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Evaluation Of Analgesic Potential Of Methanolic Extracts Of Paddy Clove Leaves In Swiss Albino Mice

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ABSTRACT

Ludwigia perennis L. (Paddy clove) is a weed of wet rice fields, banks of channels and diverse muddy places belonging to the family Onagraceae and that has not been investigated before. The present study aimed to explore the analgesic activity of *Ludwigia perennis* leaves methanolic extract of (LPLME) using acetic acid writhing reflex method formalin-induced paw licking, hot plate method and tail immersion test models. The involvement of opioid receptors in the analgesic mechanism was investigated using naloxone antagonism. Results demonstrated that LPLME exhibited a potent dose-dependent analgesic activity in all tested models for analgesia. The analgesic effect involved activation of opioid receptors in the central nervous system, where both spinal and supraspinal components might be involved.

Key Words: *Ludwigia perennis*, LPLME, Analgesic, Hot plate method.

INTRODUCTION

Despite recent discoveries in pain remedies, the medical community still required effective, safe and potent analgesic drugs for the treatment of variously painful situations mainly chronic pain.[1] A maximum number of medicinal plants in Indian are attributed to different pharmacological activities. Because it includes the different classes of phytochemicals. It is believed that recent analgesia inducing drug like opiates and non-steroidal anti-inflammatory drugs are not useful in all cases, because of their low potency and side effects.[2] Most of these herbal plants have been identified and reported by different authors for analgesic activity.[3-6] but the effect of several of these plants is yet to be scientifically evaluated and documented.

Based on these, therefore, there is the requirement for the search of bioactive compounds from various natural products mainly from medicinal plants for use as alternative analgesics with little or no adverse effect.

Paddy Clove is generally known as *ludwigia perennis* L. belonging to the family Onagraceae having various vernacular names like Bana-laung in hindi, Neerkarayambu in Malayalam, Kaere bandu gida in Kanada, Musalkathilai in Tamil, Lavangakaya mokka in Telugu and Perennial Water Primroses in English.

Fig.1: **Flower and whole plant of *Ludwigia perennis.***

Botanical description of *Ludwigia perennis.*

This yearly herb delivers an erect, many-branched plant. The leaves are on short stalks up to 5 centimetres in length and 2 cm. wide. They are lanceolate or straight lanceolate, beefy, shiny, light green on the upper surface and earthy green or violet on the underside. They develop on the other hand on a thick, rounded stem, which is generally light pink.

Ludwigia perennis blooms are little and yellow. The plant develops in moist spots, rice-fields and has been found in a couple of separated circumstances in Sri Lanka. This species has longer, lanceolate surrenders over to 34 cm. in length. It has white, light, gliding sacs (pneumatophores) which helping sterns to develop on the water surface.[7]

Table 1. Scientific Classification of *Ludwigia perennis.*

S.NO.	SCIENTIFIC CLASSIFICATION	
1	Kingdom	Plantae
2	Order	Myrtales
3	Family	Onagraceae
4	Subfamily	Ludwigioideae
5	Genus	Ludwigia
6	Species	*Ludwigia perennis*

MATERIALS AND METHODS

Collection and Certification of Plant Materials

Ludwigia perennis L. leaves having a place with the family Onagraceae were collected from Basna, dist. - Mahasamund, Chhattisgarh, India and was authenticated and identified and by M. Ahmedullah scientist 'E' from B.S.I. (Botanical Survey of India) Deccan Regional Center, Hyderabad-50048, Telangana. The plant species Number is ***BSI-DRC-2015-16-Tech.-664-06.***

Preparation of Plant Extract

The leaves of *Ludwigia perennis* were chopped into various small pieces with a knife and dried under a sunlight and pulverized into a coarse powder. The extraction was done by the Soxhlet apparatus. Later, the extract was filtered with Whatman filter papers and the filtrate concentrated to dryness in an oven at 40°C. The percentage yield was calculated using the formula below and the extract was stored in a refrigerator at 15°C until the time of use.

$$\% \text{ Yields} = \frac{\text{Weight of extract material}}{\text{Weight of original plant material used}} \times \frac{100}{1}$$

Selection of Animal Species

Albino Wistar mice weighing 21-30 g were selected for the activity. The chosen mice for dosing were 8-12 weeks matured and sufficiently. The protocol for toxicity is endorsed by Committee for the Purpose and Supervision on Experiments on Animals **(Registration number1534/PO/a/11/CPCSEA).** Every one of the animals was acclimatized to the research center condition for ten days before initiation of the investigations. The trial convention was endorsed by Institutional Animal Ethical Committee (IAEC) number - **IAEC/AU-COP/2017/14**, Anwarul Uloom College of Pharmacy, New Mallepally, Hyderabad-Telangana, India.

