

# Antinociceptive, Immunomodulatory and Antipyretic Activity of Nymphayol Isolated from *Nymphaea stellata* (Willd.) Flowers

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## Abstract

In the present study, we aimed to analyze the antinociceptive, immunomodulatory and antipyretic activities of nymphayol were investigated in wistar rats and mice. Antinociceptive effect was evaluated by acetic acid induced writhing, formalin induced paw licking and hot-plate tests. Immunomodulatory activity was assessed by neutrophil adhesion test, humoral response to sheep red blood cells, delayed-type hypersensitivity, phagocytic activity and cyclophosphamide induced myelosuppression. Antipyretic activity was evaluated by yeast induced hyperthermia in rats. Nymphayol produced significant ( $p < 0.05$ ) antinociceptive activity in acetic acid induced writhing response and late phase of the formalin induced paw licking response. Pre-treatment with nymphayol (50 mg/kg, oral) evoked a significant increase in neutrophil adhesion to nylon fibres. The augmentation of humoral immune response to sheep red blood cells by nymphayol (50 mg/kg) was evidenced by increase in antibody titres in rats. Oral administration of nymphayol (50 mg/kg) to rats potentiated the delayed-type hypersensitivity reaction induced by sheep red blood cells. Treatment with nymphayol showed a significant ( $p < 0.05$ ) reduction in pyrexia in rats. The results suggest that nymphayol possesses potent anti-nociceptive, immunomodulatory and antipyretic activities.

**Key Words:** Antinociceptive, Immunomodulatory, Antipyretic, *Nymphaea stellata*, Nymphayol, Writhing response

## INTRODUCTION

The immune system is known to be involved in the etiology as well as pathophysiological mechanisms of many diseases. During an inflammatory process, infectious agents, toxins, and tissue fluids enter the circulation and cause fever. The production of fever by prostaglandins is supported by the fact that anti-inflammatory agents can inhibit prostaglandin production may also be antipyretic (Futaki *et al.*, 1994). We have studied the different pathological conditions with common mechanism called prostaglandin synthesis. Accompanying the inflammatory process is often pain or hyperalgesia, and fever. It is not, therefore, surprising that both pain and fever are the first signs of inflammation to be relieved by antiprostaglandin therapy. Plants have also been evaluated for their anabolic, antistress/adaptogenic, nootropic, antioxidant and antiageing effects. Such as steroids, flavonoids and tannins could account for the antinociceptive and antiinflammatory actions by means of inhibiting the synthesis of prostaglandin or products of arachidonic acid metabolism and the production of reactive oxygen

species (Kianbakht and Ghazavi, 2011).

Several classes of antioxidant dietary compounds have been suggested to present health benefits, and there are evidences that consumption of these products leads to a reduction of the expression of various pro-inflammatory and/or oxidative stress biomarkers (Halliwell, 2002). The use of traditional medicine is widespread and plants still represent a large source of natural antioxidants that might serve as leads for the development of novel drugs. Several anti-inflammatory, digestive, antinecrotic, neuroprotective, and hepatoprotective drugs have recently been shown to have an antioxidant and/or radical scavenging mechanism as part of their activity (Lin and Huang, 2002). The phytochemicals from Indian medicinal plants are claimed to induce paraimmunity, the non-specific immunomodulation of macrophages, granulocytes, NK cells, lymphocytes and complement functions. The isolation, purification and chemical characterization of the immunoactive phytochemicals have been carried out in some of the plants (Wagner, 1984).

*Nymphaea stellata* Willd. (Nymphaeaceae), a medicinal plant

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has been mentioned for the treatment of liver disorders in Ayurveda, an ancient system of medicine. The leaves, roots and flowers have a wide range of pharmacological activities and are used for diabetes, eruptive fevers and as cardiogenic, emollient, diuretic, narcotic and as an aphrodisiac (Nadkarni, 1954). It has hepatoprotective, anti-inflammatory, and particularly antidiabetic activity (Das *et al.*, 2012). In our previous study, we have isolated nymphyol (25, 26-dinorcholest-5-en-3 $\beta$ -ol) a sterol from *N. stellata* Willd. flowers which reverse the damaged endocrine tissue and stimulate secretion of insulin (Subash-Babu *et al.*, 2009). But yet the compound has not been subjected to systematic scientific investigation to assess its immunomodulatory potential. Therefore we aimed to investigate its antinociceptive and antipyretic effect through immunomodulatory potential. Indeed, published biological actions of phytosterol and terpenoids identified from *Bryophyllum Pinnatum* appear to justify the findings of the present study, as well as the folkloric uses of the plant, especially in the control and management of painful and inflammatory conditions, and diabetes mellitus (Ojewole, 2005). Through this study, we report the use of nymphyol as anti-nociceptive, immunomodulatory and antipyretic agent using animal models. Possible mechanisms of action are also examined.

