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Original Article

HRLC-MS Metabolite Profiling and Evaluation of Analgesic and Anti-inflammatory Activity of *Ficus nervosa* Heyne ex Roth

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ABSTRACT

Background: Medicinal plants are one of the major sources for the development of new drugs, which creates the interest on searching biologically active molecules from the medicinal plants. Medicinal plants have been used as folkloric medicine for decades. The medicinal plant *Ficus nervosa* used in the Eastern Ghats for various health care preparations. However, the plant has not been scientifically validated with concern to the pharmacological aspects to undertake the current study. Objective: The study aimed to identification of phytoconstituents from *Ficus nervosa* leaves extract and exploring the analgesic and anti-inflammatory effects. **Materials and Methods:** The phytoconstituents were analysis through LCMS/MS method. Further, extract activity was evaluated using tail flick, hot plate and acetic acid-induced writhing methods. **Results:** The study identified top 20 compounds (10 from each +ve and -ve mode analysis). The extract found to be safe up to 2000 mg/kg and there were no mortalities were observed. In contrast, the NM extract showed anti-inflammatory effect at the dose of 500 mg/kg compared to reference compound. The NM dose 500 mg/kg showed a significant analgesic effect with a maximum latency response and inhibition of acetic acid-induced writhing. **Conclusion:** These study findings suggest that *Ficus nervosa* may have potential analgesic and anti-inflammatory activity meriting further biological evaluations.

Keywords: Acetic acid induced writhing response, Carrageenan-induced paw edema method, *Ficus nervosa* leaves, Hot plate, Tail flick.

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INTRODUCTION

The pain is unpleasant sensation and emotional experience brough on by actual or probable tissue injury or something that equal of such complication. The serves as a valuable mechanism for promoting healing, but it compels the sufferer to rest the injured area and seek medical attention.[1] More than 30% of people influenced by pain and inflammation which are the most difficult and increasing health complications in the worldwide.[2] These common symptoms of various medical conditions, injuries and diseases. Inflammation is characterized by swelling, redness, heat and pain in the affected area.[3] Anti-inflammatory drugs suppress the inflammatory response, alleviating symptoms and promoting healing (Figure 1). The importance of analgesic and anti-inflammatory agents cannot be overstated due to their significant roles in healthcare and patient well-being.[4-6]

The medicinal plants have been used in traditional medicines for many decades for primary healthcare treatments.[7] These plants are medicinally advantageous for the management of various diseases due their chemical composition. World health Organization reported that 80% of people in the developing countries depended on traditional medicines for their primary health care needs.[8] This is due to availability and affordability of the herbal medicines and also lack of modern medical facilities. The plant *Ficus nervosa* (Family: Moraceae), commonly known as the "shiny-leaved fig" or "Java fig." It is characterized by its glossy, dark green leaves with prominent veins and its small, round fig fruits that turn from green to purple-black when ripe (Figure 2). *Ficus nervosa* is a species of fig tree that includes other well-known species like the common fig (*Ficus carica*) native to Southeast Asia, mainly found in countries like India, Indonesia, Malaysia and the Philippines. The various parts of plant *Ficus nervosa* for traditional medicinal purposes, although specific practices and uses may vary between regions.[9,10] It is reported to have various pharmacological activities like anti-oxidant, anti-inflammatory, cytotoxicity, anti-bacterial, antimalaria and antidiabetic activities.[11,12] The plant extracts were reported to have followed chemical constituents are alkaloids, flavonoids,

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coumarins, lignans, phenylpropanoids, chromones and terpenoids.[12] Based on the reports of plant activity on pain and inflammation complications, the present study is aimed to evaluate phytochemical which may be responsible for the management of the analgesic and anti-inflammatory properties.

MATERIALS AND METHODS

Plant material and chemicals

Ficus nervosa leaves were collected from Rajahmundry region, Andhra Pradesh, India. Further plant materials were authenticated by Prof. L. Rasingam, Head of the Department, Ministry of Environment, Forest and climate change, Botanical Survey of India, Deccan regional center, Hyderabad, Telangana. The plant specimen was deposited (BSI/DRC/2021-22/Tech./402) for future reference. All of the laboratory-grade chemicals, reagents and standard medications were procured from Sigma Aldrich.

Preparation of extracts

The plant leaves were shade dried and made into coarse powder form using mechanical grinder. The powdered leaves were extracted by Soxhlet extraction method using various solvents hexane, chloroform and methanol at 40-60°C for 12 hr. The extracts were concentrated using a rotary evaporator (Buchi, India).[13] Further extracts were dried then transferred into desiccators to prevent the moisture. The extracts yields were calculated using formula, % yield=(obtained extract/powdered plant material)×100. The obtained extracts *Ficus nervosa* Hexane (NH) extract, *Ficus nervosa* Chloroform (NC) extract and *Ficus nervosa* Methanol (NM) extracts were stored at room temperature.

