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## Research Article

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### ANTINOCICEPTIVE AND ANTI-INFLAMMATORY ACTIVITY OF THE EXTRACT OF *LYCOPUS EUROPAEUS* ON LABORATORY ANIMALS

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#### ABSTRACT

**Aims:** To evaluate the analgesic and anti-inflammatory effects of methanolic extract of *Lycopus europaeus* on experimental animal models.

**Methodology:** The analgesic activity was evaluated by hot plate, acetic acid induced writhing and formalin induced writhing method in Swiss Albino mice divided into 4 different groups (control, standard diclofenac sodium and extract at two different doses of 250 and 500 mg/kg BW). The extract was also investigated for the anti-inflammatory effect on Long Evans rats using carrageenan induced rat paw edema method. For anti-inflammatory study, 24 rats were divided into 4 different groups each receiving either distilled water, standard drug or the extract at the doses of 250 and 500 mg/kg BW.

**Results:** Phytochemical analysis of the extract revealed the presence of tannins, flavonoids and terpenoids. The extract elicited a highly significant ( $p < 0.001$ ) analgesic activity in a dose dependent manner on hot plate method, acetic acid induced writhing test and also on both the early and late phases of formalin test at the doses employed. In the hot plate method, the extract increased the reaction time of heat sensation to 60.81% and 66.52% at the doses of 250 and 500 mg/kg BW respectively while that of the standard drug was 57.40% at the 3rd hour of study. In acetic acid induced writhing test, the percent inhibition of writhing response by the extract was 62.87% and 70.66% at 250 and 500 mg/kg doses respectively ( $p < 0.001$ ) which were even better than the standard drug diclofenac sodium (50.30%). The extract also significantly inhibited the licking response at the dose of 500 mg/kg in both the early phase (55.11%,  $p < 0.01$ ) and the late phase (66.43%,  $p < 0.01$ ) of formalin test while the standard drug inhibited by 52.27% and 72.03%, respectively. The oral administration of the extract significantly ( $p < 0.001$ ) inhibited inflammatory response induced by carrageenan in a dose dependent fashion. The most prominent inhibition of 61.68% (250 mg/kg) and 73.65% (500 mg/kg) were observed at the 4th hour of study.

**Conclusion:** The central and peripheral analgesic as well as anti-inflammatory effect of the methanolic extract of *Lycopus europaeus* may be due to the presence of various chemical constituents specially flavonoids, tannins, alkaloids or terpenoids. These experimental findings would further establish the scientific basis of the traditional uses of the plant in the management and/or control of pain as well as inflammatory conditions. **Keywords:** Basic needs, biodiversity conservation, women's basic understanding.

**Keywords:** *Lycopus europaeus* , phytochemical screening; analgesic; antiinflammatory; acetic acid; formalin; carrageenan.

#### INTRODUCTION

With onset of scientific research in herbals, it has become clearer that the medicinal herbs have a

potential in today's synthetic era, as numbers of medicines are becoming resistant. According to one

estimate only 20% of the plant flora has been studied and 60% of synthetic medicines owe their origin to plants (Ayurvedaherbs, 2005). Moreover, in some Asian and African countries, 80% of the population depends on traditional medicine for primary health care (WHO, 2008). Due to its importance and availability, herbs are staging a comeback and herbal 'renaissance' is happening all over the globe. The herbal products today symbolize safety in contrast to the synthetics that are regarded as unsafe to human and environment. More than 30% of the entire plant species, at one time or another, was used for medicinal purposes (Joy et al., 1998).

Inflammation is a pathophysiological response of mammalian tissues to a variety of hostile agents including infectious organisms, toxic chemical substances, physical injury or other noxious stimuli leading to local accumulation of plasma fluid and blood cells (Sobota et al., 2000; Medzhitov, 2010). Although it is a defense mechanism, the complex events and mediators involved the inflammatory reaction can induce, maintain or aggravate many diseases (Sosa et al., 2002) and so it has become the focus of global scientific research. Anti-inflammatory and analgesic therapy is dominated by opioids and non steroidal anti-inflammatory drugs (NSAIDs) but both classes of drugs produce serious side effects (Park et al., 2004). The search for pharmacological agents to overcome these shortcomings has become a major goal in pain research. Medicinal plants are considerably useful and economically essential. They contain active constituents that are used in the treatment of many human diseases. The effectiveness of phytochemicals in the treatment of various diseases may lie in their analgesic and anti-inflammatory effects (Akinmoladun et al., 2007).

