Antiinflammatory Activity of Hot Water Infusion of *Nyctanthes arbo-tristis* Flowers

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Ratnasooriya, *et al.*: Antiinflammatory Activity and *Nyctanthesarbo-tristis* Flowers

In Sri Lankan ethnomedicate it is claimed the flowers of *Nyctanthes arbo-tristis* is effective in the treatment of inflammatory conditions but this has not been scientifically validated. This experiment was carried to investigate the antinflammatory potential of hot water infusion of *Nyctanthes arbo-tristis* flowers. Oral antiinflammatory activity of hot water infusion of *Nyctanthes arbo-tristis* flowers (concentrations: 3.75, 7.5, 12.5 and 18.75 mg/kg) was assessed in rats using both acute (carrageenan-induced paw oedema assay) and chronic (formaldehyde induced-paw oedema and cotton pellet-granuloma tests) inflammatory models. In an attempt to investigate its mode of action, antihistamine activity (by wheal test), inhibition of prostaglandin synthesis (by enteropooling test), inhibition of Tumor necrosis factor$_\alpha$ secretion (using human mononuclear cells), and suppression of vascular permeability (acetic acid-induced vascular permeability test) and cytotoxicity (Evans blue test) were assessed. In the carrageenan-induced paw oedema test, hot water infusion simultaneously suppressed both initial and late stages of inflammation in an inversely dose related manner. Hot water infusion also inhibited paw oedema in formalin and cotton pellet granuloma tests. In addition, this infusion exhibited marked anti histamine activity, prostaglandin synthesis inhibition and suppression of vascular permeability. These findings scientifically support the traditional use of *Nyctanthes arbo-tristis* flowers in treatment of inflammatory conditions.

Key words: *Nyctanthes arbo-tristis* flowers, antiinflammatory activity, hot water infusion, inflammation

Nyctanthes arbo-tristis Linn, night-flowering Jasmine, is a small tree up to 10 m tall found in central India but now cultivated elsewhere including Sri Lanka[1,2]. In Sri Lanka, it is commonly found in Buddhist temples and lone gardens almost throughout the country[3]. The tree flowers at night, all the year around, and these start to fall from midnight. The flowers are bisexual, fragrant and bears six white petals and a characteristic orange coloured, slender, cylindrical, glabrous corolla about 12 mm long[3].

Traditionally, *Nyctanthes arbo-tristis* flowers are used to provoke menstruation and as a diuretic[4], and to treat scabies and other skin diseases[5]. In addition, some Sri Lankan traditional practitioners claim that hot water infusion of *Nyctanthes arbo-tristis* flowers are therapeutically effective in the treatment of dermal inflammatory conditions[6]. This study was launched

to scientifically test the validity of the none evidence based claim made by some Sri Lankan traditional medical practitioners that hot water infusion of *Nyctanthes arbo-tristis* flowers have antiinflammatory action.

MATERIALS AND METHODS

Fresh flowers from a mature *Nyctanthes arbo-tristis* tree which were fallen on the ground, were collected between 5.00-6.00 h from a home garden at

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Hunupitiya, Sri Lanka, between July and September, 2010. The identification and authentication were done by Dr. S. Ranwala, Department of Botany, University of Colombo. A voucher specimen (WDR/Sepalika 2) is deposited at the museum of the Department of Zoology, University of Colombo.

Preparation of infusion:
The fresh flowers were oven dried (60º) for 24 h and powdered. 4.5 g of this material was soaked in 24 ml boiling distilled water (DW) for 30 min. The dark brown hot water infusion (HWI) was then filtered through muslin cloth (yield: 32% w/v), and used directly for experimental work at doses of 3.75, 7.5, 12.5 or 18.75 mg/kg, in 1 ml. These doses are identical to what we have used previously[3,7].

Animals:
Male Wistar rats (body weight, 225±15 g) and male ICR mice (body weight, 35±5 g) were used. They were maintained in the animal house at the University of Colombo under standardized conditions (temperature: 30±2⁰; photoperiod: 12±1 h day light/dark cycle and relative humidity: 55±5%) with free access to pelleted animal food and drinking water. Ethical approved for this study was obtained from the Research, Ethics and Higher Degrees committee of the Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo.

