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Evaluation of analgesic and antiinflammatory activity of *Ophiorrhiza nicobarica*, an ethnomedicine from Nicobar Islands, India

Debprasad Chattopadhyay[1,*], Sonali Das[2], Asit Baran Mandal[2], G Arunachalam[1] and SK Bhattacharya[1]

[1]ICMR Virus Unit, Infectious Diseases & Beliaghata General Hospital, GB 4, 1ST Floor, 57, Dr. Suresh C Banerjee Road, Beliaghata, Kolkata 700010, India; [2]Department of Biotechnology, Central Agricultural Research Institute, Port Blair, Andaman and Nicobar Islands, India

SUMMARY

This study reports the analgesic, anti-inflammatory and membrane-stabilizing property of alcoholic extract of *Ophiorrhiza nicobarica* (ON), a wild herb, used as an anti-infective ethnomedicine of Nicobarese and Shompen tribes of Great Nicobar Island, India. We for the first time investigated the analgesic and antiinflammatory potential of this herb in acute, subacute and chronic model of inflammation in Swiss albino mice and Wistar albino rats, along with sheep RBC-induced sensitivity and membrane stabilization. The acetic acid induced writhing, tail flick and tail immersion tests are used as a model for evaluating analgesic activity; while the carrageenin-induced paw oedema was used as the model for acute inflammation, dextran-induced oedema as sub-acute and cotton-pellate-induced granuloma as chronic inflammatory model. The probable mode by which ON mediate its effect on inflammatory conditions was studied on sheep RBC-induced sensitivity and membrane stabilization. The *in vitro* results revealed that the ON extract possesses significant ($P < 0.05$) dose dependent analgesic and antiinflammatory activity at 200 and 300 mg/kg and its fractions at 50 mg/kg, p.o. respectively, compared to the control groups. However, the extract failed to exhibit membrane-stabilizing property as it unable to reduce the level of haemolysis of RBC exposed to hypotonic solution. The acute toxicity studies of ON extract in rats and mice revealed that the extract was nontoxic even up to 3.0 g/kg body weight of the animals, with a high safety profile. We have isolated ursolic acid, β-sitosterol and harmaline respectively, from the bioactive part of the extract. The results indicated that the *O. nicobarica* is indeed beneficial in primary health care, and suggest that its anti-inflammatory activity may not be related to membrane-stabilization.

Key words: *Ophiorrhiza nicobarica*; Analgesic activity; Anti-inflammatory activity; Membrane-stabilizing property

INTRODUCTION

One of the prerequisites for success in primary health care is the availability and use of suitable drugs in time and plants have always been a common source of medicaments (Thomson, 1978), either as traditional preparation or as pure active principle (Stockwell, 1988). Ethnobotanical literature revealed that 45 indigenous plant species are used as medicaments by the different ethnic tribes (Bhargava, 1983; Chakraborty and Vasudeva Rao, 1988; Dagar and Dagar, 1991; Mandal *et al*., 2000) of Andaman and Nicobar Islands, and the follow up

*Correspondence: Debprasad Chattopadhyay, ICMR Virus Unit, Infectious Diseases & Beliaghata General Hospital, GB 4, 1ST Floor, 57, Dr. Suresh C Banerjee Road, Beliaghata, Kolkata 700010, India. Tel: +913323537425; Fax: +91332353 7424; E-mail: debprasadc@hotmail.com; debprasadc@yahoo.co.in

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study with few such medicaments showed promising results (Chattopadhyay *et al*., 1998; Chattopadhyay *et al*., 2001; Arunachalam *et al*., 2002; Chattopadhyay *et al*., 2002). The Genus *Ophiorrhiza*, sister group of Rubioideae of the family Rubiaceae contain 47 species (Manen and Natali, 1996), of which *O. infundibularis* Balakr., *O. mungos* L, *O. nicobarica* Balakr. and *O. trichocarpa* BL are recorded from Andaman and Nicobar Islands, India (Vasudeva Rao, 1986). *Ophiorrhiza nicobarica*, International plant names index (IPNI) Id: 758538-1, is an erect perennial herb with bostryx-like inflorescence, and is used by the Nicobarese tribes against ailment related to infectious diseases, especially against herpetic lesions, skin infections and irritation (Chakraborty and Vasudeva Rao, 1988; Dagar and Dagar, 1991). As infections usually accompanied with inflammation and pain, we therefore, investigated the anti-inflammatory and analgesic activity of *Ophiorrhiza nicobarica,* an endangered and threatened herb (Vasudeva Rao, 1986; Mandal *et al*., 2000) with an aim to know whether this herb possess any antiinflammatory activity that can be therapeutically useful in primary health care.

MATERIALS AND METHODS

Plant material

Ophiorrhiza nicobarica Balkr. (Rubiaceae), were collected from the rain forests of Great Nicobar Islands, India, during 2000 - 2001. The plant has been identified by Dr. Sreekumar, Senior Scientist, Botanical Survey of India, Andaman & Nicobar Circle, Port Blair and the voucher specimens have been deposited in the Herbarium collection (Herbarium No. 9227).