Phytochemical Screening by HRLC-MS

The methanolic extract was used for further HR-LCMS (LC-ESI-Q-TOF-MS, Agilent Technologies G6550A) analysis for the identification of phytoconstituents. The experiment was carried out at the Sophisticated Analytical Instrument Facility (SAIF), Indian Institute of Technology, Bombay. The gradient elusion method was used by utilizing the solvents are 0.1% formic acid in water (A) and acetonitrile (B). The flow rate was set to 0.3 ml/min. The gradient system started with 95:5% A:B, reaching 5:95% A:B in 30 min, then back to the initial composition in 15 min, which was held at the same composition for 2 min. The MS analysis was carried out both ESI-positive and ESI-negative ionization mode.[14,15] The obtained data was matched with various libraries to identify the eluted compounds.

Animals and Housing

The Swiss albino mice's (15-35 g) and Sprague-Dawley rats (120-150 g) were procured from the VAB Biosciences, Hyderabad. The animals were randomly selected and housed individually in polycarbonate cages with stainless steel and sterilized corn cob bedding. The ambient temperature (22±3°C) was maintained with a relative humidity range of 30 to 70%. Animals were housed in a 12-hr cycle of light and darkness. The food standard pellets and water were provided ad libitum to laboratory animals.[16] The animal experiments were approved (Ref. no. IAEC/GIP-1287/CS-F/Approved/14/Dec2021) for conduction of experiments as per Institutional Animal Ethics Committee (IAEC), GITAM Deemed to be University, Visakhapatnam.

Evaluation of Analgesic and anti-inflammatory activity

Tail Flick Method

The antinociceptive activity of mice was investigated using the tail-flick technique. A radiant heat automatic tail flick analgesiometer was used to evaluate reaction latencies. The basal reaction time of an animal to radiant heat was determined by placing the last 1-2 cm of the animal's tail on the source of radiant heat. Eliminating the tail from the radiant heat was referred to as the endpoint. The 15 sec threshold was implemented to prevent heat-related tail damage. Twelve groups of mice (n=5) were formed. One group was treated with the standard drug Morphine (8 mg/kg) as a positive control, one group with saline solution acted as a negative control and the other groups with extracts (NC and NM) administered to mice. The latent time of the tail-flick reaction was observed at 30, 45, 60, 75 and 90 min after the sample administration.[17]

Hot plate method in rats

The hot plate was used to check the analgesic activity of extract by calculating animal response to the pain.[18] The device temperature was adjusted between 55 and 56°C. When the temperature rises, rat starts leap, jerk and lick due their paws are heat-sensitive. The stopwatch was used to measures time of animals remain on the hot plate before jumping or beginning to lick. Total five SD rats were kept in each group to assess the analgesic activity. The test substances NC (250 and 500 mg/kg) and NM (250 and 500 mg/kg) and standard drug Pethidine (4 mg/kg) were delivered orally. Further, animals were kept on the hot plate for 60, 90, 120 and 180 min and the results were recorded.

Acetic acid-induced writhing in mice

The method writhing in mice model was also performed to assess the analgesic activity. The writing involves stretching the back legs, clenching one side, contracting the abdominal muscles and twisting the trunk. Any type of writhing is considered a positive response.

The mice were divided into seven groups and each group consists five albino mice were placed with the to assess the analgesic activity. The acetic acid (1% v/v) solution was prepared using distilled water. A diclofenac solution (10 mg/kg) was made using normal saline water. The food was withheld for 12 hr. prior to

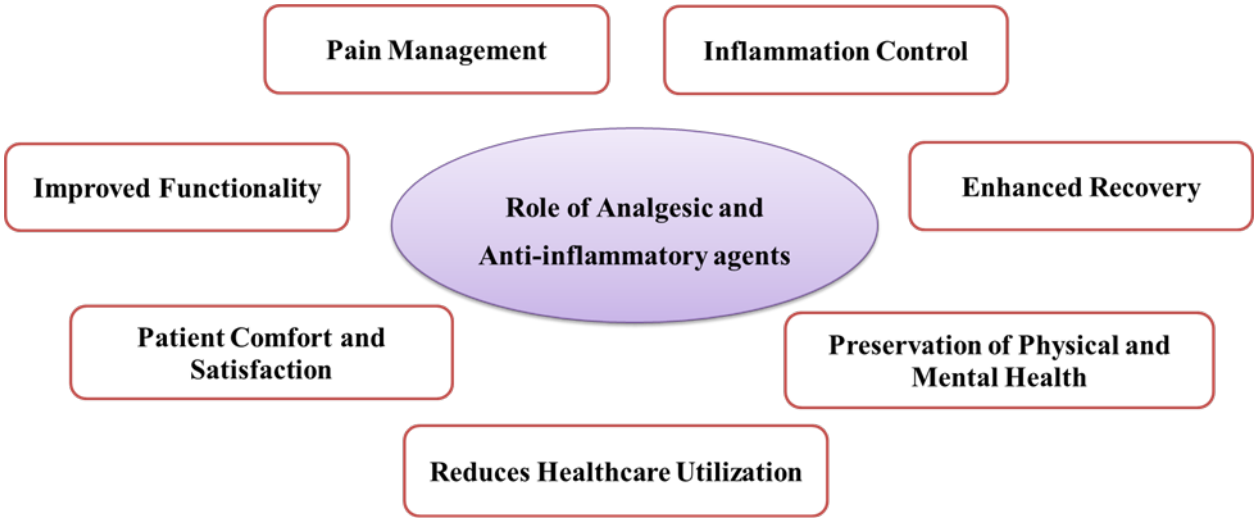

Figure 1: Role of analgesic and anti-inflammatory agents.