*Lycopus europaeus* is also known as Bugleweed, wolfstrappkraut, bitter bugle, water horehound. *L. virginicus*: Paul's betony and water bugle. *Lycopus europaeus* is a herbaceous perennial mint that grows in wet habitats. The leaves are toothed, and the small white flowers surround the square stem at the leaf axils in dense clusters. The plant has little odor; the..European species has a bitter taste, while the

American species is not bitter. The whole herb is used medicinally.

Scientists have played their important for the evaluation of traditional uses of *Lycopus europaeus* on different animals. For example extracts of *L. europaeus* administered to healthy rats reduced the weight of the thyroid, decreased thyroid hormone activity, and increased absorption and storage of iodine. The extract retarded goiter formation in propylthiouracil-treated rats. All animals treated with the extract demonstrated reduced metabolism (Hiller et al, 1954). Cardiac signs of hyperthyroidism were reduced in an experiment in rats treated with *L. europaeus* extract (Vonhoff et al, 2006). The plant was also reported for its antitussive activity (Aziz et al, 2013).

The present study was undertaken to explore any possible antinociceptive and anti-inflammatory potential of the methanolic extract of *L. europaeus* in mice and rats so as to justify the traditional uses of this plant in folklore medicine.

## MATERIALS AND METHODS

### Collection of plant and Preparation of crude extract:

The plant was collected from the tropical regions of Pakistan and was identified by a taxonomist. The plant material was made free from soil and other adulterants and vegetative debris. The dried plant material was grinded to coarse powder with the help of a special herbal grinder. The powdered plant material (1 kg) was subjected to maceration in 70% aqueous-methanol in amber coloured bottle at room temperature for 7 days with occasional vigorous shaking at room temperature and keeping the extract in the dark room. The filtrate was obtained by passing the mixture through a muslin cloth and then through a Whatman qualitative grade 1 filter paper. The filtrate was evaporated on a rotary evaporator attached to a vacuum pump at 37°C under reduced pressure to thick paste like consistency. And then the extract obtained was stored at -4°C in air tight jars.

### Animals used

Young Swiss-Albino mice aged about 4-5 weeks with average weight of 25-35 gm and adult Long Evans Rats of either sex having average weight of 100-130

gm were used for the experiment. They were housed in standard cages under standard environmental conditions of room temperature at  $24 \pm 1^\circ\text{C}$  and 55-65% relative humidity with 12 hour dark light cycle and provided with standard food for rodents and water ad libitum.

#### Method for phytochemical analysis

The freshly prepared extract of *L. europaeus* was qualitatively tested for the presence of chemical constituents. Qualitative phytochemical tests for the identification of alkaloids, flavonoids, steroids, gum and carbohydrates, saponins, tannins and terpenoids were carried out for the extract by the method described previously (Harborne, 1998; Siddiqui et al., 2009).

#### Method for the evaluation of analgesic effect

##### Hot plate test

The hot-plate test was employed for measurement of analgesic activity as previously described by Lanhers et al. and modified by Ojewole (Lanhers et al., 1992; Ojewole, 2004). The temperature was regulated at  $55^\circ \pm 1^\circ\text{C}$ .

Mice of either sex were divided into four groups consisting of six animals in each group. The mice of each group were placed in the beaker (on the hot plate) in order to obtain its response to electrical heat induced pain stimulus. Licking of the paws or jumping out of the beaker was taken as an indicator of the animal's response to heat-induced pain stimulus. The time for each mouse to lick its paws or jump out of the beaker was taken as reaction time (in second). Before treatment, the reaction time was taken once. The mean of this determination constituted initial reaction time before treatment of each group of mice. Each of the test mice was thereafter treated with either distilled water (DW), Diclofenac sodium (10 mg/kg BW) or methanol extract of *L. europaeus* at the doses of 250 and 500 mg/kg BW orally.

