Evaluation of *In-vivo* anti-inflammatory and analgesic properties of *Limnophila repens* (Benth.)

Venkateswarlu Gunji, Ganapahy Seru

[1]Department Of Pharmacognosy And Phytochemistry, A. M. Reddy Memorial College Of Pharmacy, India

[2]Department Of Pharmacognosy And Phytochemistry, Gitam Institute of Pharmacy, Gitam University, India

Abstract

The current research was accomplished to assess analgesic and anti-inflammatory activities in *Limnophila repens (L.repen)* methanolic extracts at 200 and 400 mg/kg. The entire herb was extracted with the methanol (MELR). Acute oral toxicity of the extract was according to OECD-423 recommendations. Analgesic activity was explored by utilizing hot plate, tail immersion and acetic acid-evoked writhing models. Anti-inflammatory activity (acute) of the extract was assessed by carrageenan evoked paw edema model. In acute toxicity study, no mortality was observed when each extract was orally administered with 2000 mg/kg. At the doses (200 and 400 mg/kg) MELR demonstrated considerable and dose reliant analgesic and anti-inflammatory effects. The conclusions from the present investigation confirmed the folkloric usage of *Limnophila repens* as analgesic and anti-inflammatory. This significant anti-inflammatory and analgesic impact might be because of the inhibition of any inflammatory mediators by steroids, i.e., ß-sitosterol, stigmasterol, flavonoids, i.e. quercetin and glycosides within the extract. But, further phytochemical, as well as biological tests, are needed to determine the additional active chemical constituents responsible for the antinociceptive and anti-inflammatory activities.

Özet

Mevcut araştırma, 200 ve 400 mg / kg'da *Limnophila repens* (*L. repens*) metanolik ekstrelerinde analjezik ve anti-enflamatuar etkinlikleri değerlendirmek için gerçekleştirilmiştir. Tüm bitki, metanol (MELR) ile ekstre edildi. Ekstrenin akut oral toksisitesi OECD-423 önerilerine göre yapıldı. Analjezik aktivite sıcak plaka, kuyruk daldırma ve asetik asitle uyarılmış kıvranma modelleri kullanılarak araştırılmıştır. Ekstrenin anti-enflamatuar aktivitesi (akut), karagenin ile uyarılmış pençe ödemi modeli ile değerlendirildi. Akut toksisite çalışmasında, her bir ekstre 2000 mg / kg dozda oral yolla

uygulandığında ölüm görülmemiştir. 200 ve 400 mg / kg dozlarda MELR, önemli ve doz bağımlı analjezik ve anti-enflamatuar etkiler gösterdi. Bu araştırmadan elde edilen sonuçlar *Limnophila repens*'in analjezik ve antiinflamatuar olarak folklorik kullanımını doğruladı. Bu önemli anti-enflamatuar ve analjezik etki, ekstre içindeki steroidler, yani ß-sitosterol, stigmasterol, flavonoidler ile kuersetin ve glikozitler gibi herhangi bir enflamatuar aracı maddenin inhibisyonundan kaynaklanabilir. Ancak, antinosiseptif ve antienflamatuar aktiviteden sorumlu ek aktif kimyasal bileşenlerin belirlenmesi için daha fazla fitokimyasal testlere ve ayrıca biyolojik testlere ihtiyaç duyulmaktadır.

Keywords: *Limnophila repens*, Carrageenan, Acetic acid, Phytochemical Screening, β-sitosterol.

Introduction

For a long time, natural remedies and especially medicinal plants were the main or even the only recourse of our ancestors for their medication. However, despite the development of the pharmaceutical industry, medicinal plants and remedies that could be drawn were never abandoned entirely, and people continue to resort to traditional medicine (1). Natural products are believed to be an essential source of new chemical substances with the potential therapeutic application. Several plant species were traditionally used as analgesics. In general, the herbal plant usage in the treatment of disease and pain relief is one of the essential strategies in medicine (2).

The genus *Limnophila* is frequently used in traditional medicine against cardiovascular diseases, stomach disorders, elephantiasis, diarrhoea, dyspepsia, fever, dysentery, indigestion, dysmenorrhoea and abdominal pain (3-5).

Phytochemical analysis of genus *Limnophila* revealed the presence of the number of phytoconstituents such as flavonoids, tannins, alkaloids, terpenoids, steroids, and glycosides (6). This diversity in compounds could justify the traditional use of *L. repens.*

The genus *Limnophila* is relatively abundant and widely used in folk medicine as an antioxidant (7, 8), antimicrobial (9) anticancer (10), antimycobacterial (11), as on to date no biological studies have been conducted on this plant. Consequently, this exploration was performed to evaluate the anti-inflammatory potential and antinociceptive properties of *L. repens*.

Materials and Methods

Plant Collection and Authentication

The herb, *Limnophila repens*, was collected at Tirupati during September 2017. The examined herb was recognised and verified by the botanist Dr .K. Madhava Chetty. A specimen of the herb, with the voucher number 1568, was deposited at Gitam Institute of Pharmacy, Visakhapatnam.

Preparation of Extract

The freshly gathered herb was shade dried and pulverised. The powder (1 kg) was extracted by way of petroleum ether meant for removing fatty and waxy materials. It was air-dried and then macerated by way of methanol, strained and then concentrated at 45ºC in Buchi rotavapor. Finally, the weight of methanolic extract acquired was 82g (8.2% w/w yield). The methanolic extract was revoked in distilled water within a separating funnel and then partitioned sequentially with the petroleum ether, chloroform, ethyl acetate and n-butanol to obtain fractions in these solvents. In due course, the remaining residual aqueous portion at the end was collected. The solvents were removed on a rotary evaporator at low pressure to obtain dried fractions. These extracts had been subjected to preliminary phytochemical screening, and these extracts had been kept in the refrigerator at 4ºC for additional make use of in future (12).

Phytochemical Screening

The various extract of *L. repens* was subjected to qualitative chemical analysis by using standard procedures (13-16).

Isolation of Phytoconstituents

Petroleum ether extract (PEE) was subjected to silica-gel (100-200 mesh) column (length 100 cm and diameter 3 cm) chromatography (elution rate of 2 ml min−1 flow with a total elution of 200 ml) and eluted with Petroleum ether and ethyl acetate in different proportions. The resulting fractions (Fr) were collected and spotted over pre-coated silica gel F254 plates (20 × 20 cm, Merck, Germany).