## MATERIALS AND METHODS

### Animals

Adult wistar albino rats (200-220 g) and mice (24-28 g) of either sex were used for the experiments. Animals were maintained on 12 h light/dark cycle at approximately 25  $\pm$  1°C and relative humidity of 60-70%; they had access to diet and water *ad libitum* and were acclimatized for at least 2 weeks before starting the experiments. All studies were carried out using six animals in each group. All the animal experiments were conducted according to the ethical norms approved by Ministry of Social Justice and Empowerment, Government of India and Institutional Animal Ethics Committee guidelines.

### Chemicals and drugs

Indomethacin, Sheep Red Blood Cells (SRBC), Formalin, Naloxone, Morphine, Levamisole (lev) and Cyclophosphamide (cyclo) were obtained from Sigma-Aldrich (USA). CMC (Carboxymethylcellulose) was obtained from Himedia (India).

### Plant collection, extraction and fractionation

Fresh *N. stellata* Willd. Flowers were collected from Thiruvallur district, Tamil Nadu, India. The species was identified and authenticated by Dr. D. Narasimhan, Taxonomist, Department of Botany, Madras Christian College, Chennai and the voucher specimen (MPC-186) was deposited at Institute herbarium, ERI, Loyola College, Chennai, Tamil Nadu, India. The flowers of *N. stellata* were shade dried and coarsely powdered. Three kilograms of powder was sequentially extracted with hexane, chloroform, ethyl acetate and methanol successively at room temperature for 48 h respectively. All the extracts were filtered and concentrated under reduced pressure using rotary evaporator to get completely dried extracts (Subash-Babu *et al.*, 2009).

The *N. stellata* Flower chloroform extract (25 gm) was chromatographed over silica gel column (Acme's silica gel, 100-200 mesh size, 750 gm, 3.5 i.d.  $\times$  60 cm) and successively

eluted with stepwise gradient of hexane, hexane: chloroform and hexane: ethyl acetate system (0, 5, 10, 20, 30, 50, 70 and 100%). 74 fractions (each 150 ml) were collected; each fraction was spotted on a precoated Silica gel 60 F<sub>254</sub>, 0.25 mm thick TLC plate (Merck) and eluted in hexane: ethyl acetate (4:1) system and fractions with similar R<sub>f</sub> values in TLC pattern were pooled together. Finally 17 major fractions were obtained and a crystal (30:70-Hexane: Ethyl acetate) was obtained from fraction 12 (4.8 gm). The isolated compound was subjected to spectral analysis like, <sup>1</sup>H NMR, <sup>13</sup>C NMR, IR and MASS. Tetra methyl silane (TMS) was used as standard which shows chemical shift value at zero on the  $\delta$  scale. <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded with a JEOL 300 MHz FT NMR spectrometer (H1) 75 MHz (C13) and chemical shifts were recorded in ppm. IR spectra were recorded in Shimadzu by KBr pellet method. IR spectra were taken on a Perkin Elmer FT-IR (Spectrum One) spectrophotometer. High resolution Electron Impact Mass Spectroscopy (EI-MS) was performed. Mass spectra were taken on a Jeol JMS-DX30 spectrometer (Subash-Babu *et al.*, 2009).

### Acute toxicity study

In a separate experiment, 5 groups of wistar rats (each 10) were utilized to determine the acute toxicity effect. Different doses of nymphyol (10, 20, 40, 80, 160 and 320 mg/kg b.wt.) were orally administered using 0.5% carboxy methyl cellulose (CMC) as a vehicle solution for 7 days. Behavioral changes like abnormal locomotion, respiratory distress, uncoordinated muscle movements, weight loss and mortality were observed during the experimental period.