Extraction and fractionation

The alcoholic extract was made from dried and coarsely powdered herb (500 g) successively extracted with 1 L of MeOH (95%) for 48 - 72 h in a soxhlet extractor. The extract was collected in a 5L conical flask, filtered and the solvent was evaporated *in vacuo* to a residue by a Eyela Rotary Evaporator (Japan) at 40 - 45°C, with an yield of 7.8 ± 0.2% (w/w). A part of the residue was stored in a dessicator for further study (Chattopadhyay *et al*., 2001, 2003, 2006). The phytochemical analysis (Trease and Evans, 1983; Arunachalam *et al*., 2002; Chattopadhyay *et al*., 2004) indicated the presence of tannins, flavonoids, triterpenoids, sterols, alkaloids and reducing sugars. The powdered residue (32 g) was then suspended in water and extracted with *n*-butanol. The resulting *n*-butanol and aqueous parts were separately monitored by TLC. The *n*-butanol part (24 g), were then subjected to Silica-gel CC, eluting with petroleum ether (PE), PE: $CHCl_3$, $CHCl_3$, $CHCl_3$: MeOH (at different ratio) and MeOH. The eluted fractions A (7 g), B (6 g) and C (8 g) were repeatedly Si-gel CC and were monitored by TLC (Trease and Evans, 1983; Chattopadhyay *et al*., 2002). Out of the three, fraction A was positive to terpenoid, fraction B to phytosterol and the $CHCl_3$-MeOH fraction (fraction C) was positive for alkaloid in TLC. All the fractions were isolated separately, filtered, dried, and then subjected to CC by Aluminum oxide eluted with PE, PE: $CHCl_3$ (at different ratio), $CHCl_3$, $CHCl_3$: MeOH (at different ratio) and monitored by TLC (Chattopadhyay *et al*., 2002, 2004). The fractions A and B were combined, condensed and recrystallized with suitable solvent system. The isolated compounds were confirmed by IR, ^{1}HNMR, ^{13}CNMR and Mass spectroscopy as ursolic acid (fraction A) and β-sitosterol (fraction B). A stock solution of 10 mg/ml was prepared by dissolving the powdered extract in 1ml dimethyl sulfoxide (DMSO) and diluted with sterile distilled water for further studies, as the extract was soluble to DMSO.

Isolation and identification of alkaloids

A small amount of NH_3 (25%) was added to the dried alkaloid fraction C to make its pH of 9. Then 100 ml of chloroform was added to the fraction C and slowly shake (10 min) until the alkaloid separated from water and enter to the chloroform

phase. This was repeated three times and then total chloroform phase was evaporated, yielding a total alkaloid extract (Trease and Evans, 1983; Monsef *et al*., 2004; Chattopadhyay *et al*., 2006a). The alkaloid fraction C (8 g) was chromatographed on a silica gel column (3.5 ∞ 90 cm), using a linear gradient with a $CHCl_3$-MeOH system, and collected in fractions as: 9.5-0.5, 9-1, 8.5-1.5, 8-2, 7.5-2.5. Fractions were then filtered, and concentrated at room temperature. A part of the extract was then weighted, dissolve in DMSO, diluted in water and examined for analgesic and antiinflammatory activity. The bioactive fraction was purified further and isolated with TLC using precoated silica gel plates and $CHCl_3$:MeOH: NH_3 (50: 50: 3) solvent system. TLC separation of the most active fraction demonstrated two bands with R_f of 0.33 and 0.63. The active fraction having R_f 0.33 was structurally identified as harmalin by ^{13}C NMR and ^{1}H NMR as follows: MP: 229°C, ^{1}H NMR ($CDCl_3$): δ 8.05 (bs, 1H, H-1), 7.47 (d, J = 8.8 Hz, 1H, H-9), 6.85 (d, J = 2.4 Hz, 1H, H-12), 6.82 (dd, J = 8.8 Hz, J = 2.4 Hz, 1H, H-10), 3.86 (s, 3H, OCH_3), 3.8 (t, J = 8.4 Hz, 2H, H-5), 2.82 (t, J = 8.4 Hz, 2H, H-6), 2.34 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$): 158.3 (C-3), 157 (C-11), 137 (C-13), 128.5 (C-2), 128 (C-8), 120.5 (C-9), 116.5 (C-7), 110.5 (C-10), 92 (C-12), 54.5 (OCH_3), 48 (C-5), 21.9 (CH_3), 19.4 (C-6).

