ORIGINAL ARTICLE

Analgesic and Anti-inflammatory activities of ethanolic, ethyl acetate, and hexane extracts of *Nymphoides hydrophylla* in experimental animals

K. Ravishankar[1], S. Nageswara Rao[2], V. Girija Sastry[3]

[1]Department of Pharmacology, Aditya College of Pharmacy, Aditya Nagar, A.D.B.Road, Surampalem, East-Godavari, Andhra Pradesh, India, [2]Department of Pharmaceutical Chemistry, University College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, Andhra Pradesh, India

ABSTRACT

Aim: The ethanolic, ethyl acetate, and hexane extracts of *Nymphoides hydrophylla* at the doses of 250 mg/kg and 500 mg/kg were administered for the evaluation of analgesic and anti-inflammatory activities (both *in vitro* and *in vivo*). **Materials and Methods:** Analgesic activity was evaluated by acetic acid-induced writhing, tail-flick method, and Eddy's hot plate method in albino rats. Paracetamol and tramadol were used as a standard reference drugs for analgesic activity. *In vitro* anti-inflammatory activity was evaluated by Human red blood cell membrane stabilization method and protein denaturation method. *In vivo* anti-inflammatory activity was evaluated by carrageenan-induced paw edema in albino rats. Diclofenac sodium was employed as reference drugs for anti-inflammatory studies. **Results and Discussion:** The administration of ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla* in rats with 250 and 500 mg/kg body weight (b.wt.) reduced pain and inflammation, indicating that ethanolic extract possesses better analgesic and anti-inflammatory activities compared to other two extracts. The maximum analgesic and anti-inflammatory activities were observed in rats receiving 500 mg/kg b.wt. of *N. hydrophylla* ethanolic extract. **Conclusion:** Our study indicates that *N. hydrophylla* extracts possess both anti-inflammatory and analgesic activities and it may be useful as an anti-inflammatory agent in inflammation-related disorders.

Key words: Analgesic, Anti-inflammatory, Ethanolic, Ethyl acetate and hexane extracts, *In-vivo* animal models, *Nymphoides hydrophylla*

INTRODUCTION

The process of inflammation occurs due to noxious stimuli, infection, trauma, or injury in the living tissues.[1] During this process body's white blood cells and chemicals protects the body from various types of infections, bacteria, and viruses. The process of inflammation is characterized by events including mediator release, enzyme activation, fluid extravasations, cell migration, tissue breakdown, and repair processes.[2] White blood cells are released during inflammation as a protective measure. There will be an increase in the blood flow to the area leading to redness and warmth. The process of inflammation is characterized by pain induction, redness, and rashes.[3] In the induction of inflammatory response, prostaglandins (PG) are one of the important biomolecules, as their biosynthesis is significantly increases during the process of inflammation.[4] In several diseases, including cancer, inflammation was having a key role.[5,6] In treating pathologies associated with inflammatory reactions, the agents that contain or block inflammation may play an important role.[7] Pain is defined by medical researchers as a subjective conscious experience. Natural products are used for treating various inflammatory conditions such as fever, pain, migraine, and arthritis. Herbal medicines contain pleiotropic molecules that act on

Address for correspondence:
S. Nageswara Rao, Aditya Pharmacy College, Surampalem, East Godavari, Andhra Pradesh - 533 437, India. Tel.: +91-7729995798.
E-mail: nag3_au@yahoo.com

Received: 25-09-2020
Revised: 02-12-2020
Accepted: 13-12-2020

orchestral approach, which are able to target many elements of the complex cellular pathway.[8] The medicinal plants have been a major source of a wide variety of biologically active compounds for many centuries and have been used extensively in crude form or as an active ingredient to treat various disease conditions.[9]

Nymphoides hydrophylla is an aquatic plant with a common vernacular Telugu name as Antharathaarama, cheerily, pita Kaluva belonging to the family *Menyanthaceae* is a very common plant available throughout India. The plant usually flowers and fruits in winter season. The plant is traditionally used for several medicinal purposes in India as the oil of the plant is extensively used in number of herbal preparation for curing ulcers and insect bites. Therefore, the present investigation carried out an in-depth study regard the *N. hydrophylla* ethanolic, ethyl acetate, and hexane extracts for their analgesic and anti-inflammatory properties.

MATERIALS AND METHODS

Collection of Plant

N. hydrophylla were collected from Surrounding areas of Jaggampeta, East Godavari dt., of Andhra Pradesh. The plant authentication was done by Dr. T. RAGHURAM Taxonomist, Maharani College, Peddapuram.

Preparation of Extracts

N. hydrophylla plants were subjected to shade drying at room temperature for 4–5 days. The dried plants were then powdered into a mixture. The powder was taken and weighed. From the obtained fine powder, 100 g was taken and macerated in 200 mL of ethanol, ethyl acetate, and hexane for 7 days, separately. Selected solvents have a non-toxic effect(s) or minor interfering effects on the living cells, animals, and human beings. Selection of ethanol, ethyl acetate, and hexane as a solvent would extract highly hydrophilic, moderately hydrophilic, and highly lipophilic secondary metabolites from the plants, respectively. Individual extracts were collected and filtered. The filtrate of ethanol, ethyl acetate, and hexane extracts was concentrated through distillation individually. After distillation, crude extracts were collected individually and the obtained extracts were weighed. The physical characteristics and percentage yields of various extracts are reported. The crude extracts were placed in desiccators for further studies.