### Acetic acid-induced writhing response in mice

Mice were divided into nine groups of six animals each. The study was carried out by a modified method of Mungantiwar *et al.* (1999). Each mouse was given an injection of 0.75% acetic acid aqueous solution in a volume of 0.1 ml/10 g b.wt. into the peritoneal cavity and the animals were then placed in a transparent plastic box. The number of writhes was counted for 15 min beginning from 5 min after the acetic acid injection. Test drugs nymphyol (50 mg/kg p.o.), indomethacin (10 mg/kg p.o.), morphine (5 mg/kg s.c.), morphine+naloxone (5 mg/kg s.c.+2 mg/kg i.p.), nymphyol+naloxone (50 mg/kg p.o.+2 mg/kg i.p.), indomethacin+naloxone (10 mg/kg p.o.+2 mg/kg i.p.) and control vehicle (0.5 ml of 0.5% CMC p.o.) were administered 1 h before the acetic acid injection.

### Formalin induced paw licking response in mice

This test was performed according to the method of Resine and Pasternack (1996). Mice were used and divided into two sets of nine groups of six animals. Test drugs nymphyol (50 mg/kg p.o.), indomethacin (10 mg/kg p.o.), morphine (5 mg/kg s.c.), morphine+naloxone (5 mg/kg s.c.+2 mg/kg i.p.), nymphyol+naloxone (50 mg/kg p.o.+2 mg/kg i.p.), indomethacin+naloxone (10 mg/kg p.o.+2 mg/kg i.p.) and control vehicle (0.5 ml of 0.5% CMC p.o.) were administered 1 h prior to formalin injection to animals in the first set (for early phase) and 40 min prior to formalin injection to animals in the second set (for late phase), respectively. Mice were injected subcutaneously with 50  $\mu$ l of 1% formalin in normal saline solution into the right dorsal hind paw. The time animals spent in licking of injected paw was determined during 0-5 min (the first set of mice for early phase) and during 20-30 min (the second

set of mice for late phase) after the injection of formalin.

### Hot plate test in mice

Experiments were carried out according to previously described method (Parkhouse and Pleuvry, 1979). Mice were used and divided into seven groups of six animals. For testing, mice were placed on hot plate maintained at  $55 \pm 0.5^\circ\text{C}$ . The time that elapsed until occurrence of either a hind paw licking or a jump off from the surface was recorded as the hot plate latency. Before treatment, the reaction time of each mouse (licking of the forepaws or jumping response) was done at 0 and 10 min interval. The average of the two readings was obtained as the initial reaction time. Mice with baseline latencies of  $<5$  s or  $>30$  s were eliminated from the study. The initial reaction time following the administration of nymphayol (50 mg/kg p.o.), morphine (5 mg/kg s.c.), naloxone+morphine (2 mg/kg i.p.+5 mg/kg s.c.), naloxone+ nymphayol (2 mg/kg i.p.+5 mg/kg p.o.) and vehicle (0.5 ml of 0.5% CMC p.o.) was measured at 30 min.

### Neutrophil adhesion test

The method originally described by Wilkinson (1978) was employed. Rats of Group I served as control and received 0.5% CMC. Groups II, III and IV were pretreated with nymphayol (50 mg/kg, oral). On 14th day of drug treatment, blood samples were collected (before challenge) by puncturing retro-orbital plexus into heparinized vials and analyzed for total leukocyte cell (TLC) and differential leukocyte cell (DLC) counts. After initial counts, blood samples were incubated with 80 mg/ml of nylon fibres for 15 min at  $37^\circ\text{C}$ . The incubated blood samples were again analyzed for TLC and DLC. The product of TLC and percent neutrophil gives neutrophil index of blood sample. The percent neutrophil adhesion was calculated as shown below:

$$\text{Neutrophil adhesion (\%)} = \frac{\text{Nlt} - \text{Nlu}}{\text{Nlu}} \times 100$$

where Nlu is the Neutrophil index of untreated blood samples and Nlt is the Neutrophil index of treated blood samples.