Laboratory animals

Random breed Swiss albino mice (18 - 20 g each) and Wistar albino rats (180 - 200 g each) of either sex were housed in well ventilated standard metal cages at room temperature. Animals were provided with standard feed (Hindustan Lever, India) and tap water *ad libitum*. All the animals were allowed one-week acclimatization period at room temperature (23 ± 3°C) and light: dark exposure of 12:12 h before the experiment (Arunachalam *et al*., 2002; Chattopadhyay *et al*., 2005a).

Acetic acid-induced writhing test

This was used as a model for studying analgesic activity of the extract (Koster and Anderson, 1959). Swiss albino mice of either sex were divided into eight groups of ten animals each. The animals of the first two groups received either propylene glycol (5 mg/kg) or aspirin (100 mg/kg); while the third to fifth group received crude extract of ON at doses of 100, 200 and 300 mg/kg, and the sixth to eighth groups received fraction A, B and C at 50 mg/kg i.p. respectively, 60 min before injection of 0.6% v/v acetic acid solution (10 ml/kg). Immediately after the administration of acetic acid, the numbers of writhing or stretches (a syndrome, characterized by a wave of contraction of the abdominal musculature followed by extension of hind limbs) were counted for 15 min. A reduction in the writhing number compared to the control group was considered as evidence for the presence of analgesia, which was expressed as percent inhibition of writhing. Data were calculated according to the formula: A - B/A ×100 Where A = mean number of writhes produced by the control groups, and B = mean number of writhes produced by the test groups (Nunez-Guillen *et al*., 1997; Chattopadhyay *et al*., 2005b; Zakaria *et al*., 2007).

Tail immersion test

Swiss albino mice of either sex were divided into eight groups of ten animals each. Propylene glycol at 5 mg/kg, crude extract of ON at 100, 200 and 300 mg/kg, fractions A, B and C at 50 mg/kg, and the analgesic drug pethidine at 5 mg/kg respectively, were administered i.p. The tail (up to 5 cm) was then dipped into a pot of water maintained at 55 ± 0.5°C. The time in seconds to withdraw the tail out of water was taken as the reaction time and the reading was taken after 30 min of administration of the test drug. (Ghosh, 1984).

Tail flick test

Wister albino rats of either sex weighing between 180 - 200 g were divided into eight groups of ten animals each. The tail of the rat was place on the nichrome wire of an analgesiometer (Techno, Lucknow, India) and the time taken by the animal

to withdraw (flick) its tail from the hot wire was taken as the reaction time. The crude extract of ON in doses of 100, 200 and 300 mg/kg, fractions A, B and C at 50 mg/kg and pethidine at 5 mg/kg respectively, were injected i.p. Propylene glycol at 5 ml/kg was served as vehicle control. Analgesic activity was measured after 30 min of administration of extract and standard drugs (Ghosh, 1984; Chattopadhyay *et al*., 2005b).

Carrageenan-induced rat paw oedema

The Wister albino rats were divided into eight groups of six animals each. The extract at 100, 200 and 300 mg/kg and its fractions A, B and C at 50 mg/kg doses were administered orally to six groups of rats respectively, 60 min prior to carrageenan injection. The seventh and eighth groups received 5 ml/kg propylene glycol orally as vehicle control or 10 mg/kg indomethacin as drug control respectively, for assessing comparative pharmacological significance. Thirty minutes later, oedema was induced by subplantar injection of 0.05 ml of a freshly prepared carrageenan suspension (1% w/v) in normal saline into the right hind paw of each rat (Winter *et al*., 1962; Chattopadhyay *et al*., 2005b). The paw volume (linear circumference of the injected paw) was measured at 0 h and at 3 h after the injection of carrageenan, by a plethysmometer (Winter *et al*., 1962; Arunachalam *et al*., 2002). The animals were sacrificed 4 h after the subplantar injection; both the hind paws were severed from the ankle joints and weighed. The oedema formation was determined by the difference, and the percentage inhibition of oedema was calculated (Jain and Khanna, 1981) by the formula: % Inhibition of Oedema = I_0 - $I_1/I_0 \times 100$; where I_0 = change in paw circumference in vehicle control group, and I_1 = change in paw circumference in drug treated group.

Dextran-induced rat paw oedema

The oedema was induced in the right hind paw by subplantar injection of 0.1ml of freshly prepared 1% dextran solution (Nunez-Guillen *et al*., 1997; Arunachalam *et al*., 2002; Chattopadhyay *et al*., 2005b). Paw volume was measured 30 min before and after dextran injection. The first 6 groups of rats received oral doses of 100, 200 and 300 mg/kg of crude extract and 50 mg/kg of fractions A, B and C, respectively. While the two control groups were administered with 5 ml/kg propylene glycol (vehicle) or 10 mg/kg indomethacin (drug). The percentage inhibition of oedema was calculated for both models as described by Kavimani *et al.* (1996).

