Analgesic and Anti-inflammatory Activity of Ethanolic Extract of Zizyphus nummularia

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ABSTRACT
Present study was undertaken to examine anti-inflammatory and analgesic activity of ethanolic extract of Zizyphus nummularia (EAZN). EAZN produced anti-inflammatory activity against acute paw oedema induced by carrageenan and histamine at the dose levels of 200 and 300 mg kg\(^{-1}\), at the same dose levels EAZN inhibited peritoneal leukocyte migration. Significant decrease in number of writhes and increase in tail flick latency was observed in acetic acid induced writhing and tail flick test, respectively. In conclusion, it is suggested that inhibition of pain and inflammation mediators is the possible mechanism of action.

Key words: Paw oedema, analgesic, anti-inflammatory

INTRODUCTION
Zizyphus nummularia (family: Rhamnaceae) is a thorny small bush or shrub, grows in abundance in the grazing lands of the arid and semi-arid areas of India. In Rajasthan it forms 14% of the total composition of the grassland flora. It is frequently cultivated food and fodder (Anonymous, 1989).

Tasty sweet and sour fruits of Zizyphus nummularia are consumed by all sections of society for its nutritional and medicinal value; young leaves are cooked as vegetable and used as medicine.

Zizyphus nummularia is used by the local community as analgesic, anti-inflammatory, anti-colds and anti-oughs medicine (Anonymous, 1989; Shah et al., 1990; Goyal et al., 2011; Chanda et al., 2011). Zizyphus nummularia found to contains many bioactive phytochemical constituents such as pectin, saponins, triterpenoic acids, fatty acids and cyclopeptide alkaloids.

Cyclopeptide alkaloids have been reported for sedative, antimicrobial, hypoglycemic, antiplasmodial, anti-infectious, antidiabetic, diuretic, anticonvulsant, analgesic and anti-inflammatory activities.

The extractive value of ethanolic extract of Zizyphus nummularia is found to be high and alkaloids present in Zizyphus can be extracted with alcohol (Morel et al., 2009; Ma et al., 2008). Present study was undertaken to examine analgesic and anti-inflammatory effects of ethanolic extract of leaves of Zizyphus nummularia to validate ethnomedicinal claims.

MATERIALS AND METHODS
Plant material: Zizyphus nummularia is commonly grown in Rajasthan. The leaves were collected in March 2008 from Jodhpur. Herbarium of plant was identified by
Taxonomist of Botanical Survey of India (BSI), Jodhpur, Rajasthan. A voucher specimen was deposited in the BSI.

**Preparation ethanolic extract:** One kilogram of dried and powdered leaves was extracted three times in a reflux condenser for 24 h with hexane. The residues were extracted with ethanol for 48 h and the ethanolic extract was evaporated to dryness by rotary evaporation. The yield of ethanolic extracts of *Zizyphus nummularia* was about 12% (Ma et al., 2008).

**Chemicals and drugs:** Carrageenan (S.D. Fine Chemicals Limited, Bombay), histamine (Sigma, USA), indomethacin (Recon, Bangalore), aspirin (USV Bombay), morphine (Bio E,) were used in the study.

**Animal material:** Male Wistar rats weighing 180-200 g and male Swiss mice weighing 20-24 g were used. The animals had free access to a standard commercial diet and water *ad libitum* and were kept in rooms maintained at 25°C with a 12 h light/dark cycle. The experiments were performed during the light portion (0800-1600 h). The experimental protocol and procedures used in this study were approved by IAEC of Lachoo Memorial College of Science and Technology, Pharmacy Wing, Jodhpur, Rajasthan.