Preliminary Phytochemical Screening

Preliminary phytochemical screening of *N. hydrophylla* ethanolic, ethyl acetate, and hexane extracts were performed to test the presence of the active chemical constituents such as alkaloids, flavonoids, tannins, phenolic compounds, saponins, fixed oils, and fats.[10]

Quantitative Phytochemical Testing

Aliquots of extract were prepared by dissolving 10 mg of individual extracts in 10 mL of methanol to get 1000 µg/ml.

Estimation of Phenolic Contents

The phenolic content of the ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla* (1 mg/ml, aliquots) was determined using the method Folin-Ciocalteu.[11] 0.5 ml aliquots of extract were mixed with 3 ml Folin-Ciocalteu reagent (1:10 v/v) and the mixture allowed to stand for 5 min. Four milliliters of 20% w/v of sodium carbonate solution were added into the mixture tube. The tubes were kept aside for 15 min at 30°C for color development. The absorbance was measured at 765 nm by a spectrophotometer. Phenolic content was estimated from the calibration curve using standard gallic acid in methanol and the results were expressed as gallic acid equivalent mg/100 mg dry weight of extract.

Estimation of Total Flavonoids

Total flavonoid content of the ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla* (1 mg/ml, aliquots) was determined by aluminum chloride method.[12] To 0.6 ml of aliquots of extract, 1.8 ml of methanol, 0.1 mL of 10% aluminum chloride, 0.1 ml of 1 M sodium acetate, and 3 ml of distilled water were added and left at 30°C. The absorbance was measured individually after 30 min at 415 nm. Total flavonoid was estimated from the calibration curve using standard quercetin in methanol and the results were expressed as quercetin equivalent mg/100 mg dry weight of extract.

Estimation of Alkaloids

Alkaloid content in the extracts was determined using the method of Fazel *et al*., the ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla* (1 mg/mL, aliquots) were dissolved in 2N hydrochloric acid and filtered. To the filtrate was added 0.1 N NaOH, from this solution 1 ml was transferred to a separating funnel, and then 5 ml of Bromo cresol green solution was added along with 5 ml of phosphate buffer. The mixture was shaken extracted with chloroform. The absorbance was noted at 470 nm. Alkaloid content was estimated from the calibration curve using the standard atropine calibration curve, measuring the concentration of alkaloid content in atropine equivalents using the units mg/100 mg dry weight of extract.[13]

Experimental Animals

Albino rats weighing between 150 and 200 g of either sex were used for the study. Animals were housed in colony cages at an ambient temperature of 25 ± 2°C, 12 h light/dark cycle, and 50 ± 5% relative humidity with free access to food and water *ad libitum*. To the laboratory environment, the animals were acclimatized for at least 1 week before experimentation. Animals were deprived of food except water in overnight and during the period of experimentation. The animal experiments were performed based on the Institutional Ethics Committee approval and guidelines REG.No.AIPSR/IAEC/14/01.

Acute Toxicity and Gross Behavioral Study

The experimental animals were fasted overnight, divided into groups (n = 6) and were orally fed with increasing doses (250, 500, 750, and 1000 mg/kg body weight) of ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla*. The animals were observed during 24 h to find out the percentage mortality and gross behavioral changes after administration of the extracts.[14-16]

Analgesic Activity

Tail-flick method

Analgesia was measured using a modified method of D Amour and Smith[17] called a tail-flick method. Analgesia is indicated by an increase in reaction time. Reaction time is the time between placing the tail of the rat on the radiant heat source and sharp withdrawal of the tail. Minimum cutoff time period of about 10 s was taken as maximum latency so as to avoid thermal injury to the animals while noting down the reaction time. Animals that showed a mean reaction time outside the range of 5–6 s were discarded. In all the groups, tail-flick test was performed before drug administration (at 0 min) and at 15, 30, 60, and 120 min after drug administration, and the reaction time at each time interval (test latency) was calculated.

Acetic acid-induced writhing method

The writhing model represents a chemical nociceptive test based on the induction of peritonitis like condition in animals by injecting irritant substances intraperitoneal (i.p.) After 30 min of drug administration, 0.1 ml of 1% acetic acid solution was injected i.p. Rats were kept individually in glass beakers and were allowed to elapse for a period of about 5 min. The animals were then observed for about 10 min and the numbers of writhes were recorded. For scoring purposes, writhe is indicated by stretching of the abdomen with simultaneous stretching of at least one hind limb.[18]

Percentage inhibition was calculated using the following formula:

$$\% \text{ inhibition} = \{(W_c - W_t) \times 100\}/Wc$$

Where, W_c = No. of writhes in control group, W_t = No. of writhes in test group

Compounds with <70% inhibition were considered to have minimal analgesic activity.[18]

Eddy's hot plate method

Albino rats of either sex were selected, weighed, and divided into eight groups (n = 6). The time of reaction to pain stimulus of the rats placed on the plate, heated at 55 ± 0.5°C was recorded at 0, 15, 30, 60, and 120 min after the administration of the ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla* in two different doses (250 mg/kg and 5/kg, b.w) and vehicle were administered orally before 60 min. The increase in reaction time against the control group was calculated.[19]

In Vitro Anti-inflammatory Activity[20-22]

Protein denaturation method

Procedure

Test solution (0.5 ml)

Consists of 0.45 ml of 5% w/v aqueous solution of bovine serum albumin and 0.05 ml of test samples of various concentrations (ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla* of 100 and 200 ug/ml).

Product control solution (0.5 ml)

Consists of 0.45 ml of distilled water and 0.05 ml of test samples of various concentrations (ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla* of 100 and 200 ug/ml).