Cotton pellet-induced granuloma

Seven groups of mice, eight in each group, were included for testing granuloma formation. After shaving off the fur, the animals were anaesthetized. Sterile preweighed cotton pellets (10 mg) were implanted in the axilla region of each animal through a single needle incision (D'Arcy *et al*., 1960). Crude extract (100, 200 and 300 mg/kg), fractions A, B and C (50 mg/kg) was administered orally to first 6 groups; while indomethacin (10 mg/kg) or propylene glycol (5 ml/kg) were used as control for the last two groups for seven consecutive days from the day of cotton-pellet implantation. On the eighth day, the animals were anaesthetized, the cotton pellets were removed surgically, made free from extraneous tissues, incubated at 37°C for 24 h and dried at 60°C to constant weight. The increase in the dry weight of the pellets was taken as measure of granuloma formation (Winter and Porter, 1957; Arunachalam *et al*., 2002).

Sheep RBC-induced sensitivity

Whole blood from Sheep was collected with heparinized syringes and washed three times with isotonic buffered solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4), and was centrifuged each time for 10 min at 3,000 × *g*. Swiss albino mice were immunized by injecting 1.0×10^8 sheep RBC subcutaneously in the nape of the neck. Extracts at 100, 200 and 300 mg/kg doses was given orally once daily. On 5th day the animals were challenged with 0.05 ml of sheep RBC

(1.0×10^8) in the right hind footpad, and the same volume of 0.9% saline was injected into the left hind footpad. After 24 h of challenge the animals were sacrificed, both the hind paws were severed from the ankle joints and weighed (Sur *et al.,* 2002).

Membrane stabilization

Membrane stabilizing activity of the extract was assessed by hypotonic solution-induced sheep erythrocyte haemolysis (Shinde *et al.,* 1999). The test sample consisted of 0.50 ml RBC suspension mixed with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing the extract (0.2 - 2 mg/ml) or indomethacin (0.1 mg/ml); while the control sample contain 0.5 ml of RBC mixed with hypotonic-buffered saline. The mixtures were incubated for 10min at room temperature, centrifuged at 3,000 g for 10min and the absorbance of the supernatant was measured at A_{540} nm. The percentage inhibition of haemolysis or membrane stabilization was calculated according to the formula: % Inhibition of haemolysis = $100\times\{OD_1 - OD_2/OD_1\}$, Where OD_1 = Optical density of hypotonic-buffered saline solution alone; and OD_2 = Optical density of test sample in hypotonic solution.

Acute toxicity study

Acute toxicity study was carried out according to the method described by Miller and Taniter (1944). The Swiss albino mice were divided into five groups, six in each group. The first four groups received oral doses of 250, 500, 1,000, 2,000, 3,000 mg/kg of the crude extract, while the fifth group received saline (10 ml/kg) orally. Animal mortality was assessed 24 h after administration of extract and saline. The animals were also observed for symptoms of toxicity and mortality, 24 h after treatment upto three days.

Statistical analysis

The results were expressed as mean, mean ± S.D., and S.E.M. The significance was evaluated by Student's t-test compared with control (Woodson, 1987). For comparison of data from multiple groups, one-way ANOVA followed by the post-hoc Schaffer's test was used (Chattopadhyay *et al.,* 2005a,b).

RESULTS

Acute toxicity studies

Acute toxicity study showed that the extract possessed high safety profile as no death was observed at oral doses of 3,000 mg/kg body weight in mice. The behavioral changes observed at these doses were reduced motor activity, ataxia and hyperventilation. The in vivo toxicity study also revealed that the extracts are non-toxic even at a concentration of 3,000 mg/kg body weight in mice.

Phytochemical study

The preliminary phytochemical study of the extract indicated the presence of triterpenoid, alkaloid, flavonoid, sterol, tannin and reducing sugar. The isolation and purification of the major bioactive fractions revealed the presence of ursolic acid (A), β-sitosterol (B) and alkaloid (C). The TLC separation of the fraction C demonstrated two bands with R_f of 0.33 and 0.63; and the active component with R_f 0.33 was identified as harmalin by ^{13}C NMR and ^{1}H NMR.

Analgesic activity

The results of acetic acid induced writhing test with the crude extract of ON in Swiss albino mice are presented in Table 1. The results indicated that the inhibition of writhing reflexes was 25.80%, 41.57% and 55.97% at 100, 200 and 300 mg/kg of the extract respectively; while the inhibition was 0% in vehicle control and 75.66% in aspirin treated group. The inhibition percentage was highest (55.97%) at 300 mg/kg, which is comparable to the inhibition given by the standard drug aspirin (75.66%). On the otherhand, the writhing reflex inhibitions with fractions are 50.96% (A), 40.36% (B) and 54.91% (C) respectively, compared with the