Petroleum ether extract (PEE) was subjected to silica-gel (100-200 mesh) column (length 100 cm and diameter 3 cm) chromatography (elution rate of 2 ml min^{-1} flow with a total elution of 200 ml) and eluted with Petroleum ether and ethyl acetate in different proportions and the solutions was spotted on TLC plates. Then that TLC plates had been run

by the particular solvent system and had been observed separately under UV light and also with the anisaldehyde-sulphuric acid reagent. Through many preliminary tests, it had been identified that the substances of the petroleum ether portion had been separated by the solvent system of n-hexane and ethyl acetate in the proportion of 9: 1. The petroleum ether portion, 18 g, was afflicted by column chromatography on a silica gel (60-120 mesh) with gradient elution utilising n-hexane: ethyl acetate and lastly with 100% methanol. The fractions which were found homogeneous on TLC plate are pooled out, and these were crystallized and named as LR-1(*Limnophila repens*-1) and LR-2 (*Limnophila rens*-2), respectively (17). The Ethyl acetate portion was chromatographed employing flash column on a Silica gel eluted with chloroform-methanol step-gradient (starting with 100: 0 to 4: 1), eluted part had been put together on their TLC pattern to yield eight fractions. The chloroform-methanol fraction (10:1) was chromatographed on a Sephadex LH-20 column eluted with chloroform-methanol (1:1) to yield LR-3 (*Limnophila repens*-3) (18).

Animals

Thirty adult albino rats weighing between 180 and 220 g and albino mice (25-30 g) of either sex procured from the Mahaveer Enterprises, Hyderabad, were used for this study. These were consequently housed in the Animal House of the Faculty of Basic Medical Sciences under standard environmental circumstances of the 12-h light/12-h dark cycle and allowed free access to clean drinking water and feed. The use of experimental animal complies with the OECD-423 guidelines (19). The Department Experimental Review Panel also evaluated the standard protocol before the beginning of the study. After arrival, animals were acclimatized to the animal facility for ten days before the commencement of the experiments.

Acute toxicity study

To evaluate the degree of toxicity of *L. repens* methanolic extract, the acute toxicity study was worked based upon OECD (Organization for Economic Cooperation and Development) 423 recommendations to the dose of 2000 mg/kg. The experimental animals s had been noticed for 1 h constantly after which hourly for 4 h and lastly every 24 h up to 14 days for any physical symptoms of the level of toxicity, including writhing, gasping, palpitations and lowered respiratory rate or mortality. No animals died. Therefore the LD50 is greater than 2000 mg/kg. Pre-screening investigation with 200 and 400mg per body weight was done (20).

Pharmacological activities

Analgesic Activities

The peripheral analgesic activity of *L. repens* methanolic extract was evaluated using the acetic acid-induced writhing test, while central analgesic activity was studied against thermal stimuli using the hot plate and tail immersion tests.

Hot plate method

The central analgesic activity of methanolic extract of *L. repens* was evaluated by Eddy's hot plate method (21). The rat (n=5) were divided into four groups. Group I designated as control group received vehicle orally. Group II specified as reference group was given the standard drug tramadol 10 mg/kg p.o. While animals in groups III and IV received 200 and 400 mg/kg bodyweight of methanolic extract of *L. repens* respectively. Each animal was placed separately on the hot plate maintained at temperature 55 ± 2 ºC. The reaction time (paw licking or jumping) was recorded for each mice at time periods of 30 min, 60 min and 90 min following administration of medicine or vehicle with the cut-off time 15 sec to avoid injury. The rise in reaction time in plant extracts and standard treated groups were compared to those of the control group (22).

$$\%\ Analgesic\ activity = \frac{(Ta - Tb)\ X\ 100}{Tb}$$

T_a = Average of reaction time after the administration of the extract

T_b = Average of Initial reaction time

Tail Immersion test

The lower two-thirds of the tail had been engrossed in a beaker comprising water kept at 50 ± 0.5 °C. The time in seconds before the tail was withdrawn from the water was defined as the reaction time. The animals had been pretreated 60 min before the tail immersion with the normal saline solution for the group I (negative control), 200 mg/kg acetylsalicylate acid for group II (positive control) and 200, 400 mg/kg of *L. repens* for groups III and IV respectively. The lower part of each tail was engrossed in hot water maintained at about 55 °C, leading to an excruciating response. The time, in seconds, for tail withdrawal from the water was recorded as the response period, having a cut-off time for immersion set at 15 s. The latent period of the tail immersion response was determined at 0, 30, 60, 90, 120 and 180 min after the oral administration of standard and MELR. The percentage of inhibition of heat-induced pain was computed with the following formula (23).

$$\%\ In\square ibition = \frac{Ln - Lo}{15\ s - Lo}\ \text{X100}$$

Where Lo = Latent time before drug administration in seconds

Ln = Latent time after drug administration in seconds (n= 30 to 180 min)

Acetic Acid-Induced Writhing Method

For this study acetic acid induced writhing method was acquired. Mice (n = 5) were divided into two groups for each extract of *L. repens*, in addition to that two more groups (n = 5) of mice were used for control and standard study. Control group received the vehicle; the standard group was treated with acetylsalicylic acid 100 mg/kg, p.o. Groups assigned for extracts were treated with 100 and 200 mg/kg, p.o. After 30 min, writhings were induced in mice by intra-peritoneal injection of 0.6 % v/v acetic acid. The number of writhing was counted throughout 30 min. The percentage inhibition of writhing count of the treated group was calculated from the mean writhing count of the control group by applying the formula (24, 25).

$$\% \text{ Inhibition} = \frac{\text{Mean no. of writhes (Control)} - \text{Mean no. of writhes (Treated)}}{\text{Mean no. of writhes control}} \times 100$$

Anti-inflammatory activity

Carrageenan-induced paw edema

In the rat paw edema approach, acute inflammation was produced in male Wistar rats by injecting 0.1 ml of freshly prepared carrageenan solution (1% w/v) in normal saline into the sub-plantar region of the rat's paw. Animals were divided into 4 groups (n = 5) for each extract, along with two more groups (n = 5) for control and standard. Control group was given vehicle; standard group received Diclofenac 10 mg/kg p.o and groups assigned for extracts received 200 and 400 mg/kg p.o. before 60 min of carrageenan injection. Paw volume was measured with digital plethysmometer at the time intervals of 1, 2 and 3 h after carrageenan injection. The percentage inhibition was calculated by the formula (26).

$$\% \text{ Inhibition} = T_o - Tt / T_o \times 100$$

Where, T_o = thickness of paw of rats given test extract at the corresponding time

Tt = paw thickness of rats of the control group at the same time.

Statistical Analysis

Results were expressed as mean ± SEM (standard error of the mean). The statistical analysis of the results was carried out with the ANOVA application (GraphPad Prism 5). Values of $p < 0.05$ were considered statistically significant.

