was studied as per OECD guidelines 425 using albino Swiss mice. The extract was found to be safe up to 2000 mg/kg body weight.

Pharmacological Evaluation-
In vitro Anti-inflammatory activity-
HRBC membrane stabilization assay 15, 16: Fresh human blood was collected, centrifuged and prepared 10% v/v
Table 3: % protection of aqueous extract of Gliricidia sepium flowers against carrageenan induced paw oedema

<table>
<thead>
<tr>
<th>S.No.</th>
<th>1h</th>
<th>3h</th>
<th>4h</th>
<th>8h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DICLOFENAC 10 mg/kg</td>
<td>GSAE 250 mg/kg</td>
<td>GSAE 250 mg/kg</td>
<td></td>
</tr>
<tr>
<td>1%</td>
<td>33.66±4.37</td>
<td>50.21±4.06</td>
<td>61.73±2.75</td>
<td>84.04±1.54</td>
</tr>
<tr>
<td>2%</td>
<td>5.66±1.21</td>
<td>35.12±4.37</td>
<td>51.06±5.90</td>
<td>69.81±2.93</td>
</tr>
<tr>
<td>3%</td>
<td>14.81±3.5</td>
<td>43.15±5.51</td>
<td>57.62±6.07</td>
<td>79.07±3.19</td>
</tr>
</tbody>
</table>

Results are MEAN±SEM, [n=6], *P < 0.05, **P<0.01, ***P<0.001 [Dunnett’s post hoc test] significant when compared with standard Diclofenac.

Before carrageenan injection, the paw volumes for each rat were measured separately by water plethysmometer (INCO INDIA PVT LTD). Edema caused by carrageenan was measured at 0, 1, 3, 4 and 8 hours. The anti-inflammatory potency of the extract was determined by comparing its effect with standard diclofenac sodium. Then percent inhibition of edema was calculated for each group with respect to the control group as follows. Percentage of inhibition of paw edema = [Vc – Vt / Vc] × 100

Where Vc and Vt represent average paw volume of control and drug treated animals respectively.

Statistical Analysis: Results were analyzed by using one-way ANOVA followed by post hoc Dunnett’s test using Graph pad Prism-5 v software. The results were expressed as Mean ± SEM. *P<0.05, **P<0.01 and ***P<0.001 was considered as significant.

RESULTS

The aqueous extract of the Gliricidia sepium was studied for in vitro anti-inflammatory activity by HRBC membrane stabilization method. The plant extract showed dose dependant anti-inflammatory activity and % protection of HRBC in hypotonic solution. Results were compared with standard diclofenac [Table &Figure 1]. Aqueous extract of flowers 250 & 500 mg/kg and standard drug were tested for anti inflammatory activity at different hours in carrageenan induced paw edema model using water plethysmometer. Diclofenac sodium at 10 mg/kg, Aqueous extract administered at a dose of 250 and 500 mg/kg p.o. at 1, 3, 6 and 8 hours significantly[*P<0.05,
**P<0.01, ***P<0.001** increased the volume of paw edema & % protection compared to carrageenan group and diclofenac respectively. Aqueous extract at 250 p.o. at 1, 3, 4 and 8 hours prevented the carrageenan induced paw edema with a percentage inhibition of 5.66±1.21, 35.12±4.36, 51.06±5.90, 69.8±2.93 at 1, 3, 4, 8 hour, respectively, while 14.81±3.37, 43.15±5.51, 57.6±6.07, 78.07±3.19 at dose of 500 mg/kg p.o. at 1, 3, 4, 8 hour, respectively. Diclofenac sodium decreased the carrageenan induced paw edema with a percentage inhibition of 33.66±4.37, 50.21±4.06, 61.73±2.75, 84.04±1.54 at 1, 3, 4, 8 hour, respectively [Table 1,2 & Figure 1,2].

**DISCUSSION**

Inflammation is the common problem in all the age groups irrespective of gender and the use of NSAIDS to treat inflammation produces vascular and gastrointestinal complications. Anti-inflammatory drugs available in the market produces symptomatic relief with adverse effects. Now a day’s medicinal plants and their formulations are used for various disorders in ethno medical practices as well in the traditional system of medicine in India. Furthermore, these natural remedies can be used to cure problems rather than just mask symptoms. Herbal medicines importance is increased in treatment of chronic and acute diseases with free of adverse effects. Solanum trilobatum, Plumeria acuminata, Thehesia chinensis, Mexican medicinal plant extracts and Crude Saponin Extracts also showed the anti-inflammatory effect in carrageenan induced paw edema.

In the present study the plant extract exhibited dose dependant membrane stabilization effect by inhibiting hypotonicity induced lyses of RBC membrane in *in vitro* assay. The RBC membrane is analogous to the lysosomal membrane and its stabilization indicates lysosomal membrane stabilization. It plays a vital role in reduction of pathological mechanisms involved in inflammation by augmenting the release of activated neutrophil, bactericidal enzymes and proteases, which produce further tissue inflammation and damage.

Carrageenan induced inflammation is the most sensitive and reliable model for evaluating acute phase and orally active anti-inflammatory agents. The time course of increase in paw edema is represented as biphasic event. Development of carrageenan induced inflammation during 1 hour is due to the release of histamine and serotonin along with trauma of injection, where as the second phase and third phase is attributed to the release of kinin like substances and prostaglandins, respectively

[Cyclooxygenase - 2], protease and lysosome. Mediators like histamine, serotonin and COX-2 released prostaglandins increases the vasodilatation, hyperemia, pain and edema. Table 4 & 5 represent the *in vivo* anti-inflammatory activity of extract. Significant [*P<0.05, **P<0.01, ***P<0.001*] anti-inflammatory activity was observed at 4th and 8th hours compared to standard reference diclofenac. Aqueous extract showed dose dependant anti-inflammatory activity at third phase of carrageenan induced paw edema, with maximum anti-inflammatory activity at 500 mg/kg.

In conclusion, extract showed anti-inflammatory activity in later phases in dose dependent manner and the inhibitory effect of aqueous extract may be due to the inhibition of cyclooxygenase induced prostaglandin synthesis and neutrophil mobilization. This anti-inflammatory effect of the extract observed might be due to the presence of flavonoids and saponins in the plant. The present investigation has also opened avenues for further research especially with reference to the isolation and development of potent phyto medicines from this extract for treatment of inflammation.

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