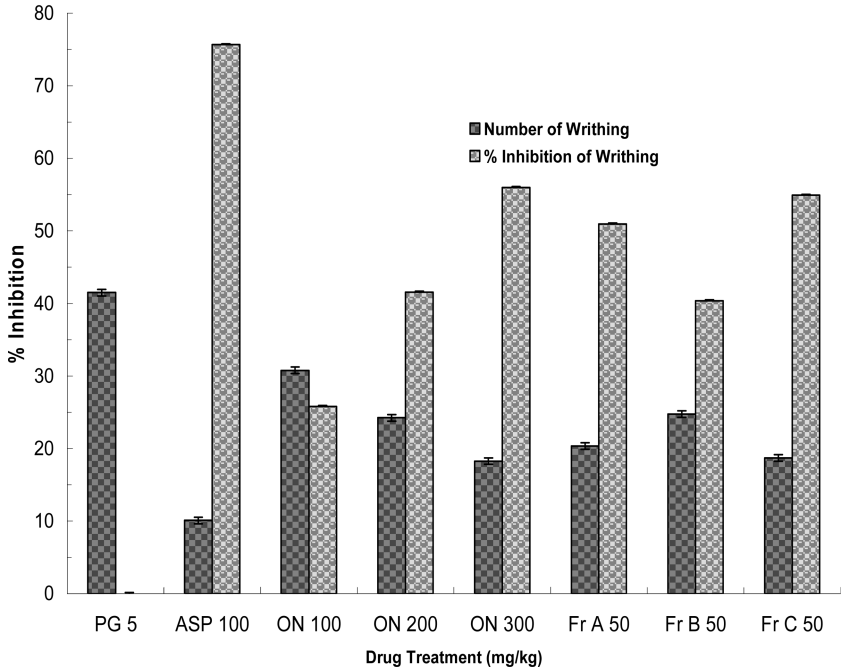

Fig. 1. Acetic acid induced Analgesic activity of *O. nicobarica* extract.

inhibition given by the drug aspirin (75.66%). Hence, the significant ($P < 0.05$) inhibition of writhing reflexes noted with the extract at 300 mg/kg dose and with the fraction A and B at 50 mg/kg, compared to the aspirin treated group (Fig. 1). The results of the Tail flick tests showed that the extract had the reaction time of 2.25 s and 3.01 s at 100 and 200 mg/kg and 3.87 s at 300 mg/kg respectively; while the reaction time for vehicle control and pethidine control group was 2.24 s and 4.21 s respectively. On the otherhand, reaction time at 50 mg/kg of fractions was 3.27 s (A), 2.01 s (B) and 3.29 s (C) respectively. Here again the extract at 300 mg/kg and fractions (A and C) at 50 mg/kg significantly ($P < 0.05$) increased the reaction time compared to the pethidine group (Table 1). The results of the Tail immersion experiment revealed that the reaction time was 2.27 s, 3.03 s and 3.54 s

Table 1. Analgesic activity of *O. nicobarica* extract and their fractions by physical and chemicals methods

Treatment	Dose (mg/kg)	Tail flick (Reaction time in s)	Tail immersion (Reaction time in s)	Number of writhing (% Inhibition)
Propylene glycol	5 ml/kg	2.24 ± 0.13	2.31 ± 0.15	41.50 ± 0.45
Aspirin	100	-	-	10.10 ± 0.43 (75.66)
Pethidine	5	4.21 ± 0.18	4.47 ± 0.12	-
O. nicobarica	100	2.25 ± 0.13	2.27 ± 0.07	30.79 ± 0.34 (25.80)
	200	3.01 ± 0.06	3.03 ± 0.02	24.25 ± 0.41 (41.57)
	300	3.87 ± 0.10	3.54 ± 0.15	18.27 ± 0.22 (55.97)
Fraction A	50	3.27 ± 0.20	3.30 ± 0.14	20.35 ± 0.42 (50.96)
Fraction B	50	2.01 ± 0.09	2.02 ± 0.10	24.75 ± 0.24 (40.36)
Fraction C	50	3.29 ± 0.05	3.27 ± 0.08	18.71 ± 0.30 (54.91)

Values are mean ± S.E. (n = 10). $P < 0.05$ vs. control, Student's *t*-test.

Table 2. Effect of *O. nicobarica* extract and its fractions on Carrageenin- and Dextran-induced paw oedema and Cotton-pellate-induced granuloma in animals

Group	Dose (p.o.)	Carrageenan-induced oedema	Dextran-induced oedema	Cotton pellate-induced granuloma
		Paw wt increase (g) with % inhibition	Paw wt increase (g) with % inhibition	Granuloma wt (mg) with % inhibition
Propylene glycol	5 ml/kg	5.26 ± 0.39 (-)	4.10 ± 0.17 (-)	70.42 ± 3.60 (-)
Indomethacin	10 mg/kg	1.71 ± 0.31* (67.49)	1.60 ± 0.03* (60.97)	32.34 ± 1.71* (54.07)
Crude extract	100 mg/kg	3.74 ± 0.11* (28.89)	2.98 ± 0.23* (27.32)	47.37 ± 2.00** (32.73)
	200 mg/kg	2.52 ± 0.12* (52.09)	2.19 ± 0.18* (46.58)	41.10 ± 1.45** (41.63)
	300 mg/kg	2.24 ± 0.09* (57.41)	1.83 ± 0.31* (55.36)	33.12 ± 1.52* (52.96)
Fraction A	50 mg/kg	2.37 ± 0.18* (54.94)	1.93 ± 0.11* (52.92)	34.50 ± 0.64*** (51.00)
Fraction B	50 mg/kg	2.77 ± 0.12* (47.34)	2.21 ± 0.21* (46.09)	35.75 ± 0.81*** (49.23)
Fraction C	50 mg/kg	2.36 ± 0.13* (55.13)	1.99 ± 0.31* (51.46)	33.82 ± 0.67*** (51.97)