Effect on rat carrageenan-induced paw oedema:
Thirty six rats were selected and divided into six groups (*n*=6/group). They were orally treated in the following manner; group 1: 1 ml of DW, group 2: 18.75 mg/kg of HWI, group 3: 12.5 mg/kg of HWI, group 4: 7.5 mg/kg of HWI, group 5: 3.75 mg/kg of HWI and group 6: 4 mg/kg of indomethacin. After 1 h, 0.05 ml of 1% carrageenan suspension was injected subcutaneously into the plantar surface of the left hind paw of each of these rats under mild ether anesthesia. The volumes of the carrageenan injected paws of these rats were measured 1 h prior to the injection of carrageenan and at hourly interval for 4 h after the injection using a plathysmometer.

Effect of formaldehyde-induced paw oedema:
Thirty six rats were randomly assigned into six groups (*n*=6/group). They were orally treated in the following manner; group 1: 1 ml of water, group 2: 18.75 mg/kg of HWI, group 3: 12.5 mg/kg of HWI, group 4: 7.5 mg/kg of HWI, group 5:

3.7 mg/kg of HWI, and group 6: 4 mg/kg of indomethacin for 7 consecutive days. On day 1 and 3 of treatment, these rats were injected with 0.1 ml of 2% formaldehyde in saline into the plantar surface of the left hind paw under mild ether anesthesia. The paw volumes of these rats were measured prior to the injection of formaldehyde, at 4 h after the injection on day 1 and at 1 h of oral treatment of HWI from days 2-7. On day 3, the paw volume was measured before the injection of formaldehyde.

Effect on cotton pellet-induced granuloma:
Thirty six rats were assigned into six groups (*n*=6/group). An autoclaved cotton pellet (7 mg) was implanted subcutaneously, in each rat above the scapula region, under ether anesthesia, using aseptic precautions. They were then orally treated in the following manner; group 1: 1 ml of DW, group 2: 18.75 mg/kg of HWI, group 3: 12.5 mg/kg of HWI, group 4: 7.5 mg/kg of HWI, group 5: 3.75 mg/kg of HWI, and group 6: 4 mg /kg of indomethacin for 7 consecutive days starting from the day of cotton pellet implantation. On day 8, these animals were killed with ether and the cotton pellets along with granulomas were removed and dried in an oven at 60º until a constant weight was obtained. The stomachs were excised, opened and macroscopically observed for any haemorrogic lesions.

Evaluation of antihistamine activity:
Thirty six rats were assigned into six groups (*n*=6/group). The left posterior lateral sides of their skins were clearly shaved under aseptic conditions. They were orally treated in the following manner; group 1: 1 ml of DW, group 2: 18.75 mg/kg of HWI, group 3: 12.5 mg/kg of HWI, group 4: 7.5 mg/kg of HWI, group 5: 3.75 mg/kg of HWI, and group 6: 0.67 mg/kg of chlorpheniramine. After 1 h, these rats were subcutaneously injected with 0.05 ml of 200 μg/ml histamine dihydrochloride into the fur removed area on the skin under mild ether anesthesia and the area of the wheel formed after 1.5 min was measured.

Effect on small intestinal secretion:
Intestinal secretion was indirectly evaluated by the enteropooling assay described by Vitali[8]. Briefly, 18 mice were assigned into three groups (*n*=6/group). Mice in group 1 were orally treated with 0.2 ml of DW, 2 with 0.2 ml of castor oil with 0.2 ml of DW and 3 with 18.75 mg/kg of HWI. Forty minutes later, mice in groups 2 and 3 were orally administered

with 0.2 ml of castor oil. After 30 min, the mice were sacrificed with ether and their small intestines were removed and weighed. The weights were then expressed as mg/20g body weight. The difference in the intestinal weight between the normal control and castor oil treated control was considered as the castor oil-induced accumulation of intestinal fluid.