Feeding and Housing Conditions

Assuming to be 30 to 70% relative humidity and 22°C ± 3°C area of experiment conditional is required for the lab animals. 12 hours day and 12 hours night and suitable, comfortable conditions to be maintained. Healthy diet and water have to be supplied continuously. For adapting to the laboratory conditions animals should be kept in cages individually which helps us in animal identity before the study of the administration of the dose.

Acute Toxicity Studies

The intense harmfulness of methanolic concentrate of *Ludwigia perennis* was resolved according to OECD rule no. 420. Because of the cut off Value of the LD_{50}, the remedially compelling dosage was derived.[8]

Preparation of Doses

The extract can be administered at different dose levels according to animal body weight and the volume must not cross 1 ml / 100 gm b.w. The normal saline or extract should not go beyond 2 ml /100gm.

Animals Requirement & Dosing Level

At Least 3 animals need for the investigation and the principal measurements level most deliver some lethal sign within the animal. On dosage can be chosen out of our settled levels of measurement 5-50-300 & 2000 milligram/kg. bw. the point of confinement test ought to be performed when the mortality isn't affirmed even at high dosage. The interim can be deferred based on danger signs.

Observations

During the 30 minutes after dosing special and individual observation required up to 4 hrs. and continue by 14 days. The duration of time can be extending based on toxic effects and recovery period. All the observations were properly documented for each animal with special attention. The observations should not be limited to changes in the skin, mucous membrane, ANS, CNS, eyes and behavioural-wise. The observation should be intended for lethargy, tremors, convulsions, coma, diarrhea and salivation.[9]

Qualitative Phytochemical Screening

The obtained crude extract from the plant source is subjected to preliminary phytochemical screening. The identification test was performed for getting knowledge about various secondary and primary metabolites such as alkaloids, glycosides, mucilage, phenolic compounds, tannins, saponins, sugars, gum, sterols, and flavonoids.[10]

Determination of Analgesic Effect

Analgesic activity of ***Ludwigia perennis*** **leaf methanolic extract** (LPLME) was determined through the following models

1. **Acetic Acid Writhing Reflex Method**
2. **Formalin-Induced Paw Licking.**
3. **Hot Plate Method**
4. **Tail Immersion Test**

Acetic Acid Writhing Reflex Method

This study was carried out using the method of Danbisya and Lee.[11]25 albino mice of both sexes were randomly divided into five groups (1-5) of five mice each group. They have fasted for 12 hrs. and after that treated as follows: Group 1 mice were given tween 20 solution 10 ml/kg (negative control group), group 2 mice were given 400mg/kg acetylsalicylic acid (Aspirin) (positive control group) while groups 3, 4 and 5 received 200, 400, 800 mg/kg of LPLME respectively all by gastric gavage.

1 hour after administration of drug and LPLME, 0.7% glacial acetic acid (10 ml/kg) was given intraperitoneally (i.p) to all the mice to induce pain characterized by abdominal writhes or constrictions. The number of writhes observed in each mouse was counted for 30 minutes and recorded. The percentage protection against abdominal writhing was used to assess the degree of analgesia and was calculated using the formula.

$$\% \text{ Protection} = \frac{\text{Mean Control} - \text{Mean Treated Groups}}{\text{Mean of Control Groups}} \times 100$$

Formalin-Induced Paw Licking

The paw licking phases were divided into two parts, the early phase (0 to 5 minutes) and the late phase (15 to 30 minutes). In the early phase, there was a reduction in the number of episodes of licking, but there was no significant difference between the control and any of the treatment groups. Twenty µ l of 2.5% formalin solution made in saline was injected intra planetary under the surface of the right hind paw.[14] After intraplantar injection of formalin, the rats were immediately placed in a glass cylinder, 20 cm in diameter and the time spent in licking the injected paw was monitored and recorded throughout 0-5 min (early phase of licking) and 15–30 min (late phase of licking). The animals were treated as Group 1 received tween 20 solutions (negative control), group 2 received 400mg/kg Aspirin and 200, 400, 800 mg/kg of LPLME for groups 3, 4 and 5 respectively.