### Humoral antibody response

The method used was similar to that described previously by Puri *et al.* (1993). On 14th and 21st day of drug treatment, each rat was immunized with  $0.5 \times 10^9$  SRBCs/ml/rat by i.p. route, including rats of control group. On 21st and 27th day of the treatment, primary and secondary antibody titres were determined. Blood samples were collected from individual animals from the orbital plexus. Two fold dilutions of sera were performed in 0.15 phosphate buffered saline (pH 7.2) and 50  $\mu\text{l}$  of each dilution was aliquoted into 96-well microtitre plates. A 25  $\mu\text{l}$  of fresh 1% SRBC suspension in the above buffer was dispersed into each well and mixed. The plates were incubated at  $37^\circ\text{C}$  for 2 h and examined visually for agglutination. The value of the highest serum dilution causing haemagglutination was taken as the antibody titre. Antibody titres were expressed in a graded manner, the minimum dilution (1/2) being ranked as 1; the mean ranks of different groups were compared for statistical significance. Each experimental group contained 6 animals.

### Delayed type hypersensitivity (DTH) response

Six animals per group (control and treated) were immunized (Nelson and Mildenhall, 1967) by i.p. administration of  $0.5 \times 10^9$  SRBCs/rat and challenged by s.c. administration of  $0.025 \times 10^9$  SRBCs/ml into right hind foot pad on day +14. To establish the effect of nymphayol and lev (levamisole) on this immune response, daily doses of nymphayol (50 mg/kg b.w.) and lev (2.5 mg/kg b.w.) were administered orally from day-14 until day +13. Simultaneously, another group of animals (control) was inoculated with 0.5 ml of 0.5% CMC under the same conditions. DTH response was measured at 24 and 48 h after SRBCs challenge on day +14. Foot pad swelling was measured with a digital vernier caliper. The difference between the means of right and left hind foot pad thickness gave a degree of foot pad swelling which was used for group comparisons.

### Phagocytic response

The method described by Hudson and Hay (1980) was followed. Groups of six rats were injected intravenously with 0.2 ml of (1.6%, w/v) suspension of carbon particles (size, 20-25  $\mu\text{m}$ ) stabilized in gelatin 30 min after the last dose of nymphayol (50 mg/kg b.w.) and lev (2.5 mg/kg b.w.) Blood samples were collected from the tip of the tail at different intervals after injection and percent transparency was determined spectrophotometrically at 675 nm until the transparency equivalent to the standard (original preinjection blood sample) was obtained. Nymphayol was administered orally for 5 days and 30 min prior to the carbon injection. The rate of carbon clearance (phagocytic index, K) was calculated from the slope of each time-concentration curve drawn by plotting 100-mean transmittance values as ordinate on semilogarithmic paper against time as abscissa.

### Cyclophosphamide induced immunosuppression

The method described by Ziauddin *et al.* (1996) was followed. Rats were divided into six groups designated as I-VI, each group containing six rats. The control Group I received 0.5 ml of 0.5% CMC. Group II was administered with only cyclophosphamide at the dose of 30 mg/kg, i.p. while Groups III, IV, V and VI rats received cyclophosphamide along with test drugs, i.e. nymphayol (50 mg/kg, p.o.) or lev (2.5 mg/kg, p.o.) for 10 days. On day 11, blood sample was collected from the retro-orbital plexus of individual animals and analyzed for haematological and serological parameters.

### Antipyretic activity in rats

Hyperthermia was induced in rats by the method of Vogel and Vogel (1997). Rats were given 10 ml/kg of 20% aqueous suspension of brewer's yeast subcutaneously. Initial rectal temperature was recorded. When the temperature was at peak, i.e. 18 h after yeast injection, only rats which developed satisfactory pyrexia ( $1^\circ\text{C}$  or more increase in rectal temperature) were used. The thermometer was inserted about 3 cm into the rectum of each rat. Nymphayol (50 mg/kg b.w.) was administered to three groups. Control group received 0.5 ml of vehicle. Paracetamol (150 mg/kg b.w.) was used as reference drug. Rectal temperature was determined at 60, 90 and 120 min after drug administration.

### Statistical analysis

Data were statistically analysed by analysis of variance (ANOVA) followed by Student's *t*-test, Tukey's test for the

stastical significance and a probability level lower than 0.05 was considered statistically significant (Tallarida and Murria, 1987).