Effect on acetic acid-induced vascular permeability:

Twelve mice were assigned into two groups (n=6/group). One group was orally administered with 12.5 mg/kg of HWI and the other with 1 ml of DW. One hour later, 1% Evans blue was injected (0.1 ml/10 g body weight) intravenously under ether anesthesia to each of these animals. Thirty minutes later, 0.7% acetic acid in saline was intraperitonially injected (0.1 ml/10 g body weight). Thirty minutes following injection of acetic acid, the animals were sacrificed with in overdose of ether. Ten milliliters of saline was injected into the peritoneal cavity and 1-2 min later the washing solution was collected[9]. The concentrations of Evans blue in washing solution was determined using a spectrophotometer at 630 nm.

Effect on acetic acid-induced protein content in the peritoneal fluid:

Twelve mice were assigned into two groups (n=6/group). One group was orally administered with 12.5 mg/kg of HWI and the other with 1 ml of DW. Thirty minutes later, 0.7% acetic acid in saline was intraperitonially injected (0.1 ml/10 g body weight). Thirty minutes following injection of acetic acid, the animals were anesthetized with ether and the peritoneal fluid was sucked using 18 G needle and plastic syringe. Protein content in the peritoneal fluid was determined using Randox kit according to manufactures instruction.

Purification of human mononuclear cells and assessment of *in vitro* toxicity:

Venous blood (5 ml) was obtained from healthy volunteers by venipuncture using EDTA as the anticoagulant. Mononuclear cells (MNC) were purified using Percoll density gradient centrifugation[10]. MNC were washed twice with incomplete culture medium (RPMI 1640 medium supplemented with 0.2% NaHCO$_3$) by centrifugation at 800 g for 5 min each time. To assess the *in vitro* cytotoxicity of HWI, MNC were treated with different concentrations

(900, 4500 and 22 500 μg/ml) and CCM (RPMI 1640 medium supplemented with 0.2% NaHCO$_3$ and 10% fetal bovine serum) with cells was used as the control. Cell counts were taken after 30 min incubation to assess the percentage of viable cells using Evans blue dye exclusion methods[10].

The effect of HWI on TNFα secretion by human MNC:

MNC were treated with 900, 4500 and 22 500 μg/ml of HWI for 30 min at 37° in 5% CO$_2$ incubator. Washed cells were cultured *in vitro* in 24 well tissue culture plates in CCM containing 0.1 μg/ml of LPS, at 37° in 5% CO$_2$ incubator for 24 h. MNC with no LPS stimulation was used as the negative control. The supernatants of each well were centrifuged at 10 000 g for 5 min and clear supernatant was stored frozen until use. The TNF$_α$ levels in culture supernatants were measured using human cytokine ELISA kits according to manufacturer's instructions. Standard curve was plotted using data obtained with the TNF$_α$ standards provided and concentrations were calculated using the standard curve.

Statistical analysis:

Data are represented as Mean±SEM. Statistical corporations were made by one way ANOVA followed by Turkey's post hoc test and Mann-Whitney U test as appropriate. Significance was set at $P<0.05$.

RESULTS

Effect on carrageenan-induced paw oedema:

As shown in Table 1, the highest dose significantly impaired the paw oedema, only at 1st h (by 53%) and 2nd h (by 44%). In contrast, the other three doses of the HWI tested significantly inhibited the paw oedema at all the time points measured; 1st h (by 59-73%), 2nd h (by 54-76%), 3rd h (by 48-57%) and 4th h (by

TABLE 1: EFFECT ON CARRAGEENAN-INDUCED PAW OEDEMA IN RATS

Dose (mg/kg)	Paw oedema (ml)			
	1 h	2 h	3 h	4 h
1 ml water	0.34±0.01	0.68±0.01	0.70±0.02	0.75±0.01
18.75	0.16±0.01*	0.47±0.02*	0.73±0.02	0.74±0.01
12.50	0.14±0.01*	0.31±0.02*	0.36±0.01*	0.46±0.02*
7.5	0.11±0.02*	0.24±0.04*	0.38±0.05*	0.42±0.05*
3.75	0.09±0.02*	0.16±0.03*	0.30±0.04*	0.41±0.04*
Indomethacin (4.00)	0.17±0.03*	0.38±0.03*	0.29±0.04*	0.33±0.04*