Thirty minutes after treatment, the reaction times of each group mice were again evaluated five times individually in one hour interval on this occasion. Percent analgesic score was calculated as,

$$\text{PAS} = \frac{\text{Ta}-\text{Tb}}{\text{Ta}} \times 100$$

Where,  $\text{Tb}$  = Reaction time (in second) before drug administration;  $\text{Ta}$  = Reaction time (in second) after drug administration.

#### Acetic acid - induced writhing method

The analgesic activity of the samples was evaluated using acetic acid induced writhing method in mice following the method of Koster et al. with slight modification (Koster et al., 1959; Owoyale et al., 2001; Altun et al., 2009). In this method, acetic acid is administered intraperitoneally to the experimental animals to create pain sensation. The animals were divided into four groups with six mice in each group. Group I animals received distilled water, Group II received Diclofenac sodium at 10 mg/kg while animals of Group III and Group IV were treated with 250 and 500 mg/kg of the methanol extract of *L. europaeus* after an overnight fast. Test samples and vehicle were administered orally 30 minutes prior to intraperitoneal administration of 0.7% v/v acetic acid solution. Animals were kept individually under glass jar for observation. Each mouse was observed individually for counting the number of writhing they made in 10 minutes commencing just 5 minutes after the intraperitoneal administration of acetic acid solution. Full writhing was not always accomplished by the animal, because sometimes the animals started to give writhing but they did not complete it. This incomplete writhing was considered as half-writhing. Accordingly, two half-writhing were taken as one full writhing. The number of writhes in each treated group was compared to that of a control group while Diclofenac sodium was used as a reference standard (positive control). The percentage inhibition of writhing was calculated as follows:

$$\% \text{ Inhibition} = \frac{(1-\text{VT}/\text{VC}) \times 100}{1}$$

VT = number of writhing motions in drug-treated mice

VC = number of writhing motions in the control group of mice

#### Formalin test

In this test, the mice were divided into four groups each containing 6 mice and were administered with either distilled water (1ml/kg, i.p.), methanolic extract of *L. europaeus* (250 and 500 mg/kg, i.p) or Diclofenac sodium (10 mg/kg, s.c). Thirty minutes after

this treatment; 50 µl of a freshly prepared 0.6% solution of formalin was injected subcutaneously under the plantar surface of the left hind paw of each mice. The mice were placed individually in an observation chamber and monitored for one hour. The time (in sec) spent in licking and biting responses of the injected paw was taken as an indicator of pain response. Antinociceptive effect was determined in two phases. The early phase (phase 1) was recorded during the first 5 minutes, while the late phase (phase 2) was recorded during the last 20-30 minutes after formalin injection.

#### Method for the evaluation of anti-inflammatory effect

The anti-inflammatory activity of the methanol extract was investigated on carrageenan induced inflammation in rat paw following an established method (Winter et al, 1962). Rats were randomly divided into four groups, each consisting of six animals, of which group I was kept as control giving only distilled water. Group II was standard which received Diclofenac sodium (10 mg/kg) as the reference standard for comparison while Group III and Group IV were given the test material at a dose of 250 and 500 mg/kg body weight respectively. Half an hour after the oral administration of the test materials, 1% carrageenan was injected to the right hind paw of each animal. The volume of paw edema was measured at 0, ½, 1, 2, 3, 4 and 6 hours using Plethysmometer after administration of carrageenan. The left hind paw served as a reference non-inflamed paw for comparison. The average percent increase in paw volume with time was calculated and compared against the control group. Percent inhibition was calculated using the formula-

$$\% \text{ Inhibition of paw edema} = \frac{V_c - V_t}{V_c} \times 100$$

Where  $V_c$  and  $V_t$  represent average paw volume of control and treated animal respectively.