Standard solution (0.5 ml)

Consists of 0.45 ml of 5% w/v aqueous solution of bovine serum albumin and 0.05 ml of various concentrations of diclofenac sodium. All the solutions were adjusted to PH 6.3 using 1N HCl. Incubation of samples was carried out for 20 min at 37°C. Later, the samples were kept for 3 min at an elevated temperature of 57°C. 2.5 ml phosphate buffer was added to the above solutions after cooling. The absorbance was recorded at a wavelength of 416 nm using an ultraviolet/visible spectrophotometer.

$$\% \text{ Inhibition of Protein Denaturation} = 100 - ([(\text{O.D of test solution} - \text{O.D of product control})/\text{O.D of test control}] \times 100)$$

The control represents 100% protein denaturation. The results were compared with diclofenac sodium.

HRBC Membrane Stabilization Method

Principle

The principle of this method is the stabilization of the human red blood cell (HRBC) membrane from the hypo-tonicity-induced membrane lysis.

Preparation of HRBC suspension

Fresh whole human blood (2 ml) was collected and was mixed with an equal volume of sterilized Alsever's solution (0.8% sodium citrate, 2% dextrose, 0.05% citric acid, and 0.42% aqueous solution of sodium chloride). The blood was subjected to centrifugation for 10 min at 3000 rpm. The compacted cells were washed thrice with isosaline solution (0.85%, pH 7.2). The volume of the blood was measured and was reconstituted as a 10% v/v suspension with isosaline.

Procedure (hypotonic solution-induced hemolysis)

The reaction mixture (of total volume 4.5 ml) is made up of 2 ml of hyposaline solution (0.25% w/v NaCl), 1 ml of 0.15 M phosphate buffer (pH 7.4), and 1 ml of test solution (ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla* of 100 and 200 ug/ml) in isosaline. After that, 0.5 ml of 10% HRBC suspension in isosaline was added. For test control, 1 ml of distilled water was used in the place of hyposaline (to produce 100% hemolysis), while the product control was made lacking the red blood cell. The mixtures were incubated for 30 min at 37°C and centrifuged for 20 min at 3000 rpm. The reference drug was diclofenac sodium. The hemoglobin content of the suspension was measured as a function of absorbance at 560 nm using a spectrophotometer. Percentage membrane stabilizing activity was calculated as follows, the percentage of HRBC membrane stabilization or protection was calculated using the following formula:

$$\text{Percentage Stabilization} = \text{Absorbance of control} - \frac{\text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

In Vivo Anti-inflammatory Activity

Carrageenan-induced paw edema[23]

Edema was induced by injecting 0.1 mL of 1% solution of carrageenan in saline to (sub plantar) right hind paw of rats. The ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla* in two different doses (250 and 500 mg/kg, b.w) and vehicle were administered orally 60 min before injection of carrageenan. The volume of edema of injected and contra collateral paws was measured at 0, 1, 2, 3, and 4 h after induction of inflammation using a plethysmograph and the percentage of anti-inflammatory activity was calculated. The values are compared with the standard drug diclofenac (10 mg/kg).

Statistical Analysis

Data were analyzed by GraphPad INSTAT® version 3.0 software and presented as mean±S.E.M. values. The statistical tests used were one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. The levels of statistical significance ranged from $P < 0.05$.

RESULTS

After extraction with different solvents such as ethanol, ethyl acetate, and hexane, the residues were dried and measured. The residue obtained was 10.2% w/w, 7.53% w/w, and 6.28% w/w for ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla*, respectively. The brownish-black, brownish-green, and black residues were obtained for ethanol, ethyl acetate, and hexane extracts of *N. hydrophylla,* respectively. The ethanol extracts were sticky in nature. The other extracts were a little gummy in nature.

Preliminary and Quantitative Phytochemical Screening

The results imply that extracts contain alkaloids, flavonoids, saponins, carbohydrates, proteins, and Amino acids which are the main phytochemical groups with biological activities. The results of quantitative phytochemical screening were tabulated [Table 1].

Acute Toxicity Studies

No toxicity or death was observed in the experimental rats when they are subjected to toxicity study. To establish the safety, extracts (ethanolic, ethyl acetate, and hexane extracts) were administered to both male and female rats. There were no significant toxic signs or death during the entire observation period. The ethanolic, ethyl acetate, and hexane

Table 1: Quantitative phytochemical determination of ethanolic, ethyl acetate, and hexane extracts of *Nymphoides hydrophylla*

S.no	Total phenolics mg/g	Total flavonoids mg/g	Total alkaloids mg/g
Ethanolic extract of *Nymphoides hydrophylla*	11.04±0.25	42.06±0.29	33.12±0.61
Ethyl acetate extract of *Nymphoides hydrophylla*	9.04±0.13	28.06±0.28	25.12±0.52
Hexane extract of *Nymphoides hydrophylla*	8.04±0.41	22.6±036	27.23±0.49

All the values are expresses ad mean±SEM, *n*=3

extracts of *N. hydrophylla* did not exhibit any mortality up to the dose level of 1000 mg/kg.

Analgesic Activity

The present study investigated the analgesic and effect of ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla* in rats. In the analgesic study, the extract produced significant analgesic effects in the three models of pain employed.

Tail-flick Method

In tail-flick method, rats are treated with *N. hydrophylla* doses (250 mg/kg and 500 mg/kg orally) significantly inhibited nociception in rats. Significant activity was exhibited by Ethanolic Extract of *N. hydrophylla* 250 mg/kg at 30 min by 42.8% and 500 mg/kg at 30 min by 69.3%, whereas paracetamol 100 mg/kg significantly inhibited pain perception at 30 min by 75.0% [Table 2].