Hot Plate Method

The Hot Plate Method explained by Franzotti.[13] was used for the study. Albino mice of both sexes were randomly categorized into 5 groups of five mice each, fasted for 12 hours with adequate clean water provided *ad libitum*.

Each of the mice was placed on a hot-plate maintained at the temperature of 55°C and the latency period examined with a stopwatch was recorded which represents the time taken for the mice to react against pain stimulus. The response to pain included; licking of the hindfoot, jumping, and raising. The cut off time was fixed for 20 sec. This was noted as control pain reaction time. The mice were then treated as follows: Group 1 received tween 20 solutions (negative control), group 2 received 400mg/kg Aspirin and 200, 400, 800 mg/kg of LPLME for groups 3, 4 and 5 respectively.

Tail Immersion Test

The method explains by Uma-Devi [12] was used for Tail Immersion Test. 25 albino mice were randomly divided into 5 groups with five mice each, all fasted for 12 hours with clean drinking water provided *ad libitum.* The animals were pre-treated 60 minutes before tail immersion with 10ml/kg tween 20 solution for group 1 (negative control), 400 mg/kg acetylsalicylate acid (aspirin) for group 2 (positive control) and 200, 400, 800 mg/kg of LPLME for groups 3, 4 and 5 respectively. Then about 2 to 3cm. of the tail of every one of the mice was dipped under a water bath containing warm water maintained at a temperature of 50°C and the time taken for the mice to flick the tail is known as the pain reaction time (PRT) and it was noted for every mice. The cut off time was put at 15 sec.

Statistical Analysis

The results are expressed as mean ± SEM. The Dunnet *t*-test was used to evaluate the significance of the difference between treated and control groups. $P < 0.05$ was considered statistically significant

RESULTS

Plant Extract

The yield of leaves of *Ludwigia perennis* extract was 5.02±1.26 w/w dry matter and was dark in colour (Table 2).

Table 2. Extractive values of *Ludwigia perennis.*

Extractive values		
1	Water soluble extractive value (% w/w)	08.22±1.12
2	Alcohol soluble extractive value (% w/w)	5.02±1.26

Acute Toxicity Study of LPLME

For the plant, *Ludwigia perennis* methanolic extract the lethal (LD_{50}) dose was estimated. The acute toxicity study was conducted

by OECD rules No.420. The extract did not create any dangerous manifestations of mortality up to the level of dosage 2000mg./k.g. body weight in the experimental animal and consequently, it was viewed as the cut-off dose. The 200 mg./k.g., 400 mg/k.g. and 800 mg./k.g. dose was taken for experiments.

Qualitative Phytochemical Screening

The qualitative compound tests were completed for the recognizable proof of the distinctive idea of phytoconstituents display in the unrefined drug of *Ludwigia perennis* by standard procedure. They are generally tried for the nearness of flavonoids, alkaloids, phenols, cardiac glycosides, tannins, steroids triterpenes, and saponins. The outcomes have appeared in Table 3.

Table 3. Phytochemical Screening of Different Extracts of *Ludwigia Perennis* Leaves.

Tested Group	Ether Extract	Chloroform Extract	Methanol Extract	Aqueous Extract
Alkaloids	---	---	+++	+++
Glycosides	---	---	+++	---
Phenolic compound	---	---	+++	+++
Steroids & Sterols	---	---	---	+++
Saponins	---	---	---	+++
Flavones & Flavonoids	+++	---	+++	+++
Proteins, Amino acids	---	---	---	+++
Carbohydrates	---	+++	+++	+++
Tannins	---	---	+++	+++