Results

Phytochemical Screening

The phytochemical screening for various extracts viz., petroleum ether, chloroform, ethyl acetate, methanol, n-butanol, and water was carried out, and results were displayed in Table 1.

Characterization of Isolated Phytoconstituents

LR-1 (*Limnophila repens* -1)

White powder, $C_{29}H_{48}O$, MW 412.69. UV λ_{max} ($CHCl_3$) nm: 257; IR (KBr) IR (KBr) ν_{max} cm^{-1}: 3418 (-OH strech), 2934 (C-H strech in CH_2 and CH_3), 2866 (=C-H strech), 2339, 1602 (C=C assymetric strech), 1566, 1461 (C-H deformation in gem dimethyl), 1409, 1383, 1251, 1191, 1154, 1109, 1089, 1053 (Cycloalkane), 1020, 791; **(**MS-ES-APCI, m/z): 409.2, 395.3, 335, 161, 144, 121.1, 105.1, 97.1, 85.1, 69, 67.2, 65, 50.2; **^{1}H NMR Data (400 MHz, $CDCl_3$)** δ : 7.25 (1H, s, OH-2), 5.34-5.35 (1H, d), 5.12-5.18 (1H, m), 4.99-5.05 (1H, m), 3.48-3.56 (1H, m), 2.18-2.31 (2H, m), 1.93-2.09 (3H, m), 1.82-1.87 (2H, m), 1.66-1.75 (1H, m), 1.37-1.54 (13H, m), 1.05-1.31 (m, 7H), 0.99-1.01 (m, 8H), 0.90-0.98 (m, 2H), 0.78-0.85 (m, 9H), 0.66-0.70 (3H, t); **^{13}C NMR Data (400 MHz, $CDCl_3$)** δ: 140.85 (C-5), 138.31 (C-22), 129.40 (C-23), 121.72 (C-6), 77.34 (C-3), 71.86 (C-14), 56.95 (C-17), 56.09 (C-24), 51.29 (C-9), 50.29 (C-13), 42.41 (C-4), 42.30 (C-20), 40.46 (C-12), 39.77 (C-10), 37.35 (C-1), 36.59 (C-7), 32 (C-8), 31.96 (C-2), 31.91 (C-27), 31.77 (C-16), 28.91 (C-15), 25.41 (C-28), 24.41 (C-29), 21.24 (C-19), 21.14 (C-11), 21.06 (C-21), 19.42 (C-18), 19.03 (C-25),

12.23 (C-26). The above spectral data (MASS, NMR) is analyzed for molecular formula $C_{29}H_{48}O$ and is similar with that of stigmasterol.

LR-02 (*Limnophila repens* -2)

White powder, $C_{29}H_{50}O$, MW 414.70; IR (KBr) ν_{max} cm^{-1}: 3424 (-OH strech), 2959 (-CH strech in $-CH_2$ and $-CH_3$), 2936, 2867 (=C-H), 1602, 1565, 1465 (C-H deformation in gem dimethyl), 1382, 1332, 1242, 1191, 1154, 1051 (Cycloalkane), 779 cm^{-1}; **^{1}H NMR Data (400 MHz, $CDCl_3$)** δ: 7.30 (1H, s), 5.34-5.35 (1H, d), 4.98-5.19 (1H, m), 3.47-3.55 (1H, m), 2.19-2.31 (2H, m), 1.03-1.30 (9H, m), 1.00 (4H, s), 0.90-0.98 (4H, m), 0.76-0.86 (9H, m), 0.68-0.69 (3H, d), 1.94-2.07 (2H, m), 1.79-1.88 (4H, m), **^{1}H NMR Data (400 MHz, $CDCl_3$)** δ: 7.30 (1H, s), 5.34-5.35 (1H, d), 4.98-5.19 (1H, m), 3.47-3.55 (1H, m), 2.19-2.31 (2H, m), 1.03-1.30 (9H, m), 1.00 (4H, s), 0.90-0.98 (4H, m), 0.76-0.86 (9H, m), 0.68-0.69 (3H, d), 1.94-2.07 (2H, m), 1.79-1.88 (4H, m); MS-ES-APCI, m/z: 411.2, 397.3, 383.3, 311.2, 161.1, 81.2. The above spectral data (MASS, NMR) is analyzed for molecular formula $C_{29}H_{50}O$ and is similar to that of β-sitosterol.

LR-03 (*Limnophila repens*-03)

Yellow powder, $C_{15}H_{10}O_7$, MW 302.23; IR (KBr) ν_{max} cm^{-1}: 3413 (-OH strech), 2340, 1607, 1565, 1523, 1462, 1408, 1383, 1320, 1263 (C-O strech), 1199, 1168, 1131, 1014, 959, 782 (=C-H bending); **^{1}H NMR Data (400 MHz, $CDCl_3$)** δ: 12.49 (1H, s), 10.77 (1H, s). 9.57 (1H, s), 9.29-9.33 (2H, d), 7.68-7.69 (1H, d), 7.53-7.69 (1H, m), 6.88-6.90 (1H, d), 6.41 (1H, d), 6.19 (1H, d); **^{13}C NMR Data (400 MHz, $CDCl_3$)** δ: 175.81 (C-4), 163.85 (C-7), 160.70 (C-5), 156.17 (C-9), 147.67 (C-2), 146.81 (C-3^1), 145.03 (C-3), 135.68 (C-6^1), 121.96 (C-5^1), 119.96 (C-4^1), 115.59 (C-2^1), 115.08 (C-10), 103.01 (C-6), 98.16 (C-8), 93.33 (C-1^1); MS-ES-APCI, m/z: 301, 301.9, 300. The above spectral data (MASS, NMR) is analyzed for molecular formula $C_{15}H_{10}O_7$ and is similar to that of quercetin.

Acute toxicity Studies

Oral administration of the highest dose of 2000 mg/kg of the methanolic extract of *Limnophila repens* did not produce any acute toxic symptoms. Moreover, no mice and rats mortality occurred during the observation period of 24 h. The extracts were found to be safe at the highest dose of 2000 mg/kg; fifth and ten-fold dilutions of the highest dose were selected for the analgesic and anti-inflammatory activities, i.e., 200 and 400 mg/kg.

Analgesic activity

Hot Plate method

The results (mean ± SEM) of hot plate showed that the MELR (200 and 400 mg/kg) exhibited an increase in basal reaction time from 9.62 ± 0.22 and 9.49 ± 0.22 at 0 min to 12.95 ± 0.62 and 14.95 ± 0.85 at 90 min respectively (Figure 1, Table 2).