#### STATISTICAL ANALYSIS

The data are expressed as the mean  $\pm$  SEM analyzed by one-way analysis of variance (ANOVA) and Dunnett's t-test was used as the test of significance. P value

<0.05 was considered as the minimum level of significance.

## RESULTS

### Phytochemical analysis

Phytochemical screening of methanol extract of *L. europaeus* revealed the presence of various bioactive components of which tannin, flavonoid and terpinoid were the most prominent.

### Analgesic activity

#### Hot plate method

Results of hot plate test are presented in Table 1 for the crude extract of *L. Europaeus*. The extract of the plant significantly increased the reaction time of heat sensation in mice at the doses of 250 and 500 mg/kg BW and the percentage protection is almost equivalent to the respective doses. In the 3rd hour of study, the extract increased the reaction time of heat sensation to 60.81% and 66.52% at the doses of 250 and 500 mg/kg BW respectively while that of the standard drug was 57.40% and the results were found to be highly statistically significant ( $P < 0.001$ ). The extract exhibited a dose dependent increase in latency time when compared with control.

#### Acetic acid-induced writhing test

Inhibition of licking response in mice due to the administration of the test drugs during acetic acid-induced writhing test is shown in Table 2. The oral administration of both doses of *L. europaeus* extract significantly ( $p < 0.001$ ) attenuated the acetic acid-induced abdominal writhes in mice in a dose dependent fashion. The percent inhibition of writhing response by the extract was 62.87% and 70.66% at 250 and 500 mg/kg doses respectively while the standard diclofenac sodium (10 mg/kg) showed 50.30% inhibition in comparison with the control.

#### Formalin-induced writhing test

The effect of the extract of *L. europaeus* on formalin induced pain in mice is shown in Table 3. The extract significantly inhibited the licking response in both the early phase (52.84% at 250 mg/kg,  $p < 0.05$  and 55.11% at 500 mg/kg,  $p < 0.01$ ) and the late phase (62.24% at 250 mg/kg,  $p < 0.05$  and 66.43% at 500 mg/kg,  $p < 0.01$ ) of the formalin test which were

comparable to those of the standard drug. Both these inhibition were dose dependent.

#### Anti-inflammatory result

The anti-inflammatory effects of the extract and standard drug are presented in Table 4. In control animals, the sub plantar injection of carrageenan produced a local edema that increased progressively to reach a maximal intensity 3 hours after injection. The oral administration of both doses of the methanolic extract of *L. europaeus* significantly ( $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$ ) inhibited inflammatory response induced by carrageenan in rats in a dose related manner. The most prominent inhibition of 61.68% at 250 mg/kg and 73.65% at 500 mg/kg were observed at the 4th hour of study after which the inhibitory activity was found to decline. The result was found to be highly statistically significant at 4th hour after administration of the sample drugs ( $p < 0.001$ ).

#### DISCUSSION

Phytochemical analysis of the methanolic extract of *L. europaeus* revealed the presence of tannins, flavonoids, saponins, gums and terpenoids. Strong occurrence of tannins in extract has been shown to possess potent anti-inflammatory properties (Fawole et al., 2010). There are also reports on the role of tannins in antinociceptive activity (Ramprasath et al., 2006). Flavonoids, also known as nature's tender drugs, possess abundant biological and pharmacological activities. Analgesic and anti-inflammatory effects have been observed in flavonoids (Rao et al., 1998; Kim et al., 2004). It is also reported that flavonoids such as rutin, quercetin and luteolin produced significant antinociceptive and anti-inflammatory activities (Pathak et al., 1991). Certain flavonoids possess strong inhibitory activity against a wide range of enzymes such as protein kinase C, protein tyrosine kinases,