Note: (+++) Present (---) Absent

Analgesic Activity

1. Acetic Acid Writhing Reflex Method

The effect of LPLME on the acetic acid-induced abdominal constrictions in mice is presented in Table 4. The result shows that the extract (200, 400, 800 mg/kg), and the reference drug aspirin (400 mg/kg) significantly ($P < 0.0001$) reduced abdominal writhing in mice when compared to the negative control group reducing the mean number of writhing from 30.6 ± 7.96 in the negative group to 8.0 ± 4.13 at the dose of 800 mg/kg. The reduction was in a dose-dependent manner. The extract caused a dose-dependent increase in inhibition of abdominal writhing, increasing it from 0% in the negative control group to 70% at the dose of 800 mg/kg. Furthermore, posthoc analysis did not detect any significant difference between the extract at the doses of 200 versus reference drug (aspirin) and control. Also, no significant differences occurred between 400mg/kg versus reference drug and 800mg/kg versus reference drug.

Table 4. Effect of Methanolic Extract of *Ludwigia perennis* on Acetic Acid-Induced Writhing Reflex in Mice.

Group	Treatment mg/kg.	Mean Number of Writhing	% Protection
1	Tween 20 Solution	30.6 ± 7.96	0
2	Aspirin (400)	15.87 ± 7.49	47
3	LPLME 200	27.0 ± 6.57	7
4	LPLME 400	11.3 ± 8.14	58
5	LPLME 800	8.0 ± 4.13	70

2. Formalin-Induced Paw Licking.

The time spent in licking the injected paw with formalin (early phase) was 48.41±7.06 and 61.99±4.52, respectively, in the control animal. LPLME did not reduce the time spent in licking the injected paw in both the tests. However, morphine reduced the duration of licking in capsaicin-induced neurogenic pain. Paw licking time was not significantly reduced by Aspirin in the early phase of the formalin test in animals (Table 7).

The paw licking phases were divided into two parts, the early phase (0 to 5 minutes) and the late phase (15 to 30 minutes). In the early phase, there was the reduction in the number of episodes of licking, but there was no significant difference between the control and any of the treatment groups. In the second phase, LPLME showed a significant decrease in paw licking. The percent inhibition of licking was 92.12, which was lower than the group treated with standard drug aspirin, having a percent inhibition of 87.7. The groups treated with LPLME-200 and LPLME-400 also showed a significant reduction in paw licking.

Table 7. Effect of Methanolic Extract of *Ludwigia Perennis* on Formalin-Induced Paw Licking Behaviour In Rats

Group	Treatment mg/kg.	Time spends in licking formalin injected paw	
		Early phase	Late phase
1	Control	-	-
2	Aspirin (400)	48.41±7.06	61.99±4.52
3	LPLME 200	57.40±3.30	135.11±18.17
4	LPLME 400	51.12±5.75	78.71±12.01
5	LPLME 800	49.91±4.60	69.19±4.10

*p<0.05 versus control (n=6)

3. Hot Plate Method

The result of the effect of LPLME on the hot plate method is shown in Table 5. The result shows that there was no significant difference in the PRT during the pre-drug testing time. After drug and extract administration, comparing the pre-and post-drug PRT using T-test showed that the reference drug aspirin at dose 400 mg/kg and the extract at the doses of 400 and 800 mg/kg significantly (P = 0.048) increased the PRT. The extract at the dose of 200 mg/kg did not show any significant increase in the mean PRT.

Table 5. Effect of Methanolic Extract of *Ludwigia perennis* on Hot Plate Induced Pain in Mice

Group	Treatment mg/kg.	Mean pre-drug reaction time ± S.E.M. (Second)	Mean post drug reaction time ± S.E.M. (Second)
1	Tween 20 Solution	2.33 ± 0.16	2.12 ± 0.57
2	Aspirin (400)	2.54 ± 0.59	4.08 ± 0.77
3	LPLME 200	2.32 ± 0.17	3.08 ± 0.33
4	LPLME 400	1.62 ± 0.44	3.22 ± 0.48
5	LPLME 800	1.36 ± 0.13	4.55 ± 1.15

4. Tail Immersion Test

The result of the tail immersion test in mice is presented in Table 6. The result shows that the extract at the dose of 800mg/kg and the reference drug aspirin significantly (P = 0.03 and P = 0.02 respectively) increased the PRT when compared to the negative group (group 1). At the doses of 200 and 400 mg/kg, the extract did not show any significant increase in PRT, although there was a small increase in the mean PRT. (P =0.28 and 0.42 respectively).

