Turk J Pharm Sci 2020;17(3):329-336
DOI: 10.4274/tjps.galenos.2018.60566

ORIGINAL ARTICLE

Anti-inflammatory and Analgesic Effects of *Limnophila repens* (Benth.)

Limnophila repens (Benth.) Anti-enflamatuvar ve Analjezik Etkileri

Venkateswarlu GUNJI[1]*, Ganapaty SERU[2]

[1]A.M. Reddy Memorial College of Pharmacy, Department of Pharmacognosy and Phytochemistry, Andhra Pradesh, India
[2]GITAM Institute of Pharmacy, Department of Pharmacognosy and Phytochemistry, Andhra Pradesh, India

ABSTRACT

Objectives: The analgesic and anti-inflammatory effects of methanolic extract of *Limnophila repens* (MELR) were assessed at 200 and 400 mg/kg.

Materials and Methods: In carrageenan-mediated paw edema, the anti-inflammatory effect of MELR was investigated and analgesic activity was assessed by central and peripheral models

Results: MELR had strong analgesic and anti-inflammatory effects at different dosages (200 and 400 mg/kg). The study results confirmed the use of *Limnophila* as both an analgesic and anti-inflammatory. The strong anti-inflammatory and analgesic effects can be caused by anabolic steroids, i.e. β-sitosterol and stigmasterol; and flavonoids, i.e. quercetin and glycosides, in the extraction of some kind of inflamed arbitrators.

Conclusion: Based on the study, we can conclude that *Limnophila repens* had analgesic and anti-inflammatory activity. In addition to organic studies, however, additional phytochemicals are required to evaluate the extra energetic chemicals responsible for the antinociceptive and anti-inflammatory effects.

Key words: *Limnophila repens*, carrageenan, phytochemical screening, β-sitosterol

ÖZ

Amaç: *Limnophila repens* (MELR) metanol ekstresinin analjezik ve anti-enflamatuvar etkileri 200 ve 400 mg/kg dozda değerlendirildi.

Gereç ve Yöntemler: Karagenin ile indüklenen pence ödeminde MELR'nin anti-enflamatuvar etkisi araştırıldı ve analjezik aktivite merkezi ve periferik modellerle değerlendirildi.

Bulgular: MELR, farklı dozlarda (200 ve 400 mg/kg) güçlü analjezik ve anti-enflamatuvar etki göstermiştir. Çalışma sonuçları, *Limnophila*'nın hem analjezik hem de anti-enflamatuvar olarak kullanımını doğrulamıştır. Güçlü anti-enflamatuvar ve analjezik etkilerden anabolik steroidlerin (β-sitosterol ve stigmasterol gibi); flavonoidlerin (kuersetin ve glikozitler gibi) sorumlu olabileceği değerlendirilmiştir.

Sonuç: Çalışmaya dayanarak *Limnophila repens*'in analjezik ve anti-enflamatuvar aktiviteye sahip olduğu sonucuna varabiliriz. Bununla birlikte, organik çalışmalara ek olarak, antinosiseptif ve anti-enflamatuvar etkilerden sorumlu ekstra enerjik kimyasalları değerlendirmek için ek fitokimyasallar gereklidir.

Anahtar kelimeler: *Limnophila repens*, karagenin, fitokimyasal tarama, β-sitosterol

***Correspondence: E-mail:** gvr9885@gmail.com, **Phone:** 9000079873 **ORCID-ID:** orcid.org/0000-0001-7076-5090
Received: 04.09.2018, **Accepted:** 01.11.2018

INTRODUCTION

Herbal treatments, particularly therapeutic plants, were our ancestors' primary or only source of healthcare. Despite the rise of the healthcare industry, therapeutic plants and medicines that can be developed from them have never been totally discarded and people still resort to traditional medicine.[1] Basically, the use of natural flora in the treatment of illnesses and pain management is a key remedy.[2]

Limnophila is used to treat heart attacks, elephantiasis, diarrhea, dyspepsia, high temperature, dysentery, acid indigestion, dysmenorrhea, and stomach pain.[3-5]

Phytochemical examination of *Limnophila* shows a number of primary and secondary phytoconstituents.[6] This variety of substances justifies the traditional use of *L. repens*.