Values are means±SEM (*n*=6), *$P<0.05$ as compared with the control (Mann-Whitney, U-test). SEM: standard error of mean

38-45%). Overall, the antioedema effects of HWI was curvilinearly and inversely dose-dependent (r^2=-1.0, P<0.05). Further, the overall antioedema effects of HWI during the 1st phase (1st and 2nd h; 62%) was almost twice that of the 2nd phase (3rd and 4th h; by 31%). EC_{50} value of this antioedema action was 7.89 mg/ml. Indomethacin induced significantly impairment of oedema at all time points measured (by 44-56%).

Effect on formaldehyde-induced paw oedema:
As shown in Table 2, all doses of HWI significantly reduced (overall, by 44-61%) the paw oedema induced by two injections of formaldehyde from days 2 to 7.

Effect on cotton pellet test:
As shown in Table 3, low and mid doses of HWI significantly inhibited (by 48% and by 34%) the dry weight of granuloma. Indomethacin also significantly impaired the granuloma formation (by 39%). Further, no macroscopic haemorrgic lesions were evident in any of the treated rats.

Effect on antihistamine activity:
As shown in Table 4, all doses of HWI significantly reduced (by 21-55%) the area of the wheel formed following injection of histamine. Indomethacin, also significantly impaired the histamine-induced wheel formation (by 26%).

Effect on small intestine secretion:
As shown in Table 5, oral administration of castor oil significantly and profoundly increased (by 152%) the intestine weight compared with the normal control group. In contrast, the highest dose of HWI significantly and markedly reduced (by 84%) the castor oil induced intestinal weight gain.

Effect on acetic acid-induced vascular permeability and protein content in the peritoneal fluid:
The high dose of HWI profoundly and significantly impaired (by 75%) the amount of Evans blue leaked in to the peritoneal cavity. (control vs treatment: 3.23±0.21 vs 0.81±0.06 µg/dl). The highest dose of HWI induced a moderate impairment (by 32%) of the amount of proteins leakage into the peritoneal fluid (control vs treatment; 24.21±0.45 vs 16.42±1.14 mg/dl).

Effect on viability of rat leucocytes:
None of the doses of HWI tested significantly altered secretion of TNF_{α} from human blood mononuclear cells *in vitro* (data not shown).

DISCUSSION

The results showed, for the first time, that HWI of *Nyctanthes arbo-tristis* flowers possess marked oral antiinflammatory activity against both acute and chronic inflammation. The antiinflammatory activity in carrageenan-induced paw oedema test and cotton plate granuloma assay were inversely dose related whilst in the formaldehyde-induced paw oedema test, it was not dose dependent. Inverse dose relationships with antiinflammation has been previously shown with plant extracts such as *Vitex negundo*[11]. The inverse dose relationship shown may be due to reduction of the effectiveness of the active principle at its high concentrations as has been proposed for *Vitex negundo*[11]. However, further studies are essential to confirm this suggestion. Nevertheless, overall results of this study scientifically substantiate the claim made by Sri Lankan traditional practitioners that HWI *Nyctanthes arbo-tristis* flowers have antiinflammatory activity. The HWI of *Nyctanthes arbo-tristis* is shown to be non cytotoxic and well tolerated[3]. Further, in this study, no macroscopic gastric haemorrhagic lesions were evident unlike with most of the clinically used antiinflammatory drugs[12]. Another important feature about *Nyctanthes arbo-tristis* flowers is that these can be obtained freely all the year round[7]. On the other hand, in developing countries, allopathic antiinflammatory agents are expensive. Taken together,