Values are mean ± S.E.M. (n = 10). *$P < 0.05$; **$P < 0.05$ compared with control, Student's *t*-test's.

with 100, 200 and 300 mg/kg respectively, compared to the vehicle (2.31 s) and the drug control (4.47 s) group. The reaction time for fractions was 3.30, 2.02 and 3.27 s respectively at 50 mg/kg dose. All these data are significant with respect to the control group (Table 1).

Anti-inflammatory activity

The results presented in Table 2 showed that the methanol extract of ON at 100, 200 and 300 mg/kg and its fractions at 50 mg/kg, p.o. exhibited significant ($P < 0.05$) dose-dependent anti-inflammatory activity in all the three experimental models. In carrageenin-induced paw oedema model methanol extract at 300 mg/kg dose exhibited 57.41% inhibition of paw oedema; and the fractions at 50 mg/kg showed 54.94% (A), 47.34% (B) and 55.13% (C) inhibition respectively; while indomethacin produced 67.49% inhibition of oedema volume after 4 h of drug treatment (Table 2). In dextran-induced paw oedema test the inhibition of oedema swelling at 300 mg/kg was 55.36%, while with fractions (50 mg/kg) it was 57.92% (A), 46.09% (B) and 51.46% (C) respectively ($P < 0.05$) compared to indomethacin (60.97%). Similarly in cotton pellet-induced granuloma test the extract at 300 mg/kg exhibited 52.96% inhibition of granuloma weight; while the fractions showed 51.0% (A), 49.23% (B) and 51.23%(C) inhibition ($P < 0.05$) respectively, compared with 54.07% inhibition by indomethacin (Fig. 2).

Effect of extract on sheep RBC-induced sensitivity and membrane stabilization

The results on sheep RBC induced sensitivity test, presented in Table 3, indicated that the weight increase with the extract was not statistically significant. Here the mean weight in control group was 19.8 mg while with indomethacin it was 8.9 mg. On the otherhand, the weight increase with extract and its fractions varies from 15.7 - 19.7 mg, which is much closer to the control group. The extract at 250 - 2,000 μg/ml did not significantly protect the rabbit erythrocyte membrane against lyses induced by hypotonic solution. In contrast, indomethacin at 10 μg/ml offered significant protection of the rabbit RBC against the damaging effect of hypotonic solution. At a concentration of 2.0 mg/ml the extract produced 23.88% inhibition of RBC haemolysis as compared with 50% produced by indomethacin (Table 3).

DISCUSSION

In acetic acid induced writhing experiments, the numbers of writhing movements were significantly

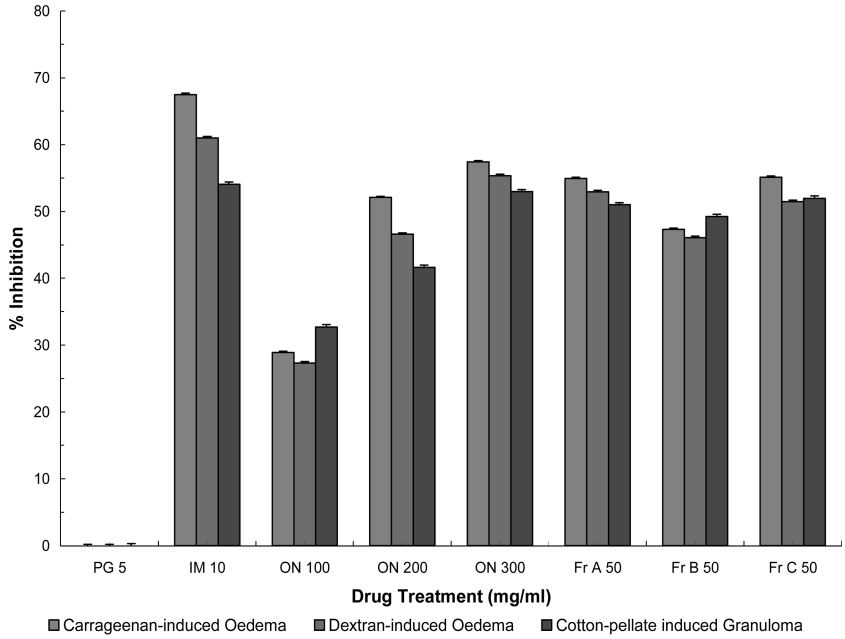

Fig. 2. Dose-dependent Antiinflammatory activity of *O. nicobarica* Extract (inhibition in Oedema and Granuloma volume).