Figure 2: *Ficus nervosa* leaves.

the commencement of the drug administration and until the conclusion of the trial. The standard drug (diclofenac sodium) and test extracts (NC and NM) were administered orally (500 mg/kg, p.o.). After 60 min, writhing was induced using an intraperitoneal (i.p.) injection of 1% acetic acid in 0.1 ml/10g body weight. The writhing response was recorded for 30 min and stretching gestures such as back arches, torso lengthening and hind limb extensions were counted.[19,20] The calculations were done using formula, % inhibition= (NW_C- NW_T/ NW_C)×100. Where, NW_C =Number of writhes in the control group, NW_T =Number of writhes in the test group.

Carrageenan-induced paw edema

The rats were made to experience pedal inflammation using carrageenan induced paw edema model. The rats were divided into four groups and each group containing five animals. In contrast, the control and reference groups were given saline and indomethacin (5 mg/kg, p.o.) respectively. The other two groups were received 500 mg/kg, p.o. dose of NC and NM respectively. Each animal received an intraplantar injection of 0.1 mL of 1% carrageenan under the sub plantar aponeurosis in the left hind paw one hour after extracts, indomethacin, or saline were administered. Paw size was measured by encircling the paw with a length of cotton thread and then measuring the circumference using the meter rule. Paw measurements were taken before and 1-4 hr after the carrageenan injection.[21,22] The edema inhibitory activity was determined using, % inhibition=(Ct-Co) Control-(Ct-Co) treated/(Ct-Co) Control x100. Where, Ct=paw circumference at time t, Co=paw circumference before carrageenan injection and Ct - Co=edema.

RESULTS

Extraction of plant material

The extracts were prepared using solvents hexane, chloroform and methanol by Soxhlet extraction method. The obtained extractive yields for NH, NC and NM are 0.8% w/w, 1.2% w/w, 6.82% w/w respectively.

Table 1: **Phytochemical screening of *F. nervosa* leaves extracts.**

Phyto-constituents	NH	NC	NM
Alkaloids	-Ve	+Ve	+Ve
Glycosides	-Ve	-Ve	+Ve
Tannins	+Ve	+Ve	+Ve
Saponins	-Ve	-Ve	-Ve
Flavonoids	-Ve	+Ve	+Ve
Phytosterols	+Ve	+Ve	+Ve
Carbohydrates	+Ve	+Ve	+Ve
Volatile Oils	-Ve	-Ve	-Ve
Proteins	-Ve	-Ve	-Ve