Eddy's Hot Plate Method

In Eddy's hot plate method, rats are treated with ethanolic extract of *N. hydrophylla* whole plant doses (250 mg/kg and 500 mg/kg orally) significantly inhibited nociception in rats. Significant activity was exhibited by ethanolic extract of *N. hydrophylla* 250 mg/kg at 60 min by 75% and 500 mg/kg at 60 min by 80.2%, whereas tramadol 5 mg/kg significantly inhibited pain perception at 60 min by 84.2% [Table 3].

Acetic Acid-induced Writhing Response

In acetic acid-induced writhing responses method, rats are treated with ethanolic extract of *N. hydrophylla* doses (250 mg/kg and 500 mg/kg orally) shows significantly inhibited nociception in rats. Significant activity was observed by ethanolic extract of *N. hydrophylla* was found to be 62.35% at 250 mg/kg and 76.47% at 500 mg/kg, whereas tramadol (5 mg/kg) showed 92.94% protection against acetic acid-induced writhing in rats [Table 4].

Anti-inflammatory Activity

In-vitro studies

Protein denaturation method

In-vitro anti-inflammatory effect of ethanolic extract of *N. hydrophylla* whole plant was evaluated by protein denaturation method.

Maximum percentage inhibition of *N. hydrophylla* ethanolic extract was found to be 83.94% at 200 ug/ml, whereas standard diclofenac sodium showed the maximum inhibition 93.79% at 20 ug/ml concentration.

Table 2: Effect of ethanolic, ethyl acetate, and hexane extracts of ***Nymphoides hydrophylla*** whole plant in rats using tail-flick method

Treatment group	0 min		15 min		30 min		60 min		120 min	
	B.R.T	% inh	B.R.T	%inh	B.R.T	%inh	B.R.T	%inh	B.R.T	%inh
Control	5.7±0.67	---	6.5±0.32	---	7.1±0.97	---	5.9±0.30	---	4.0±0.81	---
Group-II Paracetamol 100 mg/kg	5.1±0.51	---	7.8±0.61	52.9%	8.9±0.12	74.5%	6.8±0.45	33.3%	6.3±0.98	23.5%
Group-III ethanolic extract of *Nymphoides hydrophylla* 250 mg/kg	6.3±0.82	---	8.5±0.82	34.9%	9.0±0.67	42.8%	7.1±0.21	12.6%	6.7±0.65	6.3%
Group-IV Ethanolic extract of *Nymphoides hydrophylla* 500 mg/kg	4.9±0.94	---	6.9±0.12	40.8%	8.3±0.92	69.3%*	7.2±0.39	46.9%	6.0±0.21	22.4%
Group-V ethyl acetate extract of *Nymphoides hydrophylla* 250 mg/kg	5.8±0.28	---	7.4±0.52	27%	8.1±0.58	39.6%	6.8±0.45	17.2%	6.1±0.65	5.1%
Group-VI ethyl acetate extract of *Nymphoides hydrophylla* 500 mg/kg	4.5±0.35	---	5.9±0.23	31.1%	7.3±0.92	62.2%*	5.5±0.42	22.2%	5.1±0.28	13.33%
Group-VII hexane extract of *Nymphoides hydrophylla* 250 mg/kg	6.5±0.28	------	8.5±0.86	23.50%	9.25±0.72	29.70%	7.5±0.28	13.30	6.8±0.34	4.61
Group-VIII hexane extract of *Nymphoides hydrophylla* 500 mg/kg	6±0.0	------	7.8±0.18	30%	8.4±0.31	40%	7.5±1.44	22.60	6.3±0.75	5

B.R.T: Basal reaction time (s) %, Inh: % Inhibition. All the values are expresses ad mean±SEM, n=6. *$P < 0.05$. Statistically significant difference in comparison with standard group

Table 3: Effect of ethanolic, ethyl acetate, and hexane extracts of *Nymphoides hydrophylla* whole plant in rats using Eddy's hot plate method

Treatment group	0 min		15 min		30 min		60 min		120 min	
	R.T	% Inh	R.T	%Inh	R.T	%Inh	R.T	%Inh	R.T	%Inh
Control	6.6±0.08		7.3±0.20		7.9±0.15		8.5±0.17		6.8±0.26	
Group-II tramadol 5 mg/kg	7.0±0.08	------	10.4±0.98	48.5%	12.2±0.34	74.2%	12.9±0.20	84.2%	10.2±0.15	45.7%
Group-III ethanolic extract of *Nymphoides hydrophylla* 250 mg/kg	7.6±0.20	------	11.0±0.20	44.7%	11.6±0.17	52.6%*	13.3±0.29	75.0%	11.8±0.23	55.2%
Group-IV ethanolic extract of *Nymphoides hydrophylla* 500 mg/kg	8.1±0.17	------	12.3±0.17	51.8%	13.4±0.17	65.4%*	14.6±0.12	80.2%	12.3±0.14	51.8%
Group-V ethyl acetate Extract of *Nymphoides hydrophylla* 250 mg/kg	9.5±0.34	------	11.50±0.36	21.05%	13±0.45	36.8%	11.20±0.63	17.8%	10.25±0.32	7.89%
Group-VI ethyl acetate extract of *Nymphoides hydrophylla* 500 mg/kg	10.5±0.28	------	15.75±0.72	33.5v	22±0.57	52%*	13.75±0.43	23%	12.25±0.43	14%
Group-VII hexane extract of *Nymphoides hydrophylla* 250 mg/kg	8.3±0.28	------	9.89±0.26	19.1%	11.21±0.45	35.06%	9.89±0.63	19.15%	9.12±0.32	9.87%
Group-VIII hexane extract of *Nymphoides hydrophylla* 500 mg/kg	8.8±0.28	------	11.15±0.29	26.70%	13±0.73	47.72%*	11.02±0.13	25.22%	9.81±0.26	11.4%

B.R.T: Basal reaction time (s) %, Inh: % inhibition. All the values are expresses ad mean±SEM, n=6. *P<0.05. Statistically significant difference in comparison with standard group

HRBC Membrane Stabilization Method

In-vitro anti-inflammatory effect of ethanolic extract of *N. hydrophylla* whole plant was carried out by HRBC membrane stabilization method.