Table 3. Effect of *O. nicobarica* extract on sheep RBC-induced sensitivity and on membrane stabilization

Group	Dose (mg/kg)	Weight increase (mg) mean ± S.E.M.	Dose (µg/ml)	Optical Density (A 560 nm)	% Inhibition of haemolysis
Control	Saline	19.8 ± 0.03	Saline	0.72 ± 0.05	-
Indomethacin	10	8.9 ± 0.04$^{a,b,c,\#}$	10	0.39 ± 0.11	50.00
Crude extract	100	15.7 ± 0.03$^{a,*,\#}$	250	0.61 ± 0.03	11.24
	200	17.9 ± 0.12$^{a,b,\#}$	500	0.59 ± 0.05	12.75
	300	19.7 ± 0.04$^{a,b,\#}$	1,000	0.60 ± 0.05	13.90
	-	-	2,000	0.54 ± 0.07	23.88
Fraction A	50	18.2 ± 0.09$^{a,b,\#}$	100	0.56 ± 0.08	20.02
Fraction B	50	16.3 ± 0.03$^{a,b,\#}$	100	0.59 ± 0.06	13.32
Fraction C	50	18.1 ± 0.11$^{a,b,\#}$	100	0.58 ± 0.08	19.18

Values are mean ± SEM (n = 6). $^{*}P < 0.01$. One-way ANOVA Schaffer's multiple comparisons F-test. Comparisons were made as: ^{a}extract vs. control; ^{b}extract vs. extract; ^{c}standard drug vs. extract; $^{\#}$fractions vs. control and extract vs. fractions.

less in the mice treated with the ON extract and its fractions A and C, compared to the vehicle control group. On the otherhand, the results revealed that the extract and its fractions might have peripheral analgesic effect when compared with the analgesic drug aspirin. However, the analgesic effect produced by the Tail flick and Tail immersion tests were comparable to that of pethidine treated group (drug control), suggesting that it may have central analgesic effect. The results of the antiinflammatory activity of ON extracts at 300 mg/kg and its lipophilic fraction A at 50 mg/kg p.o indicated strong anti-inflammatory activity; while the alkaloid fraction C have moderate antiinflammatory activity at 50 mg/kg comparable to that of indomethacin against carrageenan induced acute pedal oedema, dextran

induced oedema and cotton-pellate induced granuloma tests.

One of the cardinal sign of inflammation is the presence of oedema. The carrageenin induced oedema test is the most acceptable experimental model for the study of acute inflammation. The inflammatory condition (oedema) induced by carrageenin is believed to be biphasic, in which the step-wise release of vasoactive substances histamine and 5HT (bradykinin and serotonin) in the early phase and prostaglandins (kinin) in the acute late phase (Castro *et al*., 1968; Heller *et al*., 1998) takes place. The release of these substances results in increased vascular permeability, thereby promoting accumulation of fluid in tissues that accounts for the oedema (Williams and Morley, 1973; White, 1999). On the other hand, dextran mediated inflammation was reduced probably as a result of antihistamine effects of the extract, as dextran is known to cause inflammation through both histamine and serotonin (Ghosh *et al*., 1963). The ability of the ON extract to reduce the oedema volume produced by carrageenin and dextran, suggests that the phytochemicals present in the extract may block the release of any of those mediators, alone or in combination. The cotton pellate-induced granuloma test is reported to assess the ability of inflammatory effects of the proliferative phase of inflammation (Selye, 1953). In the present study the ON extract and its fractions A and C exhibited significant dose-dependent anti-inflammatory activity in the cotton-pellet granuloma test, probably by inhibiting the increased number of fibroblasts, synthesis of collagen and mucopolysaccharides during granuloma tissue formation (Arrigoni-Martellie, 1977).