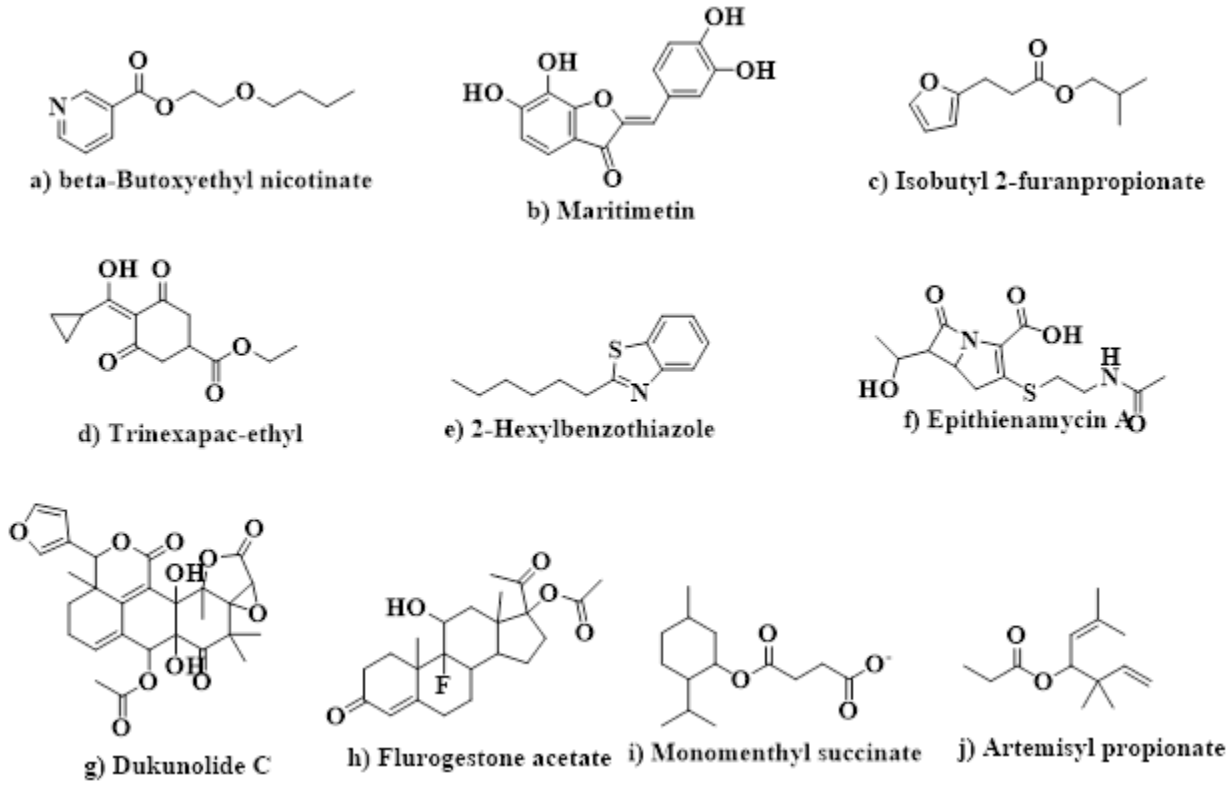

Figure 3: HR-LCMS analysis of *F.nervosa* methanol extract at positive ESI.

Preliminary Phytochemical Screening

Preliminary tests are conducted to detect the presence of major classes of phytochemicals. The extracts FH, FC and FM showed tannins, phytosterols and carbohydrates. Further NC and NM showed some additional constituents are alkaloids and flavonoids (Table 1).

Identification of Bioactive Compounds by HR-LCMS

The phytochemical profiling was performed through HR-LCMS method of NM and compound were identified by using various natural products database libraries. The HR-LCMS analysis shown multiple set of compounds although most accuracy and major 10 compounds from each analysis was listed and chemical structures (Figures 3 and 4) were presented. The top 10 compounds from each analysis may responsible for its anti-inflammatory activity.

Tail Flick Method

The NC and NM extracts were showed analgesic activity at dose of 250 mg/kg and 500 mg/kg in dose dependent manner. The dose 500 mg/kg of both NC and NM exhibited comparable analgesic activity towards morphine 8 mg/kg (Figure 5). When compared to normal control, extract treated rats showed significant difference it indicates the extracts at 500 mg/kg able to reduce the pain in the rats. Further, NM extract 500 mg/kg showed more promising results as compared to the NC extract 500 mg/kg. Which clears the NM extract is more potential compared to the NC extract.