Ethanolic extract of *N. hydrophylla* whole plant prevents hypotonicity-induced membrane lysis (HRBC membrane stabilization method) to the extent of 52.58% at the concentration of 200 ug/ml, which is comparable to that of the standard drug diclofenac sodium 73.7% (20 ug/ml). The anti-inflammatory activity of the extract was concentration-dependent.

In-vivo Studies

Carrageenan-induced paw edema in rats

In-vivo anti-inflammatory effect of ethanolic extract of *N. hydrophylla* whole plant was evaluated using Carrageenan-induced paw edema in rats. It was observed that rats are treated with ethanolic extract at 250 mg/kg and 500 mg/kg showed significant anti-inflammatory activity and caused a significant inhibition in the percentage increase Carrageenan-induced rat paw edema when compared with standard diclofenac sodium. Ethanolic extract of *Nymphoides* decreased the paw diameter when compared with other groups [Table 5].

DISCUSSION

In the present study, the putative analgesic and anti-inflammatory activities of ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla* were performed to demonstrate their pain- and inflammation-relieving effects. Analgesics mainly acts on the central or peripheral nervous system to relieve from pain without altering consciousness.[24] Centrally acting analgesics mainly act by increasing the threshold for pain and altering the pain physiological response. Peripherally acting drugs mainly involve the mechanism of inhibiting the pain generation impulses at the chemoreceptor level.[25] Three different analgesic screening methods were carried out for evaluating the possible peripheral and central effects of the ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla* such as tail-flick, Eddy's hot plate, and acetic acid-induced writhing tests, which are the major pharmacological models for evaluating the analgesic effect exerted by natural products.[26] Both tail-flick and hot plate methods are used for screening of centrally acting analgesics,[27] while peripherally acting drugs are more sensitive to acetic acid-induced writhing test.[28]

In the visceral pain model, that is, acetic acid-induced writhing response, the analgesic mechanism of abdominal writhing was induced by acetic acid, which mainly involves the arachidonic acid (AA) release through cyclooxygenase (COX) and PG biosynthesis.[29]

Table 4: Effect of ethanolic, ethyl acetate, and hexane extracts of *Nymphoides hydrophylla* whole plant in rats using acetic acid-induced writhing responses

Treatment	Number of writhing	% Inhibition
Control (Group I)	85±0.92	----------
Tramadol (5 mg/kg) (Group II)	6±0.14	92.94
Group-III ethanolic extract of *Nymphoides hydrophylla* 250 mg/kg	32±0.29	62.35
Group-IV ethanolic extract of *Nymphoides hydrophylla* 500 mg/kg	20±0.32	76.47*
Group-V ethyl acetate extract of *Nymphoides hydrophylla* 250 mg/kg	35±0.56	58.82
Group-VIethyl acetate extract of *Nymphoides hydrophylla* 500 mg/kg	29±0.23	65.88*
Group-VII hexane extract of *Nymphoides hydrophylla* 250 mg/kg	40±0.41	52.94
Group-VIII hexane extract of *Nymphoides hydrophylla* 500 mg/kg	34±0.18	60.00*

All the values are expressed as Mean±SEM, n=6. *P<0.05. Statistically significant difference in comparison with standard group

Table 5: *In-vivo* anti-inflammatory effect of ethanolic, ethyl acetate, and hexane extracts of *Nymphoides hydrophylla* whole plant in carrageenan-induced paw edema in rats

Treatment	Mean increase in paw diameter mm				
	0 h	1 h	2 h	3rd h	4th h
Group-I Control	1.7±0.03	3.2±0.18	3.7±0.08	4.1±0.2	2.2±0.1
Group-II diclofenac sodium (10 mg/kg)	1.8±0.1	2.3±0.12	2.6±0.1	2.9±0.96	2.3±0.81
Group-III ethanolic extract of *Nymphoides hydrophylla* (250 mg/kg)	1.87±0.28	1.99±0.17	2.13±1.24	2.27±1.51	2.16±0.81
Group-IV ethanolic extract of *Nymphoides hydrophylla* (500 mg/kg)	1.86±1.81	1.98±1.12	2.15±0.38	2.30±1.08	2.20±0.79
Group-V ethyl acetate extract of *Nymphoides hydrophylla* 250 mg/kg	1.87±0.89	1.96±0.18	2.08±1.26	2.19±0.24	2.11±0.87
Group-VI ethyl acetate extract of *Nymphoides hydrophylla* 500 mg/kg	1.88±0.78	1.97±1.25	2.11±0.76	2.24±0.84	2.15±1.15
Group-VII hexane extract of *Nymphoides hydrophylla* 250 mg/kg	1.88±0.87	1.94±0.74	2.03±1.24	2.14±0.29	2.03±0.17
Group-VIII hexane extract of *Nymphoides hydrophylla* 500 mg/kg	1.87±1.15	1.97±0.47	2.06±0.58	2.18±1.26	2.09±074

Ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla* at the oral dose of 250 and 500 mg/kg significantly decreased the acetic acid-induced writhing response in rats. Compared to the other two extracts, ethanolic extract has exhibited significant analgesic activity of 62.35% at 250 mg/kg and 76.47% at 500 mg/kg, whereas tramadol (5 mg/kg) showed 92.94% protection against acetic acid-induced writhing in rats and the results are tabulated in Table 4. In tail-flick test, ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla* exhibited significant antinociceptive activity by increasing the latency time of responses in rats in a concentration-dependent manner, in all tested doses of 250 and 500 mg/kg can be seen in Table 2. In comparison to control, paracetamol (standard drug) produced the highest analgesic activity among all tested samples.