Limnophila is already very prevalent and frequently used in herbal remedies as an antimycobacterial, antioxidant, antineoplastic, and antimicrobial,[7-11] but no natural research studies have been performed on this herb. Subsequently, the present experiment was performed to determine the anti-inflammatory and antinociceptive activities of *L. repens*.

MATERIALS AND METHODS

Plant selection and authentication

During September 2017, *L. repens* was collected at Tirupati. Dr. K. Madhava Chetty identified and tested the examined herb. GITAM Institute of Pharmacy, Visakhapatnam, deposited an herb specimen with the voucher number 1568.

Preparation of extract

The powder (1 kg) was obtained by petroleum ether method suggested for removing both fatty and waxy materials. The methanol extract was initially extracted in alcoholic water through a splitting-up channel and then sequentially segmented together with petrol ether, chloroform, ethyl acetate, and n-butanol to obtain portions of these solvents. These extracts had been subjected to preparatory phytochemical evaluation and had, in addition, been kept in the fridge at 4°C for potential further use.[12]

Phytochemical screening

Various extracts of *L. repens* were subjected to qualitative chemical assessment using uniform criteria.[13-16]

Separation of phytoconstituents

Column chromatography on silica gel (60-120 mesh) using n-hexane, ethyl acetate, and 100% methanol afforded an 18 g petroleum ether portion. The fractions on the thin-layer chromatography (TLC) plate were pooled and crystallized, and named *Limnophila repens* (LR-1) and LR-2.[17] A silica gel column eluted from the chloroform-methanol phase gradient (from 100:0 to 4:1) chromatographed the ethyl acetate section and put eight sections on their TLC. In the Sephadex LH-20, chloroform methanol (1:10) was chromatographed with methanol to provide LR-3.[18]

Animals

All the experimental animals used for this research were acquired from Nicholas Piramal India Limited, Mumbai. They were subsequently put in the Animal House of A.M. Reddy Memorial College of Pharmacy with IAEC Approval no. AMRMCP/05/IAEC/18-19/PHD. While in the Animal House they were allowed to consume water and eat. The animal usage complied with OECD-423 guidelines.[19]

Acute toxicity study

Test methanolic extract of *Limnophila repens* (MELR) toxicity in an acute toxicity study was based on OECD 423 recommendations for 2000 mg/kg dose. The test animals were regularly checked at 1 h, then 4 h, and finally every 24 h for 14 days for body signs and symptoms of poisoning, consisting of squirming, gulping, or pulsation as well as decreased respiratory system rate or even impermanence. No fatality was observed in this study.[20]

Grouping of animals and selection of dose

Furthermore, male rodents were randomly divided into four groups (control, regular, and pair of examination groups) composed of 5 animals each for analgesic and anti-inflammatory study. The first group was initially designated as the control, with 10 mL/kg distilled water. Group II specified as reference group was given the standard drug tramadol 10 mg/kg p.o. Groups III and IV received MELR (200 mg/kg and 400 mg/kg, respectively) in distilled water.

Pharmacological activity

Antinociceptive activity

The peripheral study behavior of MELR was evaluated using acetate-induced acid while the central analgesic function was investigated using the hot plate and tail-flick techniques.

Hot plate technique

Each rodent was individually placed independently on the hot plate at 55±2 °C. The response time was videotaped for each mouse at 30 min, 60 min, and 90 min, monitoring medicine or vehicle administration along with 15 s cut-off to avoid injury. Increased response time and extracts were matched to the control group.[21,22]

Percentage of analgesic activity was calculated by using the formula

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

Ta: Average reaction time after extract; Tb: Average initial reaction time

Tail immersion test

The lower part of the rodent tail was immersed in warm water, around 55 °C, which caused a painful reaction. 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. In addition, the percentage of inhibition was calculated using the formula[23]

$$\text{Inhibition} = \frac{\text{Ln-Lo}}{15 \text{ s-Lo}} \times 100 ,$$

where Lo: Latent time before drug administration in seconds, Ln: Latent time after drug administration in seconds (n=30 to 180 min).