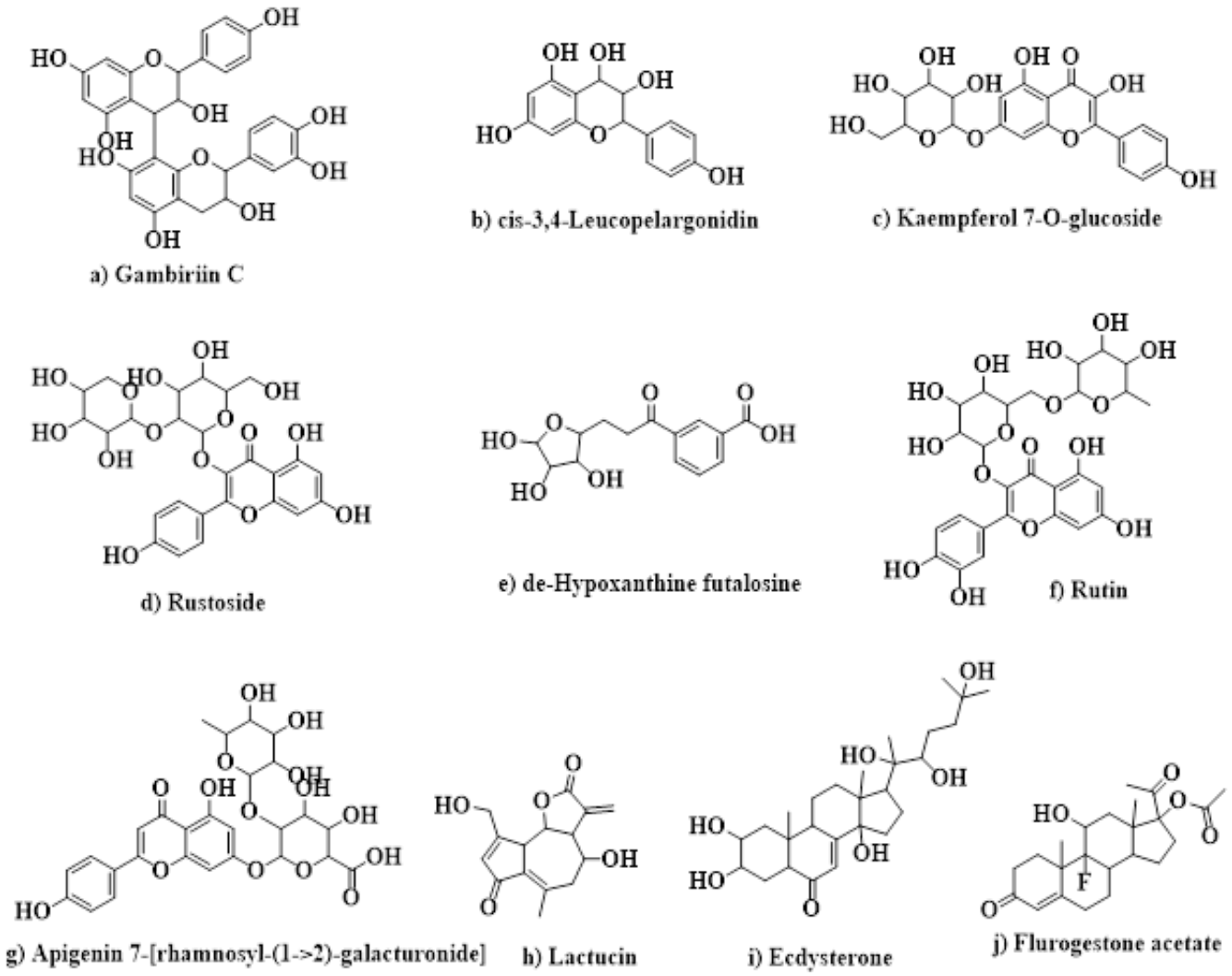

Figure 4: HR-LCMS analysis of *F. nervosa* methanol extract at negative ESI.

Hot plate method

The NC and NM extracts were showed latency period is more at dose of 250 mg/kg and 500 mg/kg in dose dependent manner (Figure 6). The dose 500 mg/kg of both NC and NM exhibited comparable analgesic activity towards pethidine 4 mg/kg. when compared to normal control, treatment group animals were showed more latency time which indicates the analgesic activity of NC and NM extracts at 500 mg/kg. All the extracts and standard drugs significantly (p<0.01) increase the paw withdrawal from the hot plate, indicating analgesic activity.

Acetic acid-induced writhing method

The extracts NC (500 mg/kg) and NM (500 mg/kg) significantly (p<0.01) reduce the number of abdominal constrictions and stretching of the hind limb induced by acetic acid injection as compared to the standard drug diclofenac sodium. The results suggest both NC and NM extracts are effective at a dose of 500 mg/kg and specifically NM extract showed more promising results (Figure 7).

Carrageenan-induced paw edema

The extracts NC and NM at a dose of 500 mg/kg showed least paw edema and results were significant (p<0.01) as compared to standard drug indomethacin 5 mg/kg (Figure 8). The results indicate the NC and NM extracts at a dose of 500 mg/kg able to reduce the edema which clears that extracts have anti-inflammatory activity.

DISCUSSION

The extraction and evaluation of bioactive compounds from plant materials such as NC (chloroform extract) and NM (methanol extract) are pivotal in discovering therapeutic agents with potent analgesic and anti-inflammatory properties. Through this study, we employed Soxhlet extraction using hexane, chloroform and methanol to obtain NH, NC and NM extracts with yields of 0.8%, 1.2% and 6.82% w/w, respectively. The findings indicate that the polarity of methanol contributed to its high extractive yield, potentially due to its enhanced solubility for a broader range of phytochemical constituents.