## RESULTS

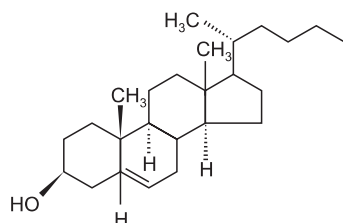
### Identification of Nymphayol

MASS: EIMS (m/z): 358 [m+], 343 [m+\_me], 273 [m-side chain]+, 325 [m-CH<sub>3</sub>-H<sub>2</sub>O], 287 [m+-Ring B cleavage], 231 [m-side chain-ring D cleavage]+, 329 [m-CH<sub>3</sub>-CH<sub>2</sub>]+, 315 [m-CH<sub>3</sub>-(CH<sub>2</sub>)<sub>2</sub>]+. IR: IR c KBr/max cm<sup>-1</sup>: 3,433 (hydroxyl); 2,936, 2,866, 1,645 (trisub double bond); 1,464, 1,377, 1,231, 1,054, 801 (trisub double bond); Molecular formula is C<sub>25</sub>H<sub>42</sub>O. Based on the above spectral data the isolated compound was confirmed as 26, 27-dinorcholest-5-en-3-β-ol, a sterol (Fig. 1). It was named as Nymphayol (Subash-Babu *et al.*, 2009).

### Toxicity and effective dose evaluation

There were no behavioural changes in the rats. Mortality was not observed during acute toxicity study. The results revealed that nymphayol up to an oral dose of 320 mg/kg b.wt. was nontoxic and safe in animal models.

To determine the effective dose, different doses of nymphayol *viz.* 12.5, 25, 50, 100 and 200 mg/kg b.wt. were screened for anti-nociceptive, immunomodulatory and antipyretic activity. 50 mg/kg b. w. dose showed significant effect compared to 12.5 and 25 mg/kg b.wt. 100 and 200 mg/kg b.wt. doses of nymphayol also produced the same effect. So 50 mg/kg body



**Fig. 1.** Chemical structure of a nymphayol.

weight was selected as the upper limit for further studies.

### Antinociceptive effects

Evidence of antinociceptive activities of nymphayol was detected in the three different models for nociception used to investigate the antinociceptive effect.

Nymphayol significantly reduced writhings and stretchings induced by acetic acid (Table 1). The protective effect of nymphayol was dose dependent with 76.7% ( $p < 0.05$ ) reduction observed in 50 mg/kg b.wt. Indomethacin (10 mg/kg b.w.) inhibited 78.5% ( $p < 0.05$ ) and morphine (a centrally acting analgesic) inhibited 94.6% ( $p < 0.05$ ). Statistically there's no difference between the nymphayol-alone group and the naloxone+ nymphayol group. On the other hand naloxone completely arrested morphine activity. Nymphayol exerted its action only on the second phase (20-30 min) compared to the first phase (0-5 min). These phases corresponded to neurogenic and inflammatory pains respectively. The dose 50 mg/kg b.wt. inhibited 20.0% ( $p < 0.05$ ) in the first phase and 62.1% ( $p < 0.05$ ) in the second phase. Indomethacin was significantly active (72.9%,  $p < 0.05$ ) on the second phase whereas morphine acted in both the phases (Table 1). The opioid antagonist naloxone inhibited the action of morphine at both the phases, but the activity of nymphayol was not interrupted by naloxone. In both tests (acetic acid induced writhing and formalin induced paw licking) the activity of indomethacin was not disrupted by naloxone.

In the hot plate test, nymphayol did not show any activity. Morphine sulphate at 05 mg/kg b.wt. manifested its maximum latent time of 36.01 ( $p < 0.05$ ). Action of morphine was completely arrested by naloxone (2 mg/kg b.w.) (Table 2).

### Neutrophil adhesion test

Pretreatment of nymphayol (50 mg/kg, oral) evoked a significant ( $p < 0.05$ ) increase in the *in vitro* neutrophil adhesion to nylon fibres which correlated with the increase in percent neutrophils when compared with respective control (Table 3).

### Haemagglutinating antibody (HA) titer

The HA titre was used to assess humoral immune response. A dose-related increase in both primary and secondary anti-

**Table 1.** Effects of nymphayol, indomethacin, morphine and naloxone on acetic acid-induced writhing response and formalin-induced paw licking in mice