Tail Immersion Test

The tail immersion method revealed a well-marked increase in basal reaction time of 6.39 ± 0.15 in MELR (200 mg/kg) and 7.75 ± 0.31 in MELR (400 mg/kg) at 180 min (Figure 2). The inhibition was the highest at 180 min at 400 mg/kg dose which was lower than standard.

Writhing test

The peripheral analgesic activities of *Limnophila repens* on acetic acid-induced abdominal writhing in mice were shown in Table 4. Control group showed maximum writhing (26 ± 2.12), while MELR at a dose of 200 and 400 mg/kg demonstrated a significant antinociceptive effect against acetic acid-induced writhing, inhibiting pain by 33.07% and 49.23% as compared to the control respectively (Table 4, Figure 6). Diclofenac at 10 mg/kg had 68.46% (p<0.001) inhibition of writhing response.

Carrageenan-Induced paw edema

Table 5 shows the effect of MELR and standard drug as compared to carrageenan control at different hours in the carrageenan-induced paw edema model. MELR administered at a dose of 200 mg/kg p.o. prevented carrageenan-induced paw edema with a percentage inhibition of 19.44%, 26.61%, 33.41%, and 41.73% at 1, 2, 3,and 4 hour, respectively, while 31.94, 40.32, 50.25 and 61.44% at a dose of 400 mg/kg p.o. at 1, 2, 3.and 4 hour, respectively. Diclofenac sodium at a dose of 10 mg/kg p.o. prevented carrageenan-induced paw edema with a percentage inhibition of 52.08, 60.48, 70.46 and 73.91% at 1, 2, 3.and 4 hour, respectively.

DISCUSSION

The preliminary phytochemical analysis of the *L. repens* herb shows the presence of several compounds such as flavonoids, volatile oils, alkaloids, tannins, phytosterols, carbohydrates, glycosides, proteins and fixed oils.

It is well known that inflammation and pain are the most common diseases in human and animals, and the current treatment is to use steroidal and nonsteroidal anti-inflammatory drugs which have several side effects (27, 28). *Limnophila repens*, a well-known traditional Indian medicine, has a long history of being used for treating a lot of diseases. But its analgesic and anti-inflammatory features have never been reported. With this investigation, we demonstrated the potent analgesic and anti-inflammatory actions of *Limnophila repens* in various animal models.

The hot-plate test is astonishing in elucidating centrally mediated anti-nociceptive responses, which works mainly on changes over a spinal-cord level. The significant upsurge in pain tolerance produced by methanolic extract of *L. repens* implies the involvement of central pain pathways. Pain is centrally controlled via some intricate processes consisting of opiate, dopaminergic, descending noradrenergic and serotonergic systems. The analgesic result caused by the extract might be via central mechanisms including these types of receptor systems or through peripheral mechanisms involved in the inhibition of prostaglandins, leukotrienes, and other endogenous substances which can be crucial players in swelling and pain (29).

The abdominal constriction response evoked by acetic acid is a sensitive process to assess peripherally acting analgesics. Generally, acetic acid triggers pain by releasing endogenous substances such as serotonin, histamine, prostaglandins, bradykinins and substance P, which in turn activate nerve endings. Local peritoneal receptors will be postulated to be involved in the abdominal constrictions impulse. The technique is involving prostanoids in general, that is, elevated levels of prostaglandin-E2 (PGE2) and PGF2 in peritoneal fluids and also lipoxygenase products (30). The significant lowering of acetic acid-induced writhes through the methanolic extract of *L. repens* shows that the analgesic impact might be peripherally mediated with the inhibition of synthesis and release of prostaglandins along with endogenous substances.

Carrageenan-induced edema continues to be widely used because an experimental animal model for acute inflammation and is considered to be biphasic. The early stage (1 to 2 h) of the carrageenan model is primarily mediated by histamine, serotonin and raised synthesis of prostaglandins in the damaged tissue surroundings. The late stage (3 h) is endured by prostaglandin release and mediated by bradykinin, leukotrienes, polymorphonuclear cells and prostaglandins produced by tissue macrophages (31, 32). The

significant ($P < 0.05$) suppressive activity of the MELR in late-stage reveals its potent anti-inflammatory impact. This result is very identical to the one noticed for diclofenac at 10 mg/kg, which inhibited the edema by 61.44%. The outcome was statistically significant ($P < 0.05$). Ueno et al. (2000) found that the injection of carrageenan into the rat paw induce the liberation of bradykinin, which later causes the biosynthesis of prostaglandin and other autacoids, which are accountable for the formation of the inflammatory exudates (33). Besides, in the carrageenan-induced rat paw oedema model, the production of prostanoids has been through the serum expression of COX-2 by a positive feedback mechanism (34). PGE2, a potent vasodilator, synergises with other inflammatory vasodilators including histamine and bradykinin and contributes to the redness and improved blood circulation in regions of acute inflammation. Consequently, it is strongly recommended that the mechanism of action of the extracts might be associated with histamine and prostaglandin synthesis inhibition. The outcome indicated that the methanolic extract of plant *L. repens* (MELR) at different concentrations offers significant anti-inflammatory property. Carrageenan-induced inflammation is an important model intended for the evaluation of anti-inflammatory impact. The development of oedema in the paw of the rat following the injection of carrageenan is because of the release of histamine, serotonin, prostaglandin and the like. MELR showed significant anti-inflammatory activity. (35-37). This significant anti-inflammatory and analgesic effect may be due to the inhibition of any inflammatory mediators by the steroids, i.e., β-sitosterol, stigmasterol (38), flavonoids, i.e., quercetin (39) and glycosides present in the extract. The current result indicates the efficacy of *Limnophila repens* as an effective therapeutic agent in the treatment of acute inflammations. The result also authentifies the folklore information on the anti-inflammatory and analgesic property of the *Limnophila repens* extract. But, further phytochemical, as well as biological tests, required to determine the other active chemical constituents accountable for the antinociceptive and antiinflammatory activities.

REFERENCES

1. Metrouh-Amir H, Amir N. Evaluation in vivo of anti-inflammatory and analgesic properties of Matricaria pubescens alkaloids. South African Journal of Botany. 2018;116:168-74.

2. Husseini Y, Sahraei H, Meftahi GH, Dargahian M, Mohammadi A, Hatef B, et al. Analgesic and anti-inflammatory activities of the hydro-alcoholic extract of Lavandula

officinalis in mice: possible involvement of the cyclooxygenase type 1 and 2 enzymes. Revista Brasileira de Farmacognosia. 2016;26(1):102-8.