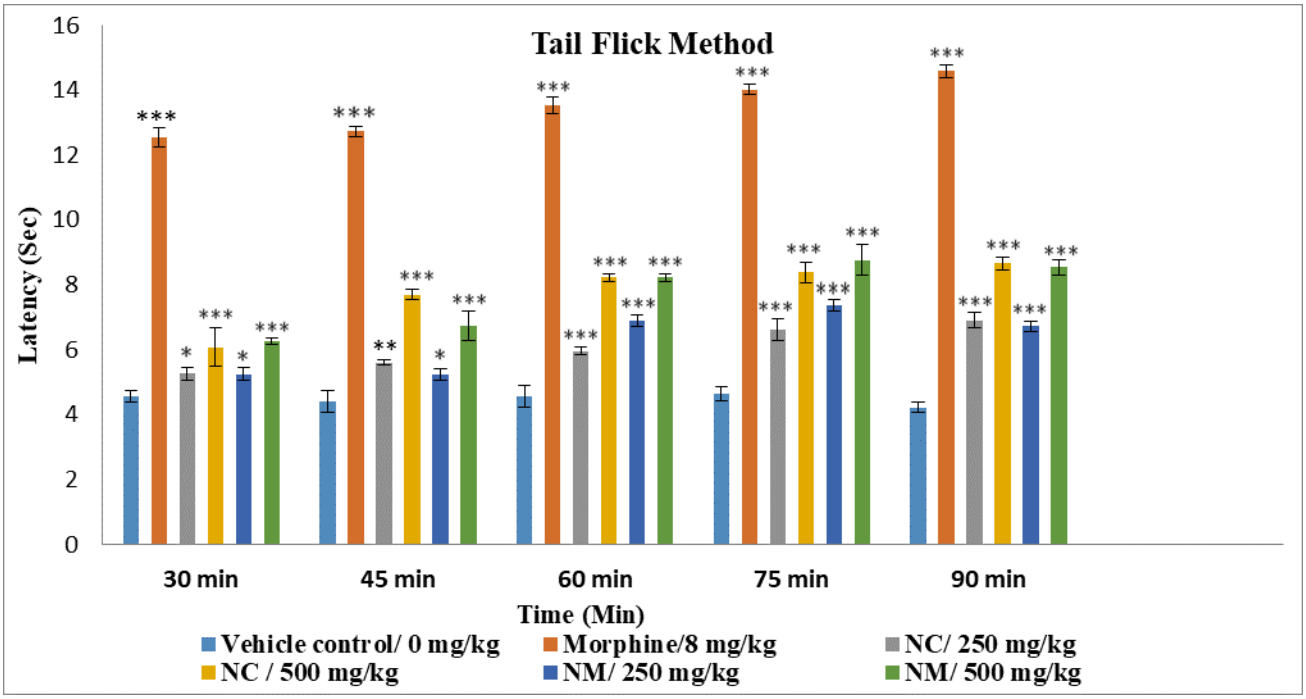

Figure 5: Tail flicking (reaction time) after administration of the extracts compared with control and standard drug. The result significantly differs from control group ($p<0.05$).

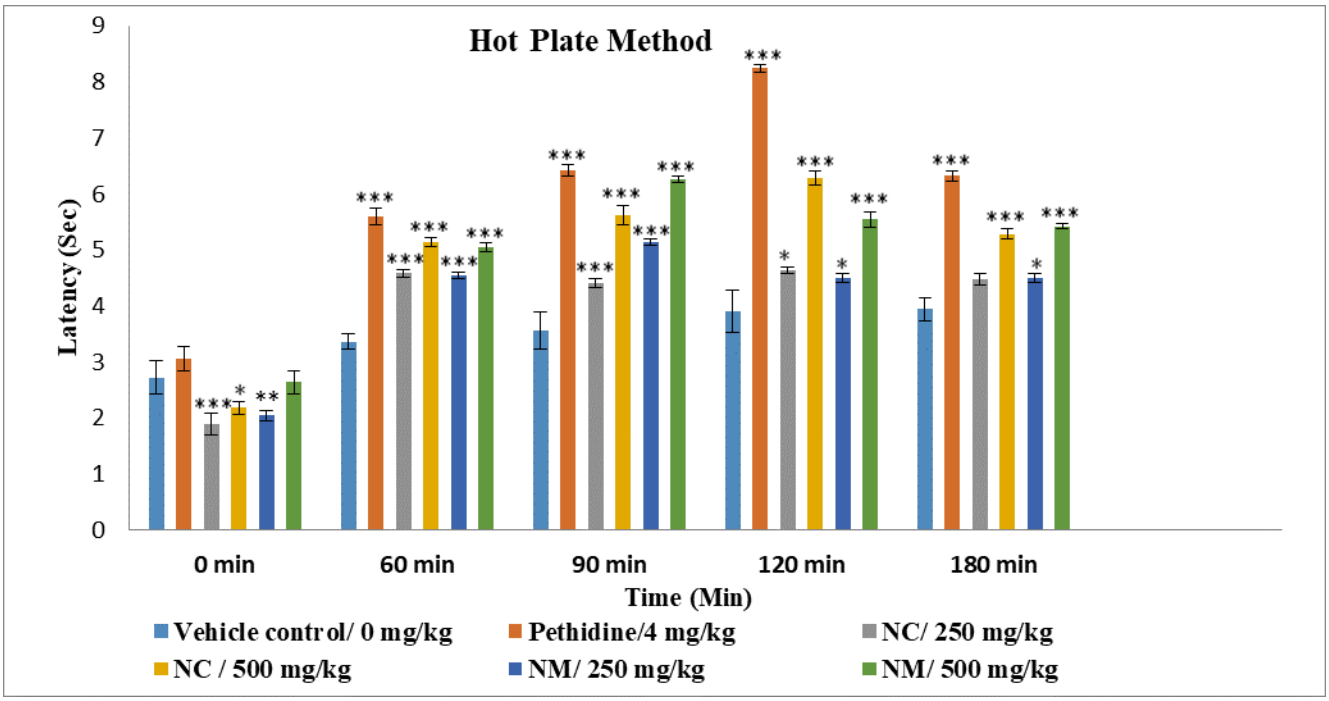

Figure 6: Result of reaction time after administration of the extracts compared with control and standard drug. Each value is the mean±SEM for 5 Rats, with a $p<0.01$ compared to the control. One-way ANOVA was used to analyze the data and then Tukey's Multiple Comparison Test was performed.

Preliminary phytochemical screening provided a foundational understanding of the bioactive profiles of the various extracts. The presence of tannins, phytosterols and carbohydrates across all extracts (FH, FC and FM) suggests that these compounds contribute to the basic pharmacological properties of the extracts. However, NC and NM were unique in containing alkaloids and flavonoids, known for their diverse bioactivities including anti-inflammatory, analgesic and neuroprotective properties. These findings align with existing literature that links flavonoids and alkaloids to pain modulation and anti-inflammatory actions, indicating that NC and NM extracts may have notable therapeutic potential.