**Anti-inflammatory activity**

**Carrageenan-induced rat paw oedema:** The rats were divided into five groups (*n* = 8). The different groups were treated with ethanolic extract of leaves of *Zizyphus nummularia* (EAZN) (100, 200 and 300 mg kg⁻¹ b.wt., p.o.), indomethacin (10 mg kg⁻¹ b.wt., p.o.) and vehicle control (5 mL kg⁻¹ b.wt., p.o.). The animals were treated with the EAZN 1 h before the administration of carrageenan. Acute inflammation was produced by the subplantar administration of 0.1 mL of 1% carrageenan in normal saline in the right hind paw of the rats. The paw volume was measured at 0 and 3 h after carrageenan injection using plethysmometer (Winter and Porter, 1957). The anti-inflammatory effect of EAZN was calculated by the following equation:

\[
\text{Anti-inflammatory activity (\%)} = (1 - D/C) \times 100
\]

where, D represents the percentage difference of paw volume of EAZN treated groups and C represents the percentage difference of volume in the control group (Suleyman et al., 1999; Aderogba et al., 2005; Awe et al., 2006).

**Histamine-induced inflammation:** The anti-inflammatory activity of the EAZN was measured against histamine which act as mediator of inflammation (Winter et al., 1982). Paw oedema was induced in rats by subplantar injection of 0.1 mL of freshly prepared histamine (1 mg kg⁻¹ b.wt.) solutions.

**Mouse carrageenan peritonitis:** The mice were divided into five groups (*n* = 6). The EAZN (100, 200 and 300 mg kg⁻¹ b.wt., p.o.), indomethacin (10 mg kg⁻¹ b.wt., p.o.) and vehicle control (5 mL kg⁻¹ b.wt., p.o.) were administered. One hour later peritonitis was induced by intraperitoneal
injection of carrageenan (0.25 mL, 0.75% in saline). After 4 h the animals were sacrificed by high dose of anesthesia and peritoneal fluid collected for total and differential leukocyte count (Griswold et al., 1987). Percentage inhibition was calculated by the following equation:

\[
\text{Percentage inhibition} = \left(1 - \frac{D}{C}\right) \times 100
\]

where, \(D\) represents the leukocyte counts of treated groups and \(C\) represents the leukocyte counts of the control group.

**Analgesic activity**

**Acetic acid-induced writhing response in mice:** Five groups of mice were selected for the study \((n = 6)\). Group one received saline \((5 \text{ mL kg}^{-1} \text{ b.wt., p.o.})\), group two received aspirin \((100 \text{ mg kg}^{-1} \text{ b.wt., p.o.})\) and remaining three groups of mice received EAZN at the dose of 100, 200 and 300 mg kg\(^{-1}\) b.wt., p.o. (Gupta et al., 2005). Thirty minutes after drug treatment, 0.1 mL of 0.6% solution of acetic acid was injected intraperitoneally and the number of writhes during the following 30 min period were counted (Koster et al., 1959; Shilpi et al., 2005; Usman et al., 2008; Arora et al., 2011; Shehab et al., 2011). Percentage inhibition was calculated by the following equation:

\[
\text{Percentage inhibition} = \left(1 - \frac{D}{C}\right) \times 100
\]

where, \(D\) represents the number of writhes of treated groups and \(C\) represents the number of writhes of the control group.

**Tail flick reaction time in mice:** Five groups of mice were selected for the study \((n = 6)\), mice which had 3.5-4.5 seconds baseline latency of tail flick were included in study. Group one received saline \((5 \text{ mL kg}^{-1} \text{ b.wt., p.o.})\), group two received morphine \((5 \text{ mg kg}^{-1} \text{ b.wt., s.c. injection})\), remaining three groups of mice received 100, 200 and 300 mg kg\(^{-1}\) b.wt., p.o. of EAZN. Morphine and EAZN were given 30 and 60 min before the test.

Mice were screened by placing their proximal third of the tail to radiant heat source maintained at 50±2°C, latency for tail flick was recorded. A cutoff time of 20 sec was used to avoid damage to the tail (D’Amour and Smith, 1941; Al-Howiriny, 2004; Chowdhury et al., 2005; Rakhshandeh et al., 2008; Gill et al., 2011). The Percentage anti-nociceptive activity was calculated following equation:

\[
\text{Percentage anti-nociceptive activity} = \left(1 - \frac{D}{C}\right) \times 100
\]

where, \(D\) represents the latency of tail flick of treated groups and \(C\) represents the latency of tail flick of the control group.