3. Les DH. Aquatic dicotyledons of North America: ecology, life history, and systematics: CRC Press; 2017.

4. Hsu H, Chen Y, Sheu S, Hsu C, Chen C, Chang H. Oriental materia medica: a concise guide: Keats Publishing, Inc.; 1996.

5. Pullaiah T. Encyclopaedia of world medicinal plants: Daya books; 2006.

6. Brahmachari G. Limnophila (Scrophulariaceae): Chemical and Pharmaceutical Aspects-An Update. The Open Natural Products Journal. 2014;7(1).

7. Do QD, Angkawijaya AE, Tran-Nguyen PL, Huynh LH, Soetaredjo FE, Ismadji S, et al. Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of Limnophila aromatica. Journal of food and drug analysis. 2014;22(3):296-302.

8. Kukongviriyapan U, Luangaram S, Leekhaosoong K, Kukongviriyapan V, Preeprame S. Antioxidant and vascular protective activities of Cratoxylum formosum, Syzygium gratum and Limnophila aromatica. Biological and Pharmaceutical Bulletin. 2007;30(4):661-6.

9. Rao JV, Aithal KS, Srinivasan K. Antimicrobial activity of the essential oil of Limnophila gratissima. Fitoterapia. 1989;60(4):376-7.

10. Nanasombat S, Teckchuen N. Antimicrobial, antioxidant and anticancer activities of Thai local vegetables. Journal of Medicinal Plants Research. 2009;3(5):443-9.

11. Suksamrarn A, Poomsing P, Aroonrerk N, Punjanon T, Suksamrarn S, Kongkun S. Antimycobacterial and antioxidant flavones from Limnophila geoffrayi. Archives of pharmacal research. 2003;26(10):816-20.

12. Ahmed D, Saeed R, Shakeel N, Fatima K, Arshad A. Antimicrobial activities of methanolic extract of Carissa opaca roots and its fractions and compounds isolated from the most active ethyl acetate fraction. Asian Pacific Journal of Tropical Biomedicine. 2015;5(7):541-5.

13. Khandelwal KR. Practical pharmacognosy : techniques and experiments. Maharashtra: Niral Prakashan; 2008.

14. Kokate C. Practical pharmacognosy, 1994. VallabhPrakashan, New Delhi.1:15-30.

15. Wallis TE. Practical pharmacognosy. 1925.

16. Yarnalkar S. Practical pharmacognosy. Pune: Nirali Prakashan. 1991:38.

17. Habib M, Nikkon F, Rahman M, Haque Z, Karim M. Isolation of stigmasterol and ß-sitosterol from methanolic extract of root. Pak J Biol Sci. 2007;10(22):4174-6.

18. Wan C, Yu Y, Zhou S, Tian S, Cao S. Isolation and identification of phenolic compounds from Gynura divaricata leaves. Pharmacognosy Magazine. 2011;7(26):101-8.
19. Oecd. OECD Guidelines for the Testing of Chemicals: Organization for Economic; 1994.
20. Kiran PM, Raju AV, Rao BG. Investigation of hepatoprotective activity of Cyathea gigantea (Wall. ex. Hook.) leaves against paracetamol-induced hepatotoxicity in rats. Asian Pacific journal of tropical biomedicine. 2012;2(5):352.
21. Vogel HG. Drug discovery and evaluation: pharmacological assays: Springer Science & Business Media; 2002.
22. Kumar Paliwal S, Sati B, Faujdar S, Sharma S. Studies on analgesic, anti-inflammatory activities of stem and roots of Inula cuspidata C.B Clarke. Journal of Traditional and Complementary Medicine. 2017;7(4):532-7.
23. Wang Y-X, Gao D, Pettus M, Phillips C, Bowersox SS. Interactions of intrathecally administered ziconotide, a selective blocker of neuronal N-type voltage-sensitive calcium channels, with morphine on nociception in rats. PAIN®. 2000;84(2-3):271-81.
24. Xu Q, Wang Y, Guo S, Shen Z, Wang Y, Yang L. Anti-inflammatory and analgesic activity of aqueous extract of Flos populi. J Ethnopharmacol. 2014;152(3):540-5.
25. Arumugam P, Murugan M, Thangaraj N. Evaluation of anti-inflammatory and analgesic effects of aqueous extract obtained from root powder of Inula racemosa Hook. f. Journal of Medicinal Plants Research. 2012;6(14):2801-6.
26. Badole SL, Zanwar AA, Ghule AE, Ghosh P, Bodhankar SL. Analgesic and anti-inflammatory activity of alcoholic extract of stem bark of Pongamia pinnata (L.) Pierre. Biomedicine & Aging Pathology. 2012;2(1):19-23.
27. Collier H, Dinneen L, JOHNSON CA, Schneider C. The abdominal constriction response and its suppression by analgesic drugs in the mouse. British journal of pharmacology and chemotherapy. 1968;32(2):295-310.
28. Deraedt R, Jouquey S, Delevallée F, Flahaut M. Release of prostaglandins E and F in an algogenic reaction and its inhibition. European journal of pharmacology. 1980;61(1):17-24.
29. Millan MJ. Descending control of pain. Progress in neurobiology. 2002;66(6):355-474.
30. Farouk L, Laroubi A, Aboufatima R, Benharref A, Chait A. Evaluation of the analgesic effect of alkaloid extract of Peganum harmala L.: possible mechanisms involved. Journal of ethnopharmacology. 2008;115(3):449-54.

31. Chavan M, Wakte P, Shinde D. Analgesic and anti-inflammatory activities of 18-acetoxy-ent-kaur-16-ene from Annona squamosa L. bark. Inflammopharmacology. 2011;19(2):111-5.

32. Oliveira de Melo J, Truiti MdCT, Muscara MN, Bolonheis SM, Dantas JA, Caparroz-Assef SM, et al. Anti-inflammatory activity of crude extract and fractions of Nectandra falcifolia leaves. Biological and Pharmaceutical Bulletin. 2006;29(11):2241-5.

33. Ueno A, Naraba H, Ikeda Y, Ushikubi F, Murata T, Narumiya S, et al. Intrinsic prostacyclin contributes to exudation induced by bradykinin or carrageenin: a study on the paw edema induced in IP-receptor-deficient mice. Life sciences. 2000;66(12):PL155-PL60.

34. Simplice FH, Arm AB, Roger P, Emmanuel AA, Pierre K, Veronica N. Effects of Hibiscus asper leaves extracts on carrageenan induced oedema and complete Freunds adjuvant-induced arthritis in rats. Journal of Cell and Animal Biology. 2011;5(5):66-8.

35. Georgewill O, Georgewill U, Nwankwoala R. Anti-inflammatory effects of Morninga oleifera lam extract in rats. Asian Pacific Journal of Tropical Medicine. 2010;3(2):133-5.