Eddy's hot plate method produces two behavioral components in response to thermal pain, with regard to their reaction times. Paw licking and jumping in rats responses are supraspinally integrated.[30] Findings from this study demonstrated that the ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla* prolonged the reaction time in the hot plate method. This might indicate higher sensitivity of the spinally mediated reflex response in Eddy's hot plate method. Ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla* at the oral dose of 250 and 500 mg/kg significantly prolonged the reaction time response in rats. Compared to the other two extracts, ethanolic extract has exhibited significant analgesic activity of 250 mg/kg at 60 min by 75% and 500 mg/kg at 60 min by 80.2%, whereas tramadol 5 mg/kg significantly inhibited pain perception at 60 min by 84.2% and the results are tabulated in Table 3.

Inflammation is a complex physiopathological response that involves the activity of inflammatory mediators such as neutrophil-derived free radical, reactive oxygen species, nitric oxide (NO), PG, and cytokines.[31] This overproduction of these mediators results in tissue injury causing damage

of macromolecules, membrane lipid peroxidation, and tissue damage that plays an important role in inflammatory diseases. NO generated by NO synthase (iNOS) mainly surpasses the physiological amount of NO in macrophages, which are usually made by a neuronal form of NOS (nNOS) or NOS constitutive form (eNOS), these NO nitrosylates macromolecules during the course of inflammatory response. NO released causes increased vascular permeability, vasodilation, tissue, and endothelial damage that leads to inflammation. In chronic inflammation, the pro-inflammatory role of NO has been inferred, based on the observation of elevated levels of nitrite in rheumatoid synovial fluid.[32] Protein denaturation is a phenomena where proteins lose their tertiary structure and secondary structure by application of external stress or compounds such as strong acid or base, a concentrated inorganic salt, and an organic solvent or heat. During the process of denaturation, many of the biological proteins lose their biological function and this is a major cause of inflammation. In the present study, on the mechanism of the anti-inflammation activity, ability of ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla* to inhibit protein denaturation was studied. Ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla* were effective in inhibiting heat-induced albumin denaturation. Maximum inhibition of 83.94% was observed at 200 µg/ml. Diclofenac, a standard anti-inflammation drug, showed the maximum inhibition 93% at the concentration of 20 µg/ml compared with control [Table 6].

Another method of *in vitro* anti-inflammatory study is the HRBC membrane stabilization method because the erythrocyte membrane is analogous to the lysosomal membrane[33,34] and its stabilization indicates that the ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla* may well stabilize lysosomal membranes. Lysosomal membrane stabilization is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, such as bacterial enzymes and proteases, that causes tissue inflammation and damage upon extracellular release causes the various disorder and are said to be related to acute or chronic inflammation. The NSAIDs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal.[35] Ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla* were effective in stabilizing the membrane. Maximum stabilization of 52.58% was observed at 200 µg/ml. Diclofenac, a standard anti-inflammation drug, showed the maximum stabilization 73.7% at the concentration of 20 µg/ml compared with control [Table 7]. Standard model of screening for anti-inflammatory activity in various experimental compounds is the Carr-induced inflammation.[36] The edema is mainly characterized by the presence of PG and other slow reaction compounds.[37] In activated inflammatory cells, COX-2 is an inducible isoform that generates inflammation prostanoid mediators.[38] For screening anti-inflammatory agents and the study of pathogenesis and pathology of the inflammatory and nociceptive processes in animal models, inhibition of COX-2 protein expression has become the most popular target.[39] In inflammatory responses, inducing innate immune responses by activating T cells and macrophages and stimulating the secretion of other inflammatory cytokines, tumor necrosis factor-alpha is a major mediator.[40] In the pathogenesis of

Table 6: *In-vitro* anti-inflammatory effect of ethanolic, ethyl acetate, and hexane extracts of *Nymphoides hydrophylla* whole plant by protein denaturation method

S. no	Concentration	% Inhibition			
		***Nymphoides hydrophylla* ethanolic extract**	***Nymphoides hydrophylla* ethyl acetate extract**	***Nymphoides hydrophylla* hexane extract**	**Diclofenac sodium**
1	10 ug/ml	-----			77.4
2	20 ug/ml	-----			93.7
3	100 ug/ml	65.4	62.3	54.8	
4	200 ug/ml	83.94	72.8	63.4	

All the values are expressed as mean±SEM, *n*=3

Table 7: *In-vitro* anti-inflammatory effect of ethanolic, ethyl acetate, and hexane extracts of *Nymphoides hydrophylla* whole plant by HRBC membrane stabilization method

S. no	Concentration	% Membrane lysis			
		***Nymphoides hydrophylla* extract**	***Nymphoides hydrophylla* Ethyl acetate extract**	***Nymphoides hydrophylla* Hexane extract**	**Diclofenac sodium**
1	10 ug/ml	------			69.6
2	20 ug/ml	------			73.7
3	100 ug/ml	45.36	40.23	38.25	
4	200 ug/ml	52.58	48.35	40.92	

All the values are expressed as mean±SEM, *n*=3

inflammatory disorders, inhibition of cytokine production and action can provide therapeutic benefits. Significant correlations exist between cytokine production, COX-2 protein expression, and PG synthesis in the rats paw tissues where edema was invoked by Carr intraplantar injection.[41] Ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla* at dosages of 250 and 500 mg/kg significantly inhibited the protein expression of COX-2 level in the rats edema paw tissues. The results are tabulated in Table 5.