**Acute toxicity:** For the acute toxicity assay, two groups of three male mice \((20-24 \text{ g})\) were made. The animals were kept without access to food and water. The assay was followed as OECD Guideline 423 (OECD, 2001). The control group received normal saline 1 mL kg\(^{-1}\) by gavage while the exposed group received 1000 mg kg\(^{-1}\) of EAZN. The safety of 1000 mg kg\(^{-1}\) dose was
subsequently confirmed in another three animals as recommended in the OECD guideline. Immediately after dosing, the animals were observed continuously for symptoms of toxicity for 4 h in terms of autonomic and neurobehavioral alterations. They were then kept under observation up to 14 days in terms of weight loss and chow consumption. On day 15, the animals were euthanized and their vital organs were individually observed for overt pathology.

**Statistical analysis:** Values were expressed as Mean±SEM, statistical significance was determined by one way ANOVA followed multiple comparisons versus control group by Dunnett's Method; values with p<0.05 were considered as statistically significant.

**RESULTS**

**Rat paw oedema:** The EAZN produced anti-inflammatory activity against acute paw oedema induced by carrageenan and histamine (Table 1). The anti-inflammatory effect found to be statistically significant (p<0.05) only at the dose levels of 200 and 300 mg kg⁻¹.

**Mouse carrageenan peritonitis:** EAZN at the dose levels of 200 and 300 mg kg⁻¹ inhibited peritoneal leukocyte migration (Table 2), inhibition is found to be statistically significant (p<0.05) and dose dependent.

**Acetic acid-induced writhing in mice:** Significant (p<0.05) decrease in number of writhes was observed (Fig. 1), at dose levels 200 and 300 mg kg⁻¹. In test group maximum inhibitions of writhes was observed in group treated with 300 mg kg⁻¹ EAZN (Fig. 2).

Table 1: Effect of Ethanolic extract of *Zizyphus nummularia* and indomethacin on carrageenan and histamine-induced pedal oedema in rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose mg kg⁻¹ (p.o.)</th>
<th>Carrageenan</th>
<th>Histamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Paw oedema</td>
<td>Inhibition (%)</td>
</tr>
<tr>
<td>Control</td>
<td>5 (mL kg⁻¹)</td>
<td>0.86±0.02</td>
<td>62.79</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>10</td>
<td>0.32±0.01*</td>
<td>87.90</td>
</tr>
<tr>
<td>EAZN</td>
<td>100</td>
<td>0.86±0.02</td>
<td>61.16</td>
</tr>
<tr>
<td>EAZN</td>
<td>300</td>
<td>0.62±0.05*</td>
<td>67.90</td>
</tr>
<tr>
<td>EAZN</td>
<td>300</td>
<td>0.58±0.02*</td>
<td>68.37</td>
</tr>
</tbody>
</table>

Values are expressed as the Mean±SEM of six observations. *p<0.05 Statistical comparisons are made between: Control vs. Indomethacin, I, II and group III

Table 2: Effect of Ethanolic extract of *Zizyphus nummularia* and indomethacin on carrageenan induce peritonitis in mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose mg kg⁻¹ (p.o.)</th>
<th>Leukocytes (10⁶ mL⁻¹)</th>
<th>Leukocytes inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5 (mL kg⁻¹)</td>
<td>4.32±0.21</td>
<td>*</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>100</td>
<td>2.10±0.12*</td>
<td>61.32</td>
</tr>
<tr>
<td>EAZN</td>
<td>200</td>
<td>4.20±0.11</td>
<td>62.77</td>
</tr>
<tr>
<td>EAZN</td>
<td>300</td>
<td>3.42±0.14*</td>
<td>29.83</td>
</tr>
<tr>
<td>EAZN</td>
<td>100</td>
<td>2.50±0.08*</td>
<td>42.12</td>
</tr>
</tbody>
</table>

Values are expressed as the Mean±SEM of six observations. *p<0.05 Statistical comparisons are made between: Control versus Indomethacin, I, II and group III
Fig. 1: Effect of Ethanolic extract of *Zizyphus nummularia* and aspirin on acetic acid induce writhing in mice. Values are expressed as the Mean±SEM of six observations, *p*<0.001 statistical comparisons are made between: control vs. aspirin, I, II and group III.