Test samples	Treatment (mg/kg, p.o.)	Acetic acid <sup>a</sup>		Formalin test <sup>b</sup>	
		Number of writhes	Early phase Licking time (s)	Late phase Licking time (s)	
Control	-	56.01 ± 4.26	35.01 ± 2.67	37.01 ± 2.82	
Indomethacin	10	12.00 ± 0.91 (78.5)*	30.01 ± 2.28 (14.2) <sup>NS</sup>	10.00 ± 0.76 (72.9)*	
Nymphayol	50	13.00 ± 0.99 (76.7)*	28.00 ± 2.13 (20) <sup>NS</sup>	14.00 ± 1.07 (62.1)*	
Morphine	05	3.00 ± 0.23 (94.6)*	3.00 ± 0.23 (91.4)*	3.00 ± 0.23 (91.8)*	
Morphine+Naloxone	05+02	57.01 ± 4.34 (-1.78) <sup>NS</sup>	36.01 ± 2.74 (-2.8) <sup>NS</sup>	37.01 ± 2.82 (0.00) <sup>†</sup>	
Nymphayol+Naloxone	50+02	14.00 ± 1.07 (75.0)* <sup>@</sup>	28.00 ± 2.13 (20) <sup>NS</sup>	16.00 ± 1.22 (56.7)* <sup>@</sup>	
Indomethacin+Naloxone	10+02	12.00 ± 0.91 (78.5)* <sup>@</sup>	29.00 ± 2.21 (17.4) <sup>NS</sup>	10.00 ± 0.76 (72.9)* <sup>@</sup>	

NS: non significant.

Data represent mean ± S.D. (standard deviation) (n=6).

<sup>a,b</sup>Values in the parenthesis indicate writhing and paw licking inhibition percentage.

Comparison made between: \*Control with all the groups. <sup>@</sup>Morphine+Naloxone with Nymphayol+Naloxone and Indomethacin+Naloxone.

<sup>†</sup>Morphine with Morphine+Naloxone. <sup>†</sup> $p < 0.05$  significant from the control.

body titre was observed in rats treated with nymphayol. The augmentation of the humoral immune response to SRBCs by nymphayol was evidenced by increase in the antibody titres in the blood of rats (Table 4).

### Delayed-type hypersensitivity (DTH) reactions

The cell-mediated immune response was assessed by DTH reaction, i.e. foot pad reaction. Nymphayol produced a significant, dose-related increase in DTH reactivity in rats. Increase in DTH reaction in rats in response to T cell dependent antigen revealed the stimulatory effect of nymphayol on T cells (Table 5).

### Phagocytic response

The phagocytic activity of the reticulo-endothelium system (RES) was measured by the rate of removal of gelatin-stabilized carbon particles from the blood circulation. Oral admin-

istration of nymphayol (50 mg/kg) for 5 days and 30 min prior to carbon injection exhibited a dose-related increase in the clearance rate of carbon by the cells of the RES (Table 5).

### Cyclophosphamide-induced immunosuppression

Cyclophosphamide at the dose of 30 mg/kg, i.p. caused a significant reduction in the haemoglobin, RBCs and WBCs count. Combined treatment of cyclophosphamide and nymphayol (50 mg/kg, p.o.) resulted in a restoration of bone marrow activity as compared with cyclophosphamide alone treated groups (Table 6).

### Yeast-induced hyperthermia in rats

The results of the antipyretic effect of the nymphayol are presented in Table 7. Administration of brewer's yeast to rats produced a significant increase in rectal temperature 18 h after yeast injection ( $p < 0.05$ ). The results of the antipyretic study showed that oral administration of nymphayol at 50 mg/kg b.wt. caused a significant ( $p < 0.05$ ) inhibition of pyrexia induced by yeast.

**Table 2.** Effects of the nymphayol, morphine and naloxone on pain threshold of mice in the hot plate test

Test samples	Treatment (mg/kg, p.o.)	Mean latent time (sec.)	
		Initial	After 30 min.
Control	-	11.00 ± 0.84	11.00 ± 0.84
Morphine	05	10.00 ± 0.76 <sup>NS</sup>	36.00 ± 2.74*
Nymphayol	50	11.00 ± 0.84 <sup>NS</sup>	11.00 ± 0.84 <sup>NS</sup>
Morphine+	05+02	11.00 ± 0.84 <sup>NS</sup>	11.00 ± 0.84 <sup>†</sup>
Naloxone			
Nymphayol+	50+02	10.00 ± 0.76 <sup>NS</sup>	10.00 ± 0.76 <sup>NS</sup>
Naloxone			

NS: non significant.