Phenolics and flavonoids are the secondary metabolites that exert biological activities such as antioxidant, antimutagenic, and anti-inflammatory. In the expression and activation of iNOS, flavonoids are capable of inhibiting them and could be used additionally during the treatment of inflammation. The role of NO in inflammation indicates that drugs which modulate NO production are highly useful in inflammatory disease management.[42] AA is metabolized in the body through two main metabolic pathways with the enzymes: COX and 5-lipoxygenases into PG I2, PG E2, thromboxane A2, and leukotrienes.

This study demonstrated that ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla* exhibited anti-inflammatory activity against Carr-induced paw edema and analgesic activity against nociceptive responses triggered in rats by i.p. acetic acid injection injections. The exact mechanism by which ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla* exerts their analgesic effect was related to its anti-inflammatory effect, and this serves as a possible rationale for the use of *N. hydrophylla* in traditional medicine for anti-inflammation. Ethanolic extract exhibited significant analgesic and anti-inflammatory activities compared to the other two extracts which is attributed due to the presence of more phenolic and flavonoid content [Table 1].

CONCLUSION

Ethanolic, ethyl acetate, and hexane extracts of *N. hydrophylla* were selected for the study. Phytochemical studies showed the presence of alkaloids, flavonoids, saponins, carbohydrates, proteins, and amino acids. The antioxidant compounds such as phenolics, flavonoids, and alkaloids were quantitatively estimated and the ethanolic extract showed the maximum of these compounds. In conclusion, our results reveal that among all the extracts of *N. hydrophylla,* ethanolic extract exhibited significant analgesic and anti-inflammatory activities followed by ethyl acetate and hexane extracts. These findings validated the claim for the traditional use of this plant in the treatment of pain and inflammatory ailments. Further research work is needed to segregate the active constituents from the active extract exhibiting significant analgesic and anti-inflammatory activities. In addition to this research regarding, the mechanism responsible for these activities is also required which will guarantee its clinical worth. Further research work is required for the estimation of all the possible essential elements present in the plant so that we can presume the total effectiveness of the plant.

REFERENCES

1. Calixto JB, Campos MM, Otuki MF, Santos AR. Anti-inflammatory compounds of plant origin. Part II. Modulation of pro-inflammatory cytokines, chemokines and adhesion molecules. Planta Med 2004;70:93-103.
2. Risso WE, Scarminio IS, Moreira EG. Antinociceptive and acute toxicity evaluation of *Vernonia condensata* Baker leaves extracted with different solvents and their mixtures. Indian J Exp Biol 2010;48:811-6.
3. Opdenakker G, Fibbe WE, Van Damme J. The molecular basis of leukocytosis. Immunol Today 1998;19:182-9.
4. Ricciotti E, FitzGerald GA. Prostaglandins and inflammation. Arteriosc Thromb Vasc Biol 2011;31:986-1000.
5. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell 2010;140:883-99.
6. Moore MM, Chua W, Charles KA, Clarke SJ. Inflammation and cancer: Causes and consequences. Clin Pharmacol Ther 2010;87:504-8.
7. Sosa S, Balick MJ, Arvigo R, Esposito RG, Pizza C, Altinier G, *et al*. Screening of the topical anti-inflammatory activity of some central American plants. J Ethnopharmacol 2002;81:211-5.
8. Durmowicz AG, Stenmark KR. Mechanisms of structural remodeling in chronic pulmonary hypertension. Pediatr Rev 1999;20:e91-102.
9. Newman DJ, Cragg GM. Marine-sourced anti-cancer and cancer pain control agents in clinical and late preclinical development. Marine Drugs 2014;12:255-78.
10. Khandelwal KR. Practicalpharmacognosy. 11th ed. Pune: Nirali Prakashan; 2004. p. 149-56.
11. Singleton VL, Orthofer R, Lamuela-Raventós RM. [14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods Enzymol 1999;299:152-78.
12. Chang CC, Yang MH, Wen HM, Chern JC. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J Food Drug Anal 2002;10:178-82.
13. Fazel S, Hamidreza M, Rouhollah G, Verdian-Rizi M. Spectrophotometric determination of total alkaloids in some Iranian medicinal plants. Soc Adv Hortic 2010;12:69-70.
14. Kulkarni SK. Hand Book of Experimental Pharmacology. 3rd ed. New Delhi: Vallabprakasan; 1999. p. 117-71.
15. Turner RA, Hebborn P. Screening Methods in Pharmacology 2. United States: Academic Press; 1971. p. 210-45.
16. Amos S, Kolawole E, Akah P, Wambebe C, Gamaniel K. Behavioral effects of the aqueous extract of *Guiera senegalensis* in mice and rats. Phytomedicine 2001;8:356-61.
17. D'Amour FE, Smith DL. A method for determining loss