Fig. 2: Percentage inhibitions of writhes by ethanolic extract of *Zizyphus nummularia* and aspirin.

Fig. 3: Effect of ethanolic extract of *Zizyphus nummularia* and morphine on tail flick latency of mice. Values are expressed as the Mean±SEM of six observations, *p*<0.001 Statistical comparisons are made between: control vs. morphine, I, II and group III.

**Tail flick latency in mice:** Significant increase in tail flick latency (*p*<0.05) was observed at dose levels of 200 and 300 mg kg⁻¹ (Fig. 3). EAZN 300 mg kg⁻¹ produces 90% anti-nociceptive activity (Fig. 4).
Fig. 4: Percentage anti-nociceptive activity of ethanolic extract of *Zizyphus nummularia* and morphine on tail flick latency of mice

**DISCUSSION**

This study evaluated the putative analgesic and anti-inflammatory activities of the ethanolic extract of leaves of *Zizyphus nummularia* to validate ethnomedicinal claims made regarding the *Zizyphus nummularia*. Carrageenan-induced paw edema as an in vivo model of inflammation has been frequently used to assess the antiedematous effect of natural products. The EAZN showed dose-dependent anti-oedematogenic effects on paw oedema induced by carrageenan. The cellular and molecular mechanism of the carrageenan-induced inflammation well characterized. It is known that carrageenan oedema is mediated through release of inflammation mediators such as serotonin and histamine (Linardi *et al.*, 2000). The EAZN causes pronounced reduction in the paw oedema induced by histamine and reduces vascular permeability in carrageenan induced peritonitis, results suggests that anti-inflammatory activity of the EAZN is possibly backed by its anti-histaminic activity. Since histamine is important mediators of inflammation, causes vasodilation and increases the vascular permeability (Cuman *et al.*, 2001).

In acetic acid-induced abdominal writhing which is the visceral pain model, the result indicated that all the doses produced significant analgesic effect. This could be attributed, partly, to its anti-inflammatory effect as, in the visceral pain model, the processor releases arachidonic acid via cyclooxygenase and prostaglandin biosynthesis which plays a role in the nociceptive mechanism (Franzotti *et al.*, 2000). Thus, the results obtained for the writhing test are similar to those obtained for the oedematogenic test using carrageenan. Therefore, an anti-inflammatory substance may also be involved in the peripheral analgesic activity because inhibition of the acute inflammation by this extract led to their inhibitory effect on pain development.

Tail-flick test is used for screening of centrally acting analgesics, tail flick to noxious thermal stimuli are mediated via supra-spinal centers (Dewey *et al.*, 1970; Kazunaga *et al.*, 1980). EAZN was found to be effective in tail flick test in mice which indicate the analgesic activity by central mechanism.

Cyclopeptide alkaloid faction of Zizyphi Spinosi Semenis found to enhance pentobarbital-induced sleeping behaviors and this action is mediated through GABA receptors Cl⁻ channel activation. In addition, Cyclopeptide alkaloid in combination with GABAₐ receptors agonist muscimol, synergistically prolonged pentobarbital-induced sleeping time (Ma *et al.*, 2008). Muscimol also causes latency in tail flick, so, another possibility of positive results of tail flick is GABA agnostic action (Aanonsen and Wilcox, 1989).
CONCLUSION

In conclusion, since the plant extract reduced significantly the formation of oedema induced by carrageenan and histamine, as well as reduced the number of writhes in acetic acid-induced writhing models and increases tail flick latency, the EAZN exhibited anti-inflammatory and analgesic activities. The study has thus provided some justification for the folkloric use of the plant in several communities for conditions such as pain and inflammations.

REFERENCES


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