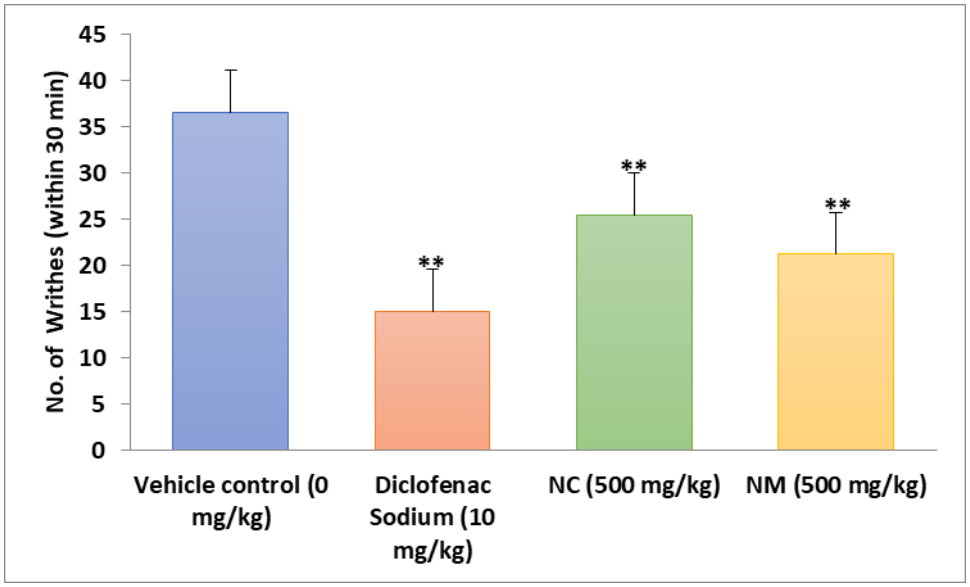

Figure 7: Acetic acid-induced writhing response of the four extracts compare to standard and control group. Each value is the mean±SEM for 5 Mice, $p<0.01$ compared with control. The data were analyzed using Tukey's Multiple Comparison tests and one-way Analysis of Variance (ANOVA).

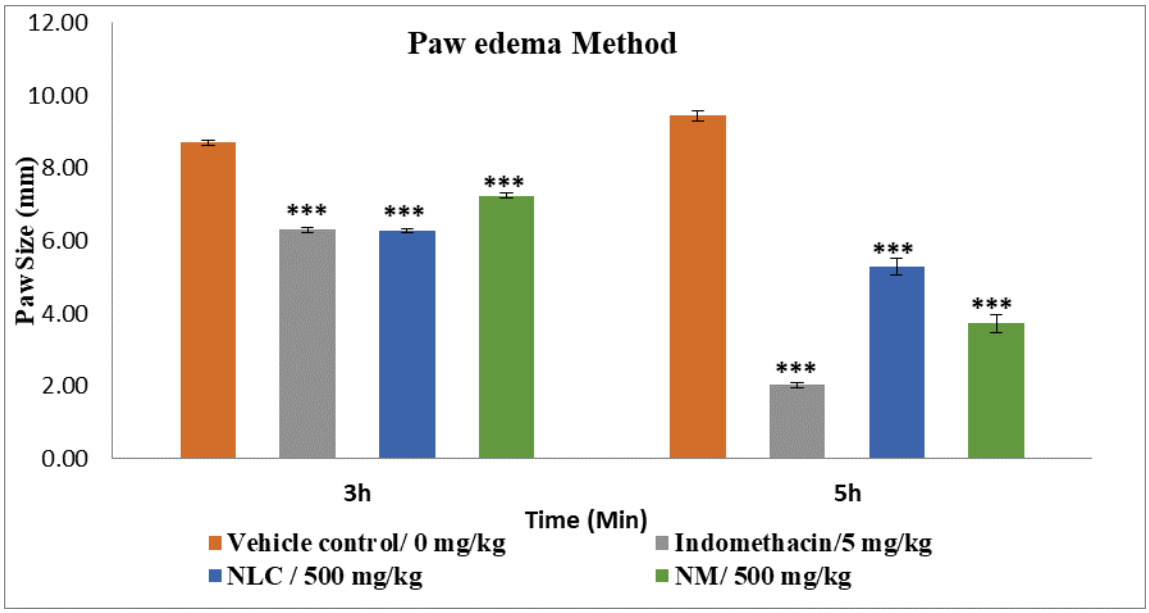

Figure 8: Percentage inhibition of the extracts and standard drug. Each value is mean±SEM for five rats, with a $p<0.01$ when compared to the control. The data were analyzed using Tukey's Multiple Comparison tests and one-way Analysis of Variance (ANOVA).

The phytochemical profiling of NM extract via High-Resolution Liquid Chromatography-Mass Spectrometry (HR-LCMS) led to the identification of a diverse array of compounds. Among these, ten major bioactive compounds were identified, whose structural elucidation was performed using natural product database libraries. This profiling is crucial, as it pinpoints specific compounds that might play a key role in the observed anti-inflammatory and analgesic activities. Identifying bioactive components helps establish a link between the phytochemical composition and therapeutic effects, especially as the presence of multiple bioactive compounds supports a synergistic mechanism of action.