of pain sensation. J Pharmacol Exp Ther 1941;72:74-9.
18. Vogel HG. Analgesic, anti-inflammatory, and anti-pyretic activity. In: Drug Discovery and Evaluation: Pharmacological Assays. Berlin, Heidelberg: Springer; 2007. p. 983-1116.
19. Kumae GS, Rajesh K, Sengottuvelu S. Evaluation of analgesic and anti inflammatory activity of methanolic extract of cocculus hirsutos leaves. Int Res J Pharm 2011;2:230-4.
20. Sakat SS, Juvekar AR, Gambhire MN. *In vitro* antioxidant and antiinflammatory activity of methanol extract of *Oxalis corniculata* Linn. Int J Pharm Pharm Sci 2010;2:146-55.
21. Reshma, Arun KP, Brindha P. *In vitro* anti-inflammatory, antioxidant andnephroprotective studies on leaves of aegle marmelos and *Ocimum sanctum*. Asian J Pharm Clin Res 2014;7:121-9.
22. Chandra S, Chatterjee P, Dey P, Bhattacharya S. Evaluation of *in vitro* anti-inflammatory activity of coffee against the denaturation of protein. Asian Pac J Trop Biomed 2012;2:S178-80.
23. Winter CA, Risley EA, Nuss GW. Carrageenin-induced edema in hind paw of the rat as an assay for antiinflammatory drugs. Proc Soc Exp Biol Med 1962;111:544-7.
24. Tripathi KD. *Essentials of Medical Pharmacology*. 5th ed. New Delhi, India: Jaypee Brothers Medical Publishers; 2004.
25. Shreedhara CS, Vaidya VP, Vagdevi HM, Latha KP, Muralikrishna KS, Krupanidhi AM. Screening of *Bauhinia purpurea* Linn. For analgesic and anti-inflammatory activities. Indian J Pharmacol 2009;41:75.
26. Hiruma-Lima CA, Gracioso JS, Bighetti EJ, Robineou LG, Brito AS. The juice of fresh leaves of *Boerhaavia diffusa* L. (*Nyctaginaceae*) markedly reduces pain in mice. J Ethnopharmacol 2000;71:267-74.
27. Carlsson KH, Jurna I. Depression by flupirtine, a novel analgesic agent, of motor and sensory responses of the nociceptive system in the rat spinal cord. Eur J Pharmacol 1987;143:89-99.
28. Chen YF, Li N, Jiao YL, Wei P, Zhang QY, Rahman K, *et al*. Antinociceptive activity of petroleum ether fraction from the MeOH extracts of *Paederia scandens* in mice. Phytomedicine 2008;15:427-36.
29. Koo HJ, Lim KH, Jung HJ, Park EH. Anti-inflammatory evaluation of gardenia extract, geniposide and genipin. J Ethnopharmacol 2006;103:496-500.
30. Vogel H. Drug Discovery and Evaluation: Pharmacological Assays. Berlin, Germany: Springer; 2007.
31. Udegbunam RI, Nwamkpa OK, Udegbunam SO, Nwaehujor CO, Offor GE. Evaluation of anti-inflammatory activities of root extracts of *Stephania dinklagei* (Engl.) Diels. Afr J Pharm Pharmacol 2012;6:834-9.
32. Guzik T, Korbut R, Adamek-Guzik T. Nitric oxide and superoxide in inflammation. J Physiol Pharmacol 2003;54:469-87.
33. Gandhidasan R, Thamaraichelvan A, Baburaj S. Anti-inflammatory action of *Lannea coromandelica* by HRBC membrane stabilization. Fitoterapia 1991;62:81-3.
34. Shenoy S, Shwetha K, Prabhu K, Maradi R, Bairy KL, Shanbhag T. Evaluation of antiinflammatory activity of *Tephrosia purpurea* in rats. Asian Pac J Trop Med 2010;3:193-5.
35. Vadivu R, Lakshmi KS. *In vitro* and *in vivo* anti-inflammatory activity of leaves of *Symplocos cochinchnensis* (Lour) Moore ssp Laurina. Bangladesh J Pharmacol 2008;3:121-4.
36. Di Rosa ML, Giroud JP, Willoughby DA. Studies of the mediators of the acute inflammatory response induced in rats in different sites by carrageenan and turpentine. J Pathol 1971;104:15-29.
37. Spector WG, Willoughby DA. The inflammatory response. Bacteriol Rev 1963;27:117.
38. Sawatzky DA, Megson IL, Rossi AG. Sildenafil offers protection against NSAID-induced gastric injury. Br J Pharmacol 2005;146:477-8.
39. Nantel F, Denis D, Gordon R, Northey A, Cirino M, Metters KM, *et al*. Distribution and regulation of cyclooxygenase-2 in carrageenan-induced inflammation. Br J Pharmacol 1999;128:853-9.
40. Beutler B, Cerami A. The biology of cachectin/TNF--a primary mediator of the host response. Ann Rev Immunol 1989;7:625-55.
41. Park WH, Park SY, Kim HM, Kim CH. Effect of a Korean traditional formulation, Hwaotang, on superoxide generation in human neutrophils, platelet aggregation in human blood, and nitric oxide, prostaglandin E2 production and paw oedema induced by carrageenan in mice. Immunopharmacol Immunotoxicol 2004;26:53-73.
42. Martel-Pelletier J, Lajeunesse D, Reboul P, Pelletier JP. Therapeutic role of dual inhibitors of 5-LOX and COX, selective and non-selective non-steroidal anti-inflammatory drugs. Ann Rheum Dis 2003;62:501-9.

Source of Support: Nil. **Conflicts of Interest:** None declared.