**Table 1: Effect of the methanol extract of *Lycopus europaeus* on latency to hot plate test**

Group	Reaction time at different time intervals (in sec)					
	0 Hr	½ Hr	1 Hr	2Hr	3 Hr	4 Hr
Control	6.780±0.611	6.080±0.948	6.320±0.773	6.140±0.644	5.960±0.614	5.680±0.608
Standard	5.300±0.889	7.180±1.223 (26.18)	9.820±1.333 (46.03)	11.100±1.07 5*** (52.25)	12.440±0.85 8*** (57.40)	8.140±1.433 (34.89)
Le.cr (250mg/kg)	5.400±0.705	9.020±1.426 (40.13)	10.340±1.959 (47.78)	11.523±0.92 6** (53.14)	13.780±1.31 2*** (60.81)	11.560±1.786* * (53.29)
Le.cr (500mg/kg)	4.989±1.337	8.400±1.346 (40.61)	9.760±0.950 (48.88)	12.420±1.46* ** (59.83)	14.900±1.02 1*** (66.52)	9.480±1.389 (47.37)

Data are represented as the mean  $\pm$  SEM, (n=6); Values in parentheses indicate percent increase in reaction time; \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 were considered significantly different in comparison with control.

**Table 2: Effect of the *Lycopus europaeus* extract on acetic acid-induced writhing in mice**

Groups	Dose	No. of writhing	% Inhibition
<b>Control</b>	10 ml/kg	41.75± 3.772	-
<b>Diclofenac sodium</b>	10 mg/kg	20.75±1.109***	50.30
<b>Le.cr 250 mg/kg</b>	250 mg/kg	15.50±1.223***	62.87
<b>Le.cr 500 mg/kg</b>	500 mg/kg	12.25± 2.250***	70.66

Data are represented as the mean ± SEM, (n=6); \*\*\*P < 0.001 was considered significantly different in comparison with control.

**Table 3: Analgesic activity of the methanol extract of *Lycopus europaeus* using formalin-induced writhing method**

Treatment	0-5 min (early phase)	% Inhibition	20-30 min (late phase)	% Inhibition
Control	44.00±9.70	-	35.75±9.51	-
Diclofenac sodium	21.00±2.89*	52.27	10.00±3.51**	72.03
Le.cr(250 mg/kg)	20.75±0.75*	52.84	13.50±1.26*	62.24
Le.cr(250 mg/kg)	19.75±3.88**	55.11	12.00±4.08**	66.43

Data are represented as the mean ± SEM, (n=6); \*P < 0.05, \*\*P < 0.01 were considered significantly different in comparison with control.

**Table 4: Anti-inflammatory activity of *Lycopus europaeus* extract using carrageenan induced rat paw edema method**

Treatment	Paw volume in ml					
	½ Hr	1 Hr	2 Hr	3 Hr	4 Hr	6 Hr
<b>Control</b>	0.63±0.048	0.75 ± 0.047	1.41 ±0.064	1.79 ±0.082	1.67 ±0.081	1.59 ±0.071
<b>Diclofenac sodium 10 mg/kg</b>	0.37 ± 0.033 (41.27)	0.37 ± 0.041* ( 50.67)	0.55 ±0.064* (60.99)	0.37 ±0.093** (79.33)	0.33 ±0.072*** (80.24)	0.66 ±0.044* (58.49)
<b>Le.cr 250 mg/kg</b>	0.47±0.049 (25.40)	0.49±0.063 (34.67)	0.72 ±0.018 (48.94)	0.73±0.133* (59.22)	0.64 ±0.063*** (61.68)	0.91 ±0.045 (42.77)
<b>Le.cr 500 mg/kg</b>	0.41 ± 0.026 (34.92)	0.44 ± 0.035 (41.33)	0.63 ±0.017* (55.32)	0.53 ±0.036** (70.39)	0.44 ±0.033*** (73.65)	0.79 ± (50.31)

Data are represented as the mean ± SEM, (n=6); Values in parentheses indicate percent inhibition of paw edema;

\* p<0.05, \*\* p<0.01, \*\*\* p<0.001 were considered significantly different in comparison with control.

phospholipase A<sub>2</sub>, phosphodiesterases and others (Middleton, 1998). Other flavonoids potently restrain prostaglandins, a group of powerful pro-inflammatory signaling molecules. Inhibition of these key enzymes provides the mechanism by which flavonoids inhibit inflammatory processes (Manthey et al., 2001). Alkaloids have been shown to possess anti-inflammatory activity by inhibiting the action of arachidonic acid metabolism via the cyclooxygenase and 5-lipoxygenase pathways (Barik et al., 1992; Chao et al., 2009).