TABLE 2: EFFECT ON FORMALDEHYDE-INDUCED PAW OEDEMA IN RATS

Dose (mg/kg)	Paw oedema (ml)						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
1 ml water	0.25±0.04	0.26±0.03	0.21±0.03	0.32±0.02	0.30±0.02	0.25±0.02	0.22±0.02
18.75	0.22±0.04	0.13±0.02*	0.08±0.02*	0.17±0.02*	0.09±0.02*	0.07±0.02*	0.06±0.01*
12.50	0.21±0.02	0.09±0.02*	0.05±0.01*	0.19±0.01*	0.10±0.02*	0.07±0.02*	0.08±0.02*
7.50	0.28±0.02	0.14±0.02*	0.09±0.02*	0.23±0.03*	0.17±0.02*	0.10±0.02*	0.09±0.02*
3.75	0.21±0.03	0.13±0.02*	0.09±0.02*	0.21±0.02*	0.11±0.03*	0.06±0.01*	0.09±0.02*
Indomethacin (4.00)	0.24±0.02	0.13±0.02*	0.08±0.02*	0.15±0.01*	0.09±0.01*	0.06±0.01*	0.04±0.01*

Values are means±SEM (*n*=6), *P<0.05 as compared with the control (Mann-Whitney, U-test). SEM: standard error of mean

TABLE 3: EFFECT ON COTTON PELLET GRANULOMA TEST IN RATS

Dose (mg/kg)	Dry weight of cotton pellet (mg)
1 ml water	24.20±5.76
18.75	17.77±2.63
12.50	20.61±1.28
7.50	15.88±1.75*
3.75	12.56±2.22*
Indomethacin (4.00)	15.28±1.25*

Values are means±SEM (n=6), *P<0.05 as compared with the control (Mann-Whitney, U-test). SEM: Standard error of mean

TABLE 4: ANTIHISTAMINE ACTIVITY

Dose (mg/kg)	Dry weight of cotton pellet (mg)
1 ml water	72.51±5.10
18.75	32.43±1.56*
12.50	52.57±2.74*
7.50	57.02±3.37*
3.75	55.06±4.64*
Indomethacin (4.00)	53.60±2.77*

Values are means±SEM (n=6), *P<0.05 as compared with the control (Mann-Whitney, U-test). SEM: standard error of mean

TABLE 5: EFFECT ON CASTOR OIL-INDUCED ENTEROPOOLING IN MICE

Dose	Small intestine weight (mg/20 g)	Castor oil-induced fluid accumulation (mg)
Normal control (water)	540.93±17.18	-
Castor oil control (0.2 ml castor oil + water)	1355.24±25.31[a]	814.31
12.50 mg/kg of HWI (0.2 ml castor oil + 12.50 mg/kg)	975.39±66.03[ab]	434.46

Values are means±SEM (n=6), [a]P<0.05 as compared to normal control, [b]P<0.05 as compared to castor oil control. SEM: standard error of mean, HWI: hot water infusion

these attributes of *Nyctanthes arbo-tristis* flowers indicates its usefulness as a cost effective and risk free herbal antiinflammatory drug. Many people in developing countries including Sri Lanka still rely on herbal medicine for their primary health care[13].

The development of oedema in the rat carrageenan-induced paw oedema test is typically a biphasic event[14,15]. The initial phase lasting up to 2 h is primarily mediated via rapid production of inflammatory mediators such as histamine, serotonin, bradykinins[14,15]. Prostaglandins and other autacoids[16]. In contrast, the late phase lasting from 3-5 h is mediated by prostaglandins and other autacoids, mobilized phagocytic cells, polymorphonuclear cells, monocytes, macrophages, reactive oxygen species, nitric oxide, proteolytic enzymes and platelet activating factor[14-18]. HWI of *Nyctanthes arbo-tristis* flowers, simultaneously inhibited both phases in

the carrageenan-induced paw oedema test, but with a higher impairment (by two fold) in the initial phase. A similar mode of action has been shown with other herbal extracts: with *Ixora coccina*[19] and *Camellia sinensis* leaves[20]. In contrast, maximum inhibition in the first phase of the carrageenan-induced paw oedema test is evident with plants like *Vitex negundo*[11] and in the second phase in the *Trichosanthes cucumerina*[21].