36. Winter CA, Risley EA, Nuss GW. Carrageenin-Induced Edema in Hind Paw of the Rat as an Assay for Antiinflammatory Drugs. Proceedings of the Society for Experimental Biology and Medicine. 1962;111(3):544-7.

37. Georgewill O, Georgewill U. Evaluation of the anti-inflammatory activity of extract of Vernonia amygdalina. Asian Pacific Journal of Tropical Medicine. 2010;3(2):150-1.

38. Saeidnia S, Manayi A, Gohari AR, Abdollahi M. The story of beta-sitosterol-a review. European journal of medicinal plants. 2014;4(5):590.

39. Li Y, Yao J, Han C, Yang J, Chaudhry MT, Wang S, et al. Quercetin, Inflammation and Immunity. Nutrients. 2016;8(3):167.

Table 1: Phytochemical analysis of various extracts of *Limnophila repens*

Phytoconstituents	Method	Pet. ether Extract	Chloroform Extract	Ethyl acetate Extract	Methanolic Extract	n-butanol Extract	Aqueous Extract
Flavonoids	Shinoda Test	-	-	+	+	-	+
	Zn+HCl test	-	-	+	+	-	+
	Lead acetate Test	-	-	+	+	-	+
Volatile oil	Stain test	+	-	-	+	-	+
Alkaloids	Wagner Test	-	+	-	+	-	+
	Hager's Test	-	+	-	+	-	+
Tannins & Phenols	$FeCl_3$ Test	-	-	+	+	+	+
	Potassium dichromate test	-	-	+	+	+	+
Saponins	Foam Test	-	-	-	-	-	-
Phytosterols	Libermann's test	+	+	-	+	-	-
Carbohydrates	Molish test	-	-	-	+	-	+
Acid compounds	Litmus test	-	-	-	-	-	-
Glycoside	Borntragers test	-	-	-	+	-	+
Amino acids	Ninhydrin test	-	-	-	+	-	+
Proteins	Biuret test	-	-	-	+	-	+
Fixed oils & fats	Spot test	+	-	-	-	-	-

Treatment	Reaction Time (s) Time after Treatment (min)				
	0	**30**	**60**	**90**	**120**
Control	9.55 ± 0.94	9.68 ± 0.97	9.72 ± 0.6	9.62 ± 0.68	9.48 ± 0.62
Aspirin (100 mg/kg)	9.34 ± 011	17.64 ± 0.46*	15.84 ± 0.39*	12.13 ± 0.84*	9.71 ± 0.54*
MELR (200mg/kg)	9.62 ± 0.22	9.46 ± 0.1$^{\#}$	10.95 ± 0.58$^{\#}$	12.95 ± 0.62$^{\#}$	10.09 ± 0.56$^{\#}$
MELR (400mg/kg)	9.49 ± 0.22	10.72 ± 0.38	12.15 ± 0.31	14.95 ± 0.85	10.64 ± 0.54

Table 2: Effect of MELR on Hot-Plate Method. All the values are expressed as mean ± SEM; n = 5 rat in each group, by one way ANOVA followed by Tukey's Multiple Comparison Test. *, p < 0.05 significant compared to control and $^{\#}$, p < 0.05 significant compared to standard.

Treatment	Reaction Time (s) Time after Treatment (min)					
	0	**30**	**60**	**90**	**120**	**180**
Control	2.31 ± 0.06	2.2 ±0.04	2.42 ± 0.11	2.51 ± 0.08	2.56 ± 0.08	2.64± 0.09
Aspirin (200 mg/kg)	2.34 ± 0.23	3.68 ± 0.28	4.7 ± 0.36	5.33 ± 0.28	6.39 ± 0.39	8.08 ± 0.17
MEAR (200 mg/kg)	2.03 ± 0.07	2.8 ± 0.15	3.6 ± 0.22	4.46 ± 0.22	5.4 ± 0.16	6.39 ± 0.15
MEAR (400 mg/kg)	2.44 ± 0.11	3.89 ± 0.23	4.89 ± 0.18	5.78 ± 0.14	6.4 ± 0.18	7.75 ± 0.31

Table 3: Protective effect of MELR on tail withdrawal reflexes induced by tail immersion method in rats. All the values are expressed as mean ± SEM; n = 5 rats in each group, by one way ANOVA followed by Tukey's Multiple Comparison Test. Results are presented as mean ± SEM, (n=5), *, p < 0.05 versus control.

Treatment	Writhing Count					Writhings (Mean±SEM)	% of Wrighting	% of Inhibition
	M-1	M-2	M-3	M-4	M-5			
Control	28	26	25	23	28	26 ± 2.12	100	0
Diclofenac Sodium (5 mg/kg)	7	9	11	6	8	8.2 ± 1.92	31.54	68.46
MELR (200 mg/kg)	16	20	12	18	21	17.4 ± 3.57	66.93	33.07
MEAR (400 mg/kg)	12	15	8	16	15	13.2 ± 3.27	50.77	49.23

Table 4: Effect of MELR on acetic acid-induced writhing behavior in mice.

M-1= Mice 1, M-2 = Mice 2, M-3 = Mice 3, M-4 = Mice 4, M-5 = Mice 5.), $, p < 0.001 versus control, #, p < 0.001 versus Aspirin and @, p < 0.001 versus MELR (200 mg/kg).

Group	Chage in Paw thickness (mm) ± SD ()				% Inhibition at hrs			
	1 hr	2 hr	3 hr	4 hr	1 hr	2 hr	3 hr	4th
Carrageenan (1%w/v of 0.1ml)	1.44 ± 0.12	2.48 ± 0.21	3.86 ± 0.18	3.45 ± 0.16	-	-	-	-
Carrageenan+Diclofenac (10 mg/kg)	0.69 ± 0.21$^{\$}$	0.98 ± 0.15$^{\$}$	1.14 ± 0.12$^{\$}$	0.9 ± 0.2$^{\$}$	52.08	60.48	70.46	73.91
Carrageenan + MELR (200 mg/kg)	1.16 ± 0.21$^{\#}$	1.82 ± 0.3$^{\#}$	2.57 ± 0.12$^{\#}$	2.01 ± 0.18$^{\#}$	19.44	26.61	33.41	41.73
Carrageenan + MELR (400 mg/kg)	0.98 ± 0.14	1.48 ± 0.18	1.92 ± 0.12	1.33 ± 0.21	31.94	40.32	50.25	61.44

Table 5: Effect of MELR on Carrageenan-Induced Paw edema method. Results are presented as mean ± SEM, (n=5), $^{\$}$, p < 0.001 versus control; #, p<0.001 versus Diclofenac (10 mg/kg).