Writhing test

The mice (n=5) were grouped into MELR teams; in addition, two more teams (n=5) were used for control and standard testing. They were divided into four teams, w here groups III and IV were given MELR at a dose of 100 and 200 mg/kg b.wt. explicitly (by i.p.), while group II was administered along with the normal aspirin 100 mg/kg drug, p.o. 1 h before acetic acid induction. The group's percentage restraint was calculated by[24,25]

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

Anti-inflammatory activity

Carrageen-induced paw edema

The rat paw edema procedure caused abrupt inflammation in the rodents by administration of 00.1 mL of prepared carrageenan fluid (1% w/v) to the subplantar area. For each sample the rodents were categorized into four sections (n=5) and control and norm groups (n=5). The control group was given vehicle; the standard group received diclofenac 10 mg/kg p.o and the groups assigned for extracts received 200 and 400 mg/kg p.o. before 60 min of carrageenan injection. Upon carrageenan infusion, paw volume was assessed with an electronic plethysmometer at 1, 2, and 3 h. % Inhibition was calculated using the formula[26]

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

where T_o: Paw thickness of rats given test extract at the same time; T_t: Paw thickness of control rats.

Statistical analysis

The data are expressed as mean ± standard error of the mean (SEM). ANOVA software (GraphPad Prism 5) was used to perform the statistical analysis. The level of statistical significance was $p<0.05$.

RESULTS

Phytochemical screening

The results of the phytochemical analysis of different extracts are shown in Table 1.

Characterization of LR-1

White powder, $C_{29}H_{48}O$, MW 412.69. ultraviolet - max ($CHCl_3$) nm: 257; IR (KBr) IR (KBr) total cm^{-1}: 3418 (-OH stretch), 2934 (C-H stretch in CH_2 and CH_3), 2866 (=C-H stretch), 2339, 1602 (C=C asymmetric stretch), 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. The above spectral data (mass, NMR) showed the molecular formula $C_{29}H_{48}O$, similar to stigmasterol (Figure 1).

LR-02

White powder, $C_{29}H_{50}O$, MW 414.70; IR (KBr) max cm^{-1}: 3424 (-OH stretch), 2959 (-CH, 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, $CDCl_3$). The above spectral data (mass, NMR) showed the molecular formula $C_{29}H_{50}O$, similar to β-sitosterol (Figure 2).

LR-03

Yellow powder, $C_{15}H_{10}O_7$, MW 302.23; IR (KBr) max cm^{-1}: 3413 (-OH bending), 2340, 1607, 1565, 1523, 1462, 1408, 1383, 1320, 1263 (C-O stretch), 1199, 1168, 1131, 1014, 959, 782 (=C-H bending); ^{1}H NMR data (400 MHz, $CDCl_3$) 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, $CDCl_3$) and others: 175.81 (C-4), 163.85 (C-7), 160.70 (C-5), 156.17 (C-9), 147.67 (C-2), 146.81 (C-31), 145.03 (C-3), 135.68 (C-61), 121.96. The above spectral data (mass, NMR) showed the molecular formula $C_{15}H_{10}O_7$, similar to quercetin (Figure 3).

Acute toxicity studies

An oral MELR dosage of 2000 mg/kg caused no immediate toxic symptoms. Furthermore, no rodents died during 24 h surveillance. The extracts have been considered to be safe at the highest allowable dose of 2000 mg/kg; the highest possible dosage was generally chosen for analgesic and anti-inflammatory activities, i.e. 200 and 400 mg/kg, 1/5th and 10-fold drops.

Analgesic activity

Hot plate tests

The mean ± SEM showed that the MELR (200 and 400 mg/kg) caused an improvement 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 4, Table 2).

Tail immersion test

The tail immersion approach showed a marked increase of 6.39±0.15 in MELR (200 mg/kg) and 7.75±0.31 in MELR (400 mg/kg) at 180 min (Figure 5). The inhibition was the strongest at 400 mg/kg dose at 180 min, lower than normal (Table 3).

Writhing test

Table 4 revealed *Limnophila*'s peripheral pharmacological behavior on visceral squirming in mice. The control group displayed maximal writhing (26±2.12), while MELR had a strong antinociceptive effect against acetic acid-induced writhing at doses of 200 and 400 mg/kg, inhibiting pain 33.07% and 49.23% relative to the control (Figure 6). At 10 mg/kg, diclofenac caused 68.46% ($p<0.001$) writhing reaction inhibition.