Curtailment of the initial phase of the carrageenan-induced paw oedema test, in this study, can be attributed, to its antihistamine activity: HWI exhibited pronounced antihistamine action[22]. In addition, impairment of prostaglandins induced by HWI is likely to play a substantial role: reduction of intestinal weight in the castor oil study[23] and inhibition of granuloma weight in the cotton pellet-induced granuloma test[24,25] suggest prostaglandin synthesis inhibition.

Inhibition of the late phase of carregeenan-induced paw oedema test by HWI can be bought about by several mechanisms. Several studies have shown that *Nyctanthes arbo-tristis* flower extracts possess antioxidant activity[3]. Antioxidants inhibit the late phase of the carregeenan-induced paw oedema test[16]. Thus, the antioxidant activity of HWI[3] can be linked to its antiinflammatory action in the late phase.

It is well known that prostaglandins and other autacoids are involved in the late phase of inflammation in the carregeenan-induced paw oedema test[16,17]. There is an indication that prostaglandin synthesis was inhibited in the study as revealed by enteropooling test[8,23] and cotton pellet-induced granuloma test[24]. Obviously, impairment of prostaglandin synthesis by HWI could play a vital role in suppressing the second phase of the carregeenan-induced paw oedema test in the study. It is now recognized that expression of cox-1 is maximal in the initial phase and cox-2 in the late phase of the carregeenan-induced paw oedema test[25]. In this study, maximum expression of inflammation was evident in the first phase, suggesting that HWI may have impaired the expression of cox-1 more than cox-2.

Stabilization of the lysosomal membrane is another potential mechanism of indicating antiinflammation[26].

The HWI possess and marked membrane stabilization effect when assessed by the rat heat-induced haemolysis test[27]. The rat red blood cell plasma membrane has close similarity with the lysosomal membrane[27] and the rat heat-induced haemolysis test is used as a biochemical index of antiinflammatory activity[27]. Accordingly, it could be postulated that membrane stabilization action of HWI may have played a role in inducing antiinflammation possibly by inhibiting the release of lysosomal enzymes: lysosomes play a key role in inflammatory reaction by releasing their enzymes[26]. An increase in vascular permeability plays an important role in inflammation[26] and agents which suppress vascular permeability has antiinflammatory actions[9]. In the acetic acid-induced vascular permeability test[9] and in the protein permeability test HWI of *Nyctanthes arbo-tristis* flowers induced a marked inhibition of vascular permeability. This indicates that antiinflammatory action of HWI may have also resulted from its impairment of leakage of inflammatory mediators.

It is known that TNF_α acts as a proinflammatory agent[28]. However, in this study, TNF_α secretion from human mononuclear cells was not significantly reduced. This suggests that antiinflammatory action of HWI may not have resulted from inhibition of TNF_α secretion. However, in the present study, it is unknown whether other proinflammatory cytokines were inhibited by HWI and contributed for the observed antiinflammatory action.

In addition to specific mechanisms, simultaneous inhibition of both phases of inflammation in the carregeenan-induced paw oedema test can result from several other nonspecific mechanisms. Diuresis is one such mechanism[26]. HWI of *Nyctanthes arbo-tristis* flowers possesses diuretic activity[7] and this mechanism is likely to be operative in this study.

HWI exerted its antiinflammatory action not only on acute inflammation but also on chronic inflammation. This is a therapeutically relevant feature since it is claimed to reflect genuine antiinflammatory action[29]. All the HWI induced mechanisms contributing for its acute antiinflammatory actions mentioned earlier can play a role in suppressing the chronic inflammation as well. Both the acute and chronic antiinflammatory action triggered by HWI may be bestowed to flavonoids[30] and organiciridoids[31] present in *Nyctanthes arbo-tristis* flowers. These two phyto constituents are known to be powerful antiinflammatory agents[32].

In conclusion, this study, for the first time, demonstrate promising antiinflammatory activity of HWI of *Nyctanthes arbo-tristis* flowers indicating its potential to be used in inflammatory conditions and also scientifically rationalize its traditional usage in Sri Lanka.

Conflicts of interest:
There are no conflicts of interest.

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