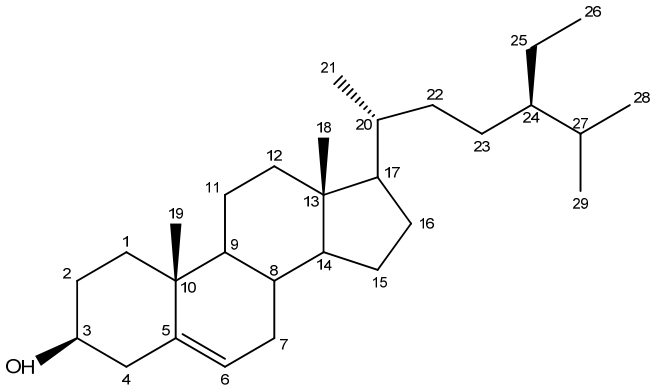

Figure 1: Structure of LR-01 (stigmasterol)

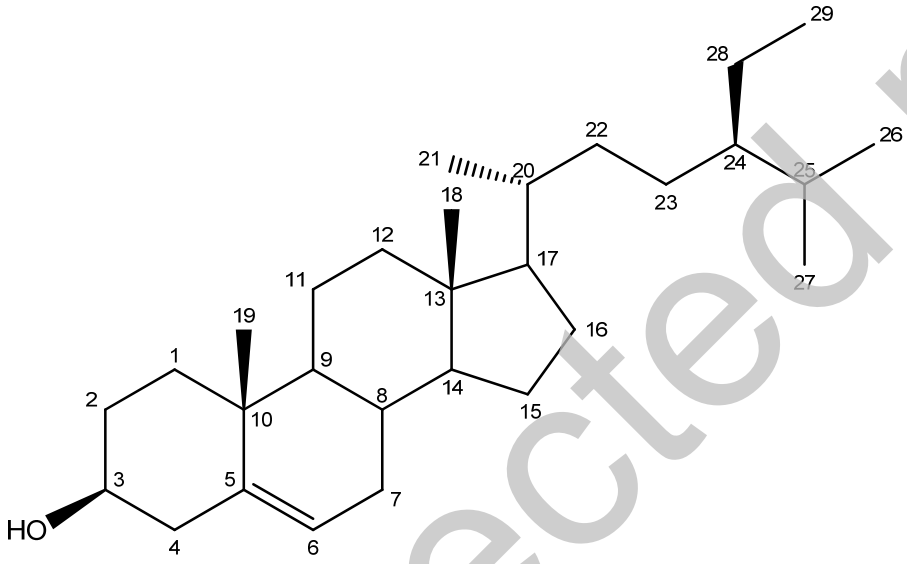

Figure 2: Structure of LR-02 (β-sitosterol)

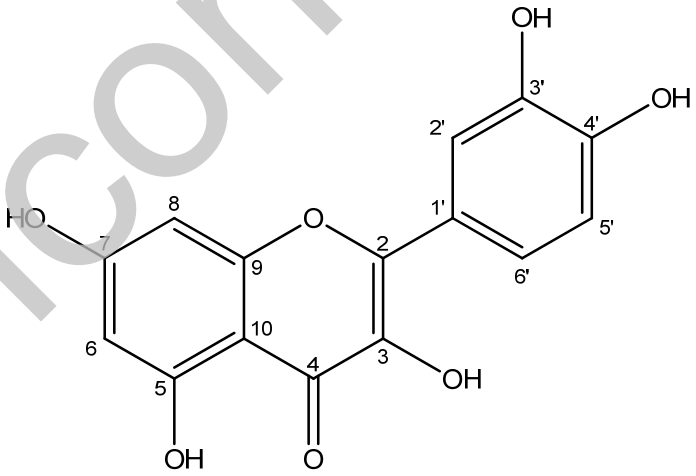

Figure3: Structure of LR-03 (Quercetin).

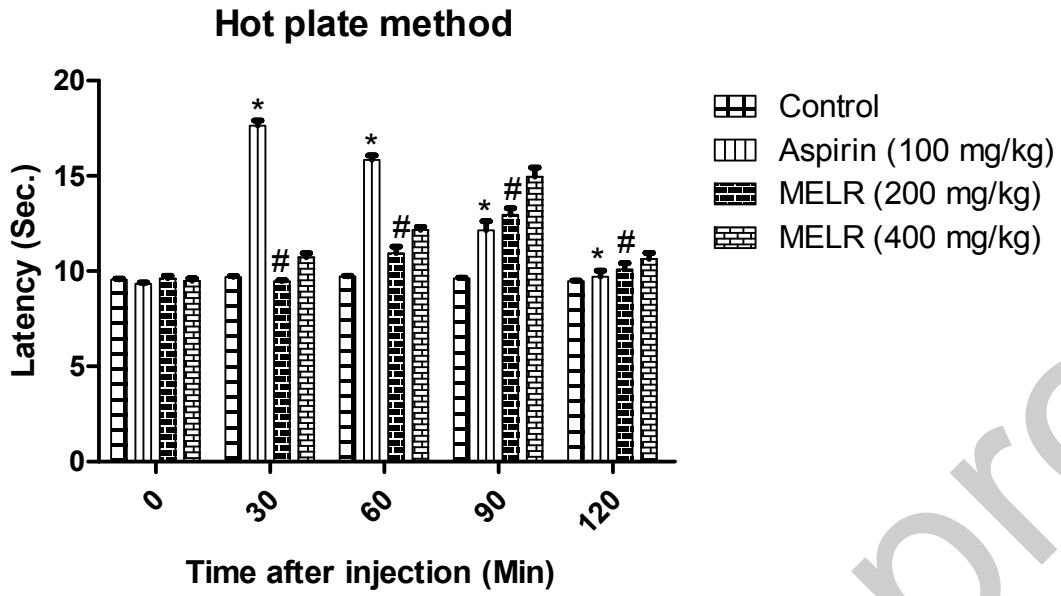

Figure 4: Effect of MELR on Hot-Plate Method. All the values are expressed as mean ± SEM; n = 5 rat in each group, by one way ANOVA followed by Tukey's Multiple Comparison Test. *, $p < 0.05$ significant compared to control and #, $p < 0.05$ significant compared to standard.