Data represent mean ± S.D. (standard deviation) (n=6). Comparison made between: \*Control with all the groups. <sup>®</sup>Morphine+Naloxone with Nymphayol+Naloxone, <sup>†</sup>Morphine with Morphine+Naloxone, <sup>†</sup> $p < 0.05$  significant from the control.

**Table 4.** Effects of nymphayol on antibody titers in antigenically challenged rats

Test samples	Treatment (mg/kg, p.o.)	Haemagglutination antibody (HA) titres	
		1° HA titre	2° HA titre
Control	-	2.00 ± 0.15	3.00 ± 0.23
Nymphayol	50	5.30 ± 0.40*	7.00 ± 0.53*
Levamisole	2.5	5.00 ± 0.38*	6.70 ± 0.51*

Data represent mean ± S.D. (standard deviation) (n=6).

\* $p < 0.05$  significant from the control.

**Table 3.** Effects of nymphayol on neutrophil adhesion test

Test samples	Treatment (mg/kg, p.o.)	Neutrophil index		Neutrophil adhesion (%)
		UB	FTB	
Control	-	254.04 ± 19.34	226.04 ± 17.21	12.00 ± 0.91
Nymphayol	50	318.05 ± 24.22*	251.04 ± 19.12*	26.00 ± 1.98*
Levamisole	2.5	328.05 ± 24.98*	260.04 ± 19.80*	29.00 ± 2.21*

Data represent mean ± S.D. (standard deviation) (n=6).

\* $p < 0.05$  significant from the control.

**Table 5.** Effects of nymphayol on foot pad reaction of antigenically challenged rats and phagocytic response

Test samples	Treatment (mg/kg, p.o.)	Paw thickness (mm)		Phagocytic index
		24 h	48 h	
Control	-	4.70 ± 0.36	3.88 ± 0.30	0.28 ± 0.02
Nymphayol	50	7.30 ± 0.56*	6.80 ± 0.52*	0.42 ± 0.03*
Levamisole	2.5	7.00 ± 0.53*	6.72 ± 0.51*	0.49 ± 0.04*

Data represent mean ± S.D. (standard deviation) (n=6).

\* $p < 0.05$  significant from the control.

**Table 6.** Effects of nymphyalol on blood cells of rats treated with cyclophosphamide for 10 days

Test samples	Treatment (mg/kg, p.o.)	RBC ( $\times 10^4/\mu\text{l}$ )	Haemoglobin (g/dl)	WBC ( $\times 10^2/\mu\text{l}$ )
Control	-	915.15 $\pm$ 69.69	10.75 $\pm$ 0.82	78.01 $\pm$ 5.94
Nymphyalol	50	1,225.20 $\pm$ 93.29*	15.24 $\pm$ 1.16*	98.02 $\pm$ 7.46*
Levamisole	2.5	1,237.21 $\pm$ 94.21*	16.10 $\pm$ 1.23*	106.02 $\pm$ 8.07*

Data represent mean  $\pm$  S.D. (standard deviation) (n=6).

\* $p < 0.05$  significant from the control.

**Table 7.** Effects of nymphyalol on yeast- induced hyperthermia in rats

Test samples	Treatment (mg/kg, p.o.)	Rectal temperature ( $^{\circ}\text{C}$ )					
		Before yeast	18 hr after yeast	Time after treatment (min)			
				30	60	90	120
Control	-	37.11 $\pm$ 2.83	39.19 $\pm$ 2.98	39.26 $\pm$ 2.99	39.23 $\pm$ 2.99	39.28 $\pm$ 2.99	39.11 $\pm$ 2.98
Nymphyalol	50	37.11 $\pm$ 2.83	39.13 $\pm$ 2.98	38.55 $\pm$ 2.94	37.83 $\pm$ 2.88*	37.51 $\pm$ 2.86*	37.34 $\pm$ 2.84*
Paracetamol	150	37.13 $\pm$ 2.83	39.27 $\pm$ 2.99	38.14 $\pm$ 2.90	37.52 $\pm$ 2.86*	37.37 $\pm$ 2.85*	37.11 $\pm$ 2.83*

Data represent mean  $\pm$  S.D. (standard deviation) (n=6).

\* $p < 0.05$  significant from the control.

## DISCUSSION

In the present study we evaluated the antinociceptive, immunomodulatory and antipyretic effects of nymphyalol, a compound isolated from *N. stellata*, employing various experimental test models.