Carrageenan-induced paw edema

Table 5 demonstrates MELR's effect on the paw edema model relative to carrageenan treatment at different stages. A dosage of 200 mg/kg MELR was given. 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 h, respectively, and 31.94%, 40.32%,

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 and 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 and fats	Spot test	+	-	-	-	-	-

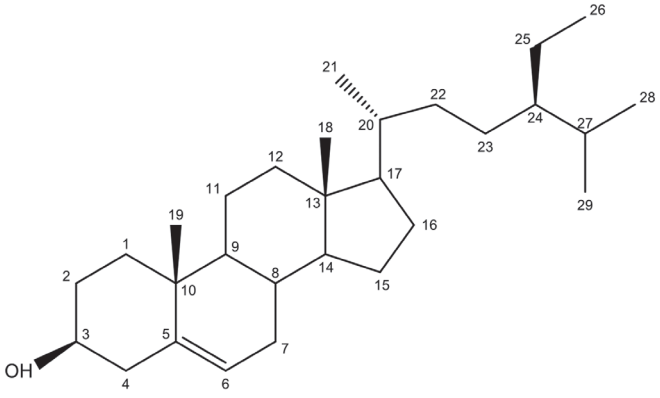

Figure 1. Structure of LR-01 (stigmasterol)
LR: Limnophila repens

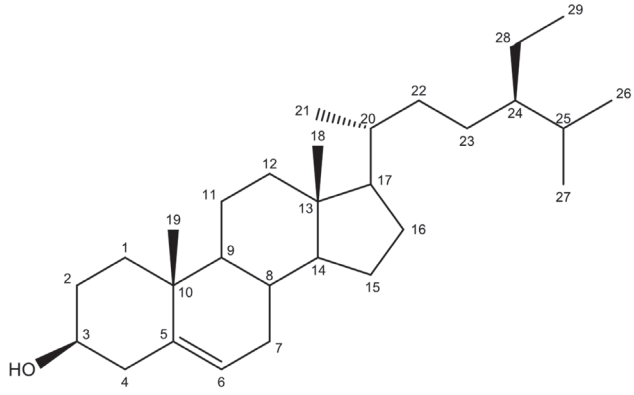

Figure 2. Structure of LR-02 (β-sitosterol)
LR: Limnophila repens

50.25%, and 61.44% at a dose of 400 mg/kg p.o. at 1, 2, 3, and 4 h, 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 h, respectively (Figure 7).

DISCUSSION

Preliminary phytochemical analysis of *L. repens* revealed many compounds including flavonoids, volatile oils, alkaloids, tannins, phytosterols, sugars, 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] *L. repens* has a long history of being used for various diseases and is a well-known Indian medicine, but its analgesic and anti-inflammatory features have never been reported. We have shown important antinociceptive and anti-inflammatory behavior of *L. repens* in various animal models. The hot-plate test exemplifies centrally moderated antinociceptive responses, which typically work on modifications over a spinal-cord degree. MELR's major discomfort-endurance implies core involvement. Some complex therapies like opiate, dopaminergic, noradrenergic, and serotonergic units usually centrally treat pain. The analgesic result due to the extract may be through major operations consisting of these types of receptors or even through specific procedures associated with prostaglandin inhibition, leukotrienes, and numerous other endogenous chemicals that may lead to swelling and discomfort.[29]