Studies have also demonstrated that terpenoids produced significant analgesic and anti-inflammatory activities (Calixto et al., 2000). The hot plate test measures the response to a brief, noxious stimulus and thus bears a closer resemblance to clinical pain. The method is considered to be selective for the drugs acting centrally. This test measures the complex response to a non-inflammatory, acute nociceptive input and is one of the models normally used for studying central nociceptive activity (Sabina et al., 2009). It is an established fact that any agent that causes a prolongation of the hot plate latency using this test must be acting centrally (Ibironke and Ajiboye, 2007). The methanolic extract of *L. europaeus* presented a longer latency time than the control group in the hot plate test in a dose related manner. Therefore, the extract might have a central activity.

Acetic acid induced writhing in mice, attributed to visceral pain, finds much attention to evaluate peripherally active analgesics (Hasan et al., 2010). Pain sensation in acetic acid induced writhing method is elicited by triggering localized inflammatory response resulting in release of free arachidonic acid from tissue phospholipid via cyclooxygenase and prostaglandin biosynthesis. The agent reducing the number of writhing will render analgesic effect preferably by inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition (Duarte et al., 1988). Results of the present study show that the plant extract produced significant analgesic effect which might be due to the presence of analgesic principles acting with the prostaglandin pathways. The formalin test is a widely used model of continuing pain involving peripheral inflammation and

central sensitization. The method shows a biphasic response comprising of an early (neurogenic) and a late (inflammatory) phase response and originates mainly from neurogenic inflammation followed by participation of kinins and leukocytes with their proinflammatory factors including PGs (Wheeler-Aceto and Cowan, 1991). It is also reported that acute inflammation induced by formalin results from cell damage which provides the production of endogenous mediators (Chen et al., 1995). In the present study, the crude extract produced antinociception against both neurogenic and inflammatory phase of formalin. The fact that the extract at the doses tested produced analgesia in all nociceptive models is indicative that it possesses both central and peripheral antinociceptive effects and the mechanism of action of the extract could, in part, be related to lipooxygenase and/or cyclooxygenase of the arachidonic acid cascade and/or opioid receptors.

Carrageenan-induced inflammation is most commonly used as an experimental model for evaluating the anti-inflammatory potency of compounds or natural products. The probable mechanism of action of carrageenan-induced inflammation is biphasic, the first phase is attributed to the release of histamine, serotonin and kinins in the first hour; while the second phase is attributed to the release of prostaglandins and lysosome enzymes in 2 to 4 hours (Brooks and Day, 1991). The second phase is sensitive to most clinically effective anti-inflammatory drugs (Vinegar et al., 1969). The results of present study indicate that the extract significantly inhibited the carrageenan-induced acute inflammation in the 4th hour of study and the finding was comparable to that of the standard diclofenac sodium. So, the anti-inflammatory effect of *L. europaeus* extract may be due to its suppressive action on prostaglandin, protease or lysosome synthesis or activity.

#### CONCLUSION

Scientific exploration and standardization of potential crude drugs is an urgent need to revolutionize our drug sector and it is possible as Pakistan is blessed with many natural forests with huge number of medicinal plants. Based on previous studies and our current

investigation, we conclude here that the central and peripheral analgesic and anti-inflammatory effect of the methanolic extract of *L. europaeus* may be due to the presence of flavonoids, tannins or terpenoids. These experimental findings lend pharmacological support to the suggested folkloric uses of the plant in the management and/or control of pain as well as inflammatory conditions. However, further studies are in progress in our laboratory to isolate the active constituents responsible for the observed effect, and to elucidate the possible mechanisms of action responsible for the analgesic and anti-inflammatory activities of the plant extract.

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