The results indicated that nymphyalol exhibited peripheral antinociceptive activity. Nymphyalol showed antinociceptive activity in acetic acid induced writhing response and formalin induced paw licking response (only on inflammatory phase). The formalin test possessed two distinctive phases which reflected different types of pain. The earlier phase reflected direct effect of formalin on nociceptors (noninflammatory pain), whereas the late phase reflected pain from inflammation (Hun-skaar and Hole, 1987). Nymphyalol showed antinociceptive activity on second phase of the formalin test, implying its effect on the synthesis and/or release of PGs and/or other pain mediators. The hot plate test is a specific central antinociceptive test (Parkhouse and Pleuvry, 1979). Nymphyalol did not show activity on hot plate test which indicated inefficiency of nymphyalol on neurogenic pain. As the antinociceptive activity of nymphyalol was not inhibited by naloxone. This indicated that nymphyalol did not act through the spinal opioid receptors such as  $\mu_2$ ,  $\kappa_1$  and  $\delta_2$ ; some other mechanism of action such as inhibition of cyclooxygenase is possible.

Modulation of the immune response through stimulation or suppression may help in maintaining a disease-free state. Agents that activate host defense mechanisms in the presence of an impaired immune responsiveness can provide supportive therapy to conventional chemotherapy (Wagner, 1984). The results obtained in the present study indicated that nymphyalol was a potent immunostimulant, stimulating both the specific and non-specific immune mechanisms.

The neutrophil, an end cell unable to divide and with limited capacity for protein synthesis, is nevertheless, capable of a wide range of responses, in particular chemotaxis, phagocy-

toxis, exocytosis and both intracellular and extracellular killing (Dale and Foreman, 1984). In the present study nymphyalol (50 mg/kg, p.o.) evoked a significant increase in percent neutrophils. This may help in increasing immunity of body against microbial infections (Benacerraf, 1978).

Antibody molecules, a product of B lymphocytes and plasma cells, are central to humoral immune responses. IgG and IgM are the major immunoglobulins which are involved in the complement activation, opsonization, neutralization of toxins, etc. (Miller, 1991). The augmentation of the humoral immune response to SRBCs by nymphyalol, as evidenced by increase in the antibody titre in rats, indicated enhanced responsiveness of T and B lymphocyte subsets involved in antibody synthesis (Benacerraf, 1978).

Cell-mediated immunity (CMI) involves effector mechanisms carried out by T lymphocytes and their products (lymphokines). CMI responses are critical to defense against infectious organisms, infection of foreign grafts, tumour immunity and delayed-type hypersensitivity reactions (Miller, 1991). Therefore increase in DTH reaction in rats in response to T cell dependent antigen revealed the stimulatory effect of nymphyalol on T cells (Table 3).

Phagocytosis is the process by which certain body cells, collectively known as phagocytes, ingest and removes microorganisms, malignant cells, inorganic particles and tissue debris (Miller, 1991). Nymphyalol appears to enhance the phagocytic function by exhibiting a dose related increase in clearance rate of carbon by the cells of the reticulo-endothelial system.

Since nymphyalol augmented the circulating antibody titre it was thought worthwhile to evaluate its effect on peripheral blood count and cyclophosphamide-induced immunosuppression. The administration of nymphyalol significantly ameliorated the total WBCs count, RBCs count and haemoglobin and also restored the myelosuppressive effects induced by cyclophosphamide.

Antipyretic activity is commonly mentioned as a character-

istic of drugs or compounds which have an inhibitory effect on prostaglandin-biosynthesis (Vane, 1987). The yeast induced hyperthermia in rats was employed to investigate the antipyretic activity of nymphayol. It was found that nymphayol caused a significant decrease in rectal temperature similar to paracetamol. This result seems to support the view that nymphayol has some influence on prostaglandin-biosynthesis, because prostaglandin is believed to be a regulator of body temperature (Milton, 1982).

The results of the present study empirically indicated that nymphayol was effective in the treatment of inflammatory diseases. Nymphayol showed potent *in vivo* antinociceptive, immunomodulatory and antipyretic effect. Inhibition of the synthesis and/or release of inflammatory mediators may be the main mechanism(s) of action of nymphayol. Due to the various biological activities of nymphayol it would be worthwhile to conduct further research in order to develop it into a drug.

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