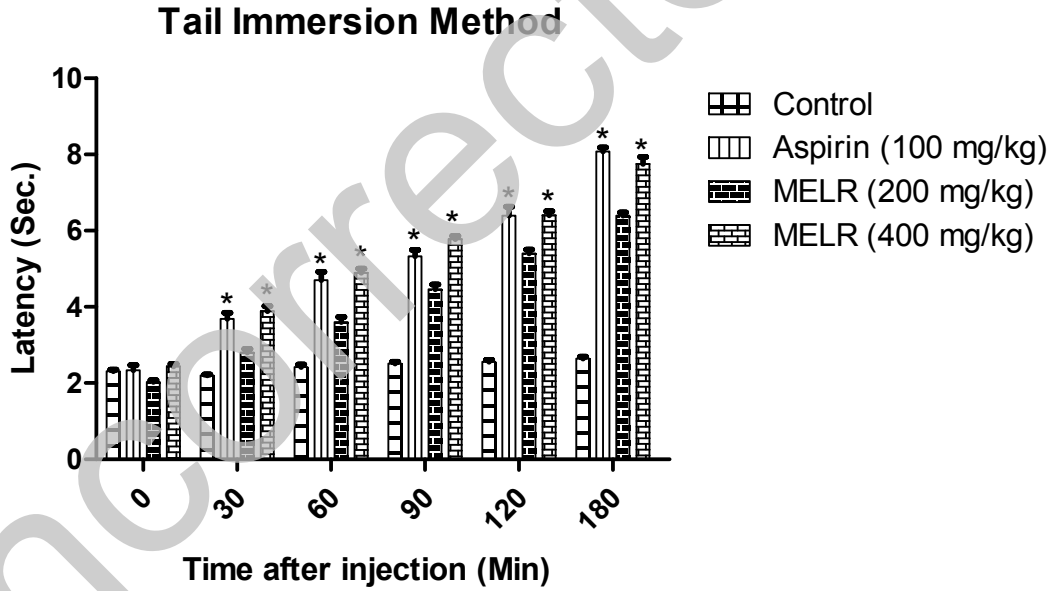

Figure 5: Protective effect of MELR on tail withdrawal reflexes induced by tail immersion method in rats. All the values are expressed as mean ± SEM; n = 5 rats in each group, by one way ANOVA followed by Tukey's Multiple Comparison Test. Results are presented as mean ± SEM, (n=5), *, $p < 0.05$ versus control.

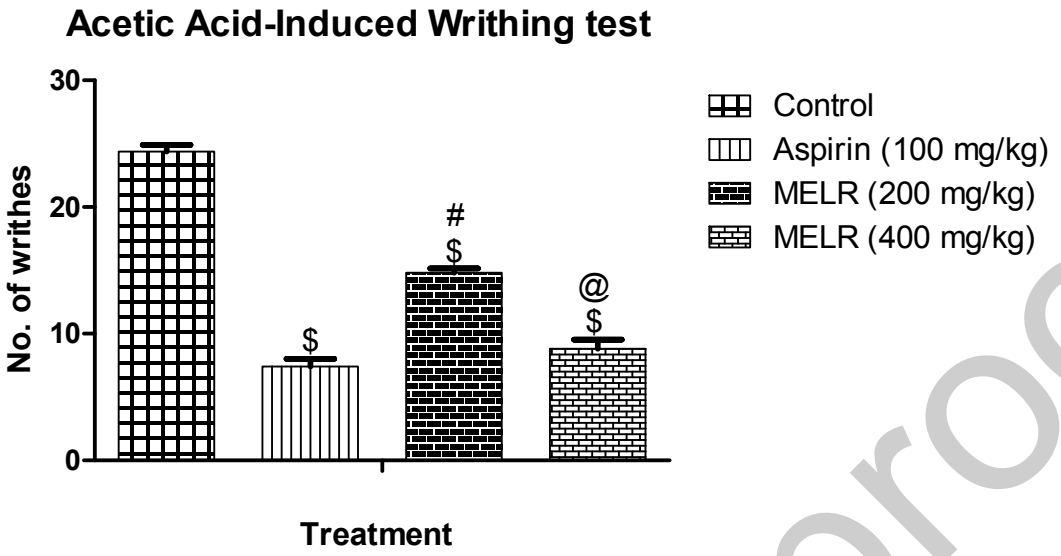

Figure 6: Effect of MELR on acetic acid-induced writhing behavior in mice.[$,] $p < 0.001$ versus control, [#,] $p < 0.001$ versus Aspirin and @, $p < 0.001$ versus MELR (200 mg/kg).

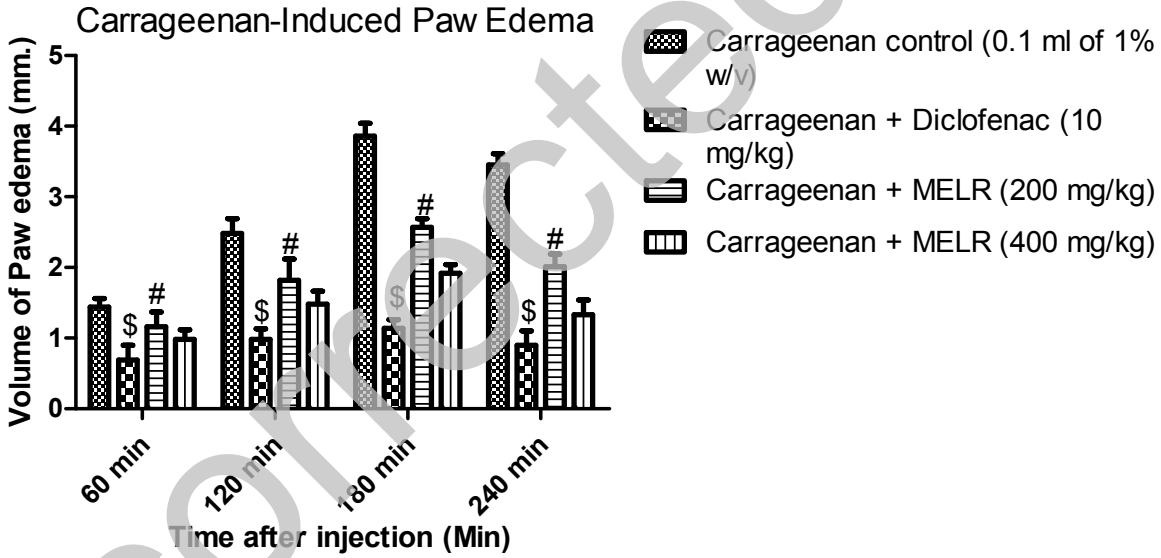

Figure 7: Effect of MELR on Carrageenan-Induced Paw edema method. Results are presented as mean±SEM, (n=5), [$,] $p < 0.001$ versus control; #, $p<0.001$ versus Diclofenac (10 mg/kg).