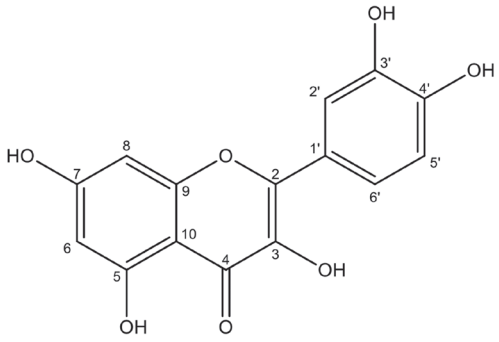

Figure 3. Structure of LR-03 (quercetin)
LR: Limnophila repens

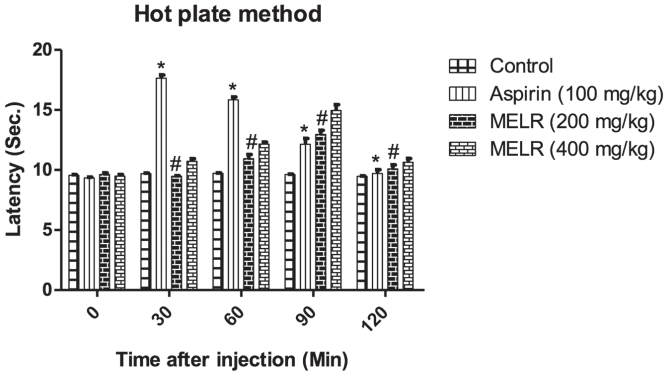

Figure 4. Effect of MELR on hot-plate method. All the values are expressed as mean ± SEM, n=5 rat in each group, by one-way ANOVA followed by Tukey's multiple comparison test *p<0.05 significant compared to control and #p<0.05 significant compared to standard
MELR: Methanolic extract of *Limnophila repens*, SEM: Standard error of the mean

The abdominal constriction response evoked by acetic acid is a sensitive process to assess peripherally acting analgesics. Acetic acid usually induces pain by releasing endogenous components such as bradykinins, histamine, serotonin, and prostaglandins, which trigger nerve endings. Peritoneal receptors are implied to communicate with stomach constraints. The strategy also requires elevated rates of prostaglandin E2 (PGE2) and PGF2 in peritoneal and lipoxygenase materials.[30] The major reduction in MELR-induced acetic acid writhes indicates that the analgesic activity may be moderated peripherally along with restriction of development and discharge of prostaglandins alongside endogenous drugs.

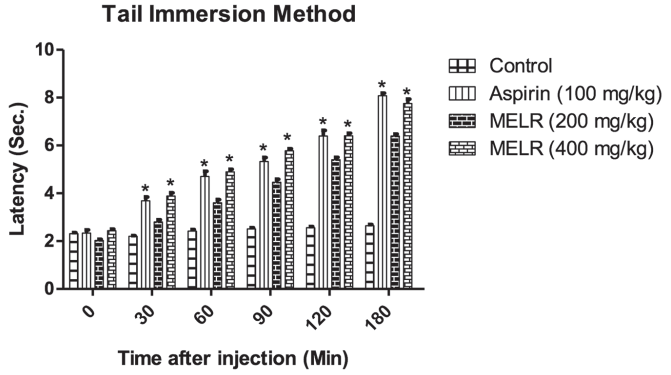

Figure 5. Protective effect of MELR on tail withdrawal reflexes induced by tail immersion method in rats. All the values are expressed as mean ± SEM, n=5 rats in each group, by one-way ANOVA followed by Tukey's multiple comparison test. Results are presented as mean ± SEM, (n=5), *p<0.05 versus control
MELR: Methanolic extract of *Limnophila repens*, SEM: Standard error of the mean

Table 2. Effect of MELR on hot-plate method

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 (200 mg/kg)	9.62±0.22	9.46±0.1#	10.95±0.58#	12.95±0.62#	10.09±0.56#
MELR (400 mg/kg)	9.49±0.22	10.72±0.38	12.15±0.31	14.95±0.85	10.64±0.54

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. *p<0.05 significant compared to control and #: p<0.05 significant compared to standard, MELR: Methanolic extract of *Limnophila repens*, SEM: Standard error of the mean

Table 3. Protective effect of MELR on tail withdrawal reflexes induced by tail immersion method in rats

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
MELR (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
MELR (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

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, MELR: Methanolic extract of *Limnophila repens*, SEM: Standard error of the mean

As an animal model for extreme swelling, carrageen-induced edema remains largely unused and is actually considered

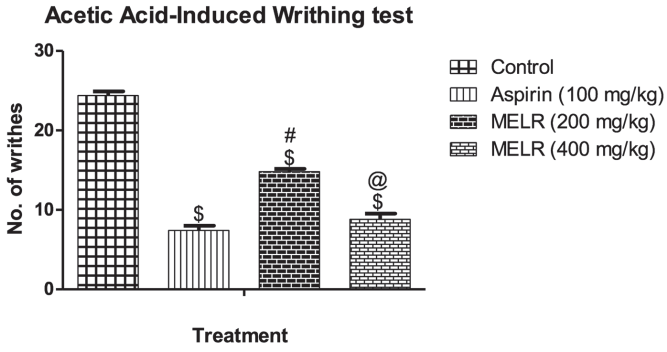

Figure 6. Effect of MELR on acetic acid-induced writhing behavior in mice
$p<0.001 versus control, #p<0.001 versus aspirin, and @p<0.001 versus MELR (200 mg/kg),
MELR: Methanolic extract of *Limnophila repens*