The analgesic activities of NC and NM were evaluated using the tail flick and hot plate methods at doses of 250 mg/kg and 500 mg/kg. The dose-dependent response observed in both methods demonstrates that as the dosage increased, so did the analgesic effect, with NM showing a more significant response than NC at 500 mg/kg. In both assays, the 500 mg/kg dose exhibited comparable analgesic effects to the reference drugs (morphine in the tail flick method and pethidine in the hot plate method). This suggests that the bioactive constituents within NM and NC interact with nociceptive pathways, potentially involving opioid receptor modulation or Central Nervous System (CNS) activity that leads to delayed pain perception.

The hot plate method specifically measures the reaction to thermal stimuli, highlighting the extracts' potential for central analgesic effects. The significant increase in latency time in treated animals compared to control indicates that both NC and NM extracts can effectively increase pain thresholds. This aligns with the pharmacological profiles of alkaloids and flavonoids, which are known to exert CNS effects, further validating the analgesic efficacy of these extracts. NM's more pronounced effect compared to NC might be attributed to a higher concentration of alkaloids and flavonoids, reinforcing its superior analgesic potential at the 500 mg/kg dose.

The acetic acid-induced writhing test further supports the analgesic potential of NC and NM. This method assesses the peripheral analgesic effect, where a reduction in the number of writhes indicates the ability of the extract to inhibit prostaglandin synthesis, a key mechanism in peripheral pain modulation. Both extracts at a dose of 500 mg/kg significantly reduced the writhing response, with NM demonstrating a stronger effect than NC. This aligns with the theory that alkaloids and flavonoids, prevalent in NM, may interact with inflammatory mediators like Cyclooxygenase (COX) enzymes, which contribute to pain and inflammation. The significant reduction in abdominal constrictions and stretching suggests that the NM extract effectively interferes with the pathways mediating peripheral pain, reinforcing its analgesic properties.

The carrageenan-induced paw edema model is a classic method to evaluate anti-inflammatory potential, where the reduction in paw swelling is indicative of the extract's capacity to mitigate acute inflammation. In this study, both NC and NM extracts at 500 mg/kg showed a significant reduction in paw edema, comparable to the standard drug indomethacin (5 mg/kg). The anti-inflammatory activity observed may be due to the inhibition of pro-inflammatory mediators, such as histamines, prostaglandins and cytokines, commonly released during carrageenan-induced inflammation. The presence of alkaloids and flavonoids in NM, known for their antioxidant and anti-inflammatory properties, likely contributes to its enhanced anti-inflammatory efficacy. Alkaloids can inhibit COX and Lipoxygenase (LOX) pathways, while flavonoids may scavenge free radicals and inhibit pro-inflammatory cytokine release.

CONCLUSION

The *F. nervosa* leaf extracts (NC and NM) can decrease central pain and peripheral analgesic action. Additionally, it demonstrated anti-inflammatory properties during both the acute and sub-acute stages of inflammation. According to these results, several endogenous inflammatory mediators and mediators of pain transmission may have been inhibited by the plant extract. The plant phytoconstituents include tannins, alkaloids, flavonoids, saponins, terpenoids and essential oils, all proven to have analgesic and anti-inflammatory properties. The research provides scientific validation for the traditional claims about *Ficus nervosa's* benefits for inflammation and other disorders. The paw withdrawal from the hot plate is significantly ($p<0.001$) increased by the extracts and standard medication, suggesting the presence of analgesic activity. Two extracts (NC and NM) and positive control (standard drug) significantly reduce the number of abdominal constrictions and hind-limb stretching caused by an acetic acid injection ($p<0.001$). The paw edema was significantly ($p<0.001$) reduced by the extracts and standard medication, suggesting the presence of anti-inflammatory activity. As indicated by the preceding data, the Methanol extract (NM) of *F. nervosa* leaves (500 mg/kg) had a considerable effect compared to the chloroform extract (NC). The study serves as a reference for further molecular level research to completely understand the analgesic and anti-inflammatory effects of extracts and identification of responsible chemical hits.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

HRLC-MS: High-Resolution Liquid Chromatography-Mass Spectrometry; **OECD:** Organisation for Economic Co-operation and Development; **i.p.:** Intraperitoneal; **p.o.:** Per oral; **RH:** Running Head; **ESI:** Electrospray Ionization; **CNS:** Central Nervous System; **COX:** Cyclooxygenase; **LOX:** Lipoxygenase; **NH:** *Ficus nervosa* Hexane extract; **NC:** *Ficus nervosa* Chloroform extract; **NM:** *Ficus nervosa* Methanol extract; **IAEC:** Institutional Animal Ethics Committee; **SEM:** Standard Error of the Mean; **ANOVA:** Analysis of Variance.

ETHICAL STATEMENT

This study was conducted in accordance with the ethical guidelines for animal research and was approved by the Institutional Animal Ethics Committee (IAEC) at GITAM Deemed to be University, Visakhapatnam (approval no. IAEC/GIP-1287/CS-F/Approved/14/Dec2021).

SUMMARY

In this study, *Ficus nervosa* constituents analyzed using LC-MS/MS method and top 20 compound were presented. The anti-inflammatory and analgesic activity if NC and NM extracts were evaluated using various *in vivo* modules. The provided scientific validation of traditional claims for the medicinal plant *Ficus nervosa*. Which can serve as further molecular level research and identification of responsible chemical constituents.

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