Table 6. Effect of Methanolic Extract of *Ludwigia perennis* on Tail Flick Response in Mice

Group	Treatment mg/kg.	Mean pain ± S.E.M. (Second)
1	Tween 20 Solution	2.08 ± 0.46
2	Aspirin (400)	4.90 ± 1.19
3	LPLME 200	3.06 ± 0.18
4	LPLME 400	3.85 ± 0.44
5	LPLME 800	5.87 ± 2.33

DISCUSSION

Total four analgesic models; Acetic acid-induced writhing reflex, Tail immersion, Hot plate method and Formalin-Induced Paw Licking models were used to evaluate the analgesic activity of *Ludwigia perennis.* The tests of analgesic drugs commonly measure nociception and involve the reaction of animals to painful stimuli.[15] The stimulus may be chemical (acetic acid-induced writhing or formalin tests) or thermal (tail immersion or hot plate tests).[16]

The methanolic leaves extract of *Ludwigia perennis* produced no death or signs of toxicity even at the dose of 2000 mg/kg which suggests that the extract was well tolerated by the mice and that the doses used were safe.

The analgesic effect of *Ludwigia perennis* was tested by an acetic acid-induced writhing test in selected animals (mice). Aspirin was used as a standard drug, because it offers relief from inflammatory pain, by inhibiting the formation of pain mediators in the peripheral tissues, in that bradykinins and prostaglandins are said to play a significant role in the pain process. Acetic acid-induced writhing model is the main test for pain sensitivity to opiates as well as to non-opiate analgesics. [17-18] The associated nociceptive response is believed to involve the release of endogenous substances such as bradykinin and prostanoids, among others, which stimulate the nociceptive endings. [19] LPLME has shown good analgesic activity in the acetic acid-induced writhing model and has produced a significant decrease in the writhing counts. Formalin-induced nociception allows evaluation of anti-nociception by NSAIDs and opioids.[20] Furthermore, it does not generate conditioned learning and has the higher prediction of anti-nociception.

Formalin pain is biphasic, an early neurogenic component, followed by the late tissue-mediated response.[21] The early response is believed to represent the direct effect of formalin on pain fibers particularly, C fibers causing the release of bradykinin and tachykinin. The late phase is due to inflammatory reactions caused by prostaglandin, bradykinin and excitatory amino acids.[22] Opioid analgesics seem to be antinociceptive for both the phases. In contrast NSAIDs such as indomethacin seem to suppress only the second phase.[23]

To test the antinociception by LPLME against formalin-induced pain is particularly relevant, as most of the NSAIDs tested, were reported to be maximum ineffective to prevent neurogenic pain response increased by formalin. The analgesic action of the extract may be due to its ability to inhibit cyclooxygenase, as in the case of NSAIDs. It is well known that NSAIDs are

largely ineffective or cause weak inhibition against both models.[24] Besides, NSAIDs can attenuate the second phase of formalin-induced licking in a dose-dependent manner.[25] In the formalin test, CBFE significantly reduced the late phase of formalin-induced nociception. These results suggest that the extract resembles mild analgesics such as, paracetamol and aspirin and induces specific modulation of tonic pain, compared with the phasic pain.[26]

The Hot plate and Tail immersion models have been used to evaluate centrally acting analgesics.[27] In Hot plate and Tail immersion models, sensory nerves sensitize the nociceptors and the involvement of endogenous substances such as prostaglandins are minimized.[28] From the results, though the LPLME showed analgesic actions in the tail immersion and hot plate models, it was not as pronounced as was seen in the acetic acid-induced model and this may suggest that the analgesic activity of LPLME may not be fully mediated through a central mechanism.

CONCLUSION

The Methanolic Extract of *Ludwigia perennis* showed significant analgesic activity and may be acting through inhibition of prostaglandin pathway and or through peripheral pain mechanism. However, more work is required in the isolation and characterization of the bioactive compound(s) and determination of the *Ludwigia perennis* extract mechanism of action.

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