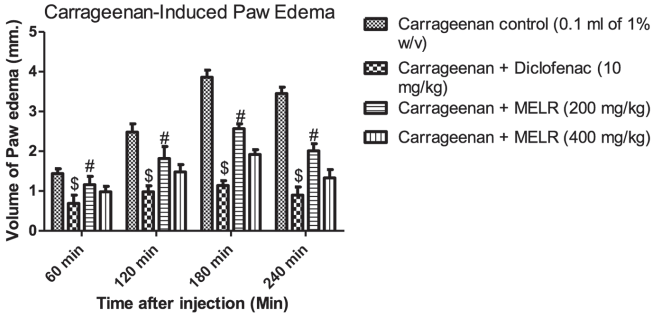

Figure 7. Effect of MELR on carrageenan-induced paw edema method. Results are presented as mean ± SEM, (n=5), $p<0.001 versus control; #p<0.001 versus diclofenac (10 mg/kg)
MELR: Methanolic extract of Limnophila repens, SEM: Standard error of the mean

biphasic. The initial stage (1-2 h) is largely solved in cell-ruined environments by histamine, serotonin, and increased prostaglandin formation. The latter stage (3 h) is liable to release prostaglandin and regulated by tissue macrophages,[31,32] bradykinin, and leukotrienes. In MELR's late-stage, substantial suppressive activity ($p<0.05$) indicates its powerful anti-inflammatory effect. It is comparable to diclofenac, which prevented edema at 10 mg/kg by 61.44%, a statistically significant finding ($p<0.05$). Ueno et al.[33] reported that rodent paw carrageen therapy results in bradykinin production that eventually leads to prostaglandin biosynthesis, as well as many other autacoids that accumulate inflammatory exudates.[34] PGE2 is a dominant vasodilator with many endogenous vasodilators, notably histamine and bradykinin, in severe inflammatory environments. Extract action mode is firmly recommended to suppress prostaglandin synthesis. Tests revealed that MELR has essential anti-inflammatory properties at various stages. Carrageenan-induced inflammation is an essential way to determine anti-inflammatory function. Edema formation in the rat paw following carrageen injection stems from histamine, serotonin, and prostaglandin release and associated substances. MELR has good anti-inflammatory behavior.[35-37] Due to anabolic steroids, i.e. β-sitosterol and stigmasterol,[38] flavonoids such as quercetin,[39] and glycosides present in the extract, this significant anti-inflammatory and analgesic impact results from the inhibition of any inflammatory mediators. The latest results indicate *Limnophila*'s efficacy in treating acute inflammation. The result also confirms the folklore information on the anti-inflammatory and analgesic property of the *L. repens* extract. Yet, additional phytochemical along with pharmacological

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

Treatment	Writhing count					Writhings (mean ± SEM)	% of writhing	% 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
MELR (400 mg/kg)	12	15	8	16	15	13.2±3.27	50.77	49.23

M-1: Mouse 1, M-2: Mouse 2, M-3: Mouse 3, M-4: Mouse 4, M-5: Mouse 5, MELR: Methanolic extract of *Limnophila repens*, SEM: Standard error of the mean

Table 5. Effect of MELR on carrageenan-induced paw edema method

Group	Change in paw thickness (mm) ± SD				% Inhibition at hours			
	1 h	2 h	3 h	4 h	1 h	2 h	3 h	4 h
Carrageenan (1% w/v of 0.1 mL)	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

Results are presented as mean ± SEM, (n=5), $: p<0.001 versus control, #p<0.001 versus diclofenac (10 mg/kg), MELR: Methanolic extract of *Limnophila repens*, SD: Standard deviation, SEM: Standard error of the mean

activity are needed to figure out the various other chemical components responsible for the anti-nociceptive and also anti-inflammatory effects.

Conflict of Interest: No conflict of interest was declared by the authors.

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