The RBC-induced sensitivity test is a well known immunological model for antiinflammatory drug testing (Dasgupta *et al*., 1988). In our study the ON extract could not prevent sheep RBC-induced sensitivity in mice, suggesting that the extract probably has no protective ability on immunological mediators or lymphokines. We also studied membrane stabilization property because the protective effect on hypotonic saline-induced RBC lysis is an index of antiinflammatory activity (Oyedapo and Famurewa, 1995). It is well known that the vitality of living cells depends on the integrity of cell membrane (Ferrali *et al*., 1992) and exposure of RBC to injurious substances (hypotonic solution, phenylhydrazine etc.) results in membrane lysis accompanied by haemolysis and oxidation of haemoglobin (Augusto *et al*., 1982; Ferrali *et al*., 1992). The haemolytic effect of hypotonic solution is related to the excessive accumulation of fluid within the cell resulting in the rupturing of cell membrane. Such injury to RBC membrane will further render the cell more susceptible to secondary damage through free radical-induced lipid peroxidation (Augusto *et al*., 1982; Ferrali *et al*., 1992). This is consistent with the observation that the breakdown of bio-membranes leads to the formation of free radicals which in turn enhance cellular damage (Halliwell *et al*., 1988; Maxwell, 1995). The progression of bone damage in rheumatoid patients for example, is due to increased free radical activity (Cotran *et al*., 1999; Pattison *et al*., 2004). It is therefore, expected that compounds with membrane-stabilizing property should offer significant protection of cell membrane against injurious substances (Liu *et al*., 1992; Maxwell, 1995; Perenz *et al*., 1995; Shinde *et al*., 1999), as membrane-stabilizing compounds are known to interfere with the prevention of the release of phospholipases (the early phase of inflammatory process) that trigger the formation of inflammatory mediators (Aitadafoun *et al*., 1996). However, the ON extract did not demonstrate significant membrane stabilizing property, suggesting that the anti-inflammatory activity observed in this study, may be related to the inhibition of the late phase of inflammatory events, i.e., the release of chemical mediators.

Earlier studies with *Ophiorrhiza* species reported that *O. filistipula*, an Australian species contain indole alkaloids (Arbain *et al*., 1993), while an

antitumor alkaloid camptothecin was isolated from *O. pimula* (Kitajima *et al*., 1997; Yamazaki *et al*., 2003), but in our study the methanol extract of *O. nicobarica*, an endangered and threatened herb, contain ursolic acid (triterpenoid), β-sitosterol (phytosterol) and harmaline (alkaloid), as major bioactive compounds, isolated by column chromatography and TLC, purified by preparative TLC and analyzed by NMR and GC-MS. The ursolic acid (fraction A) is a pentacyclic amphiphilic triterpene with hydroxylated polycyclic structure, ubiquitous in medicinal plants and has multiple activities (Chattopadhyay *et al*., 2005b). It can selectively induce apoptosis mediated by caspase-3 (Harmand *et al*., 2003) and act as an antioxidant (Kim *et al*., 1996). As a potent antiinflammatory agent, ursolic acid inhibits 5-lipooxygenase and cyclooxygenase (Ringbom *et al*., 1998), highly selective inhibitor of cyclic AMP phosphotransferase and AMP-dependent protein kinase (Wang and Polya, 1996) and thereby regulates metabolism, cell division, gene expression and development (Karin and Smeal, 1992; Chattopadhyay *et al*., 2006). Studies indicated that phytosterols contain an extra alkyl group at C-24 in the side chain, either free or with β-D glucosides. In human, sitosterol (24α-ethylcholesterol) act as plasminogen activator (Hoffmann and Klöcking, 1988) and promotes the formation of essential PUFA from linoleic acid (Leiken and Brenner, 1989) required for prostaglandin and leukotriene biosynthesis and for cell mediated immune functions (Kinsella *et al*., 1990). The β-sitosterol (fraction B), as isolated from the ON extract, is reported to have anti-inflammatory (Gupta *et al*., 1996; Chattopadhyay *et al*., 2006a), antipyretic, antiulcer and anticancerous activities (Pegel, 1980; Award *et al*., 1996). Furthermore, β-sitosterol can stimulate human peripheral leucocyte proliferation, and significantly increase the activity of helper T-cells, cytokines, interleukin 2, γ-interferon and NK cells and thereby useful in therapy of immune dysfunction diseases (Bouic *et al*., 1997).

The Harmaline (fraction C), a β-carboline alkaloid isolated in 1841 from seeds and roots of Syrian rue *Peganum harmala* and its chemical structure were established in 1919. Later this alkaloid were reported from tobacco (10 - 20 μg harman and norharman from smoke of a single cigarette), and many plants (Janiger and Dobkin de Rios, 1937), but Banisteriopsis vines and seeds of *Peganum harmala* were used in folk medicine as psychoactive agent and as dyes in Turkish and Persian rugs. The harmala alkaloid was also found in the pineal gland (in mid-forehead) of humans and several animals and is more abundant in the pineal glands of advanced yogis, leading to speculation that its presence may impart power to the "third eye" in mid-forehead. Harmala exhibits an extra ring attached to its basic indole structure, and the three-ring *p*-carboline system has an unusually placed CH_3O group. By oxidation, harmaline is converted into the psychoactive harmine, and upon reduction it yields *d*-1,2,3,4-tetrahydroharmine. Harmidine ($C_{13}H_{14}N_2O$) in moderate dose can act as antinociceptive agent (Monsef *et al*., 2004), reversible mono amine oxidase (MAO) inhibitor (Abdel-Fattah *et al*., 1997) and in combination with other β-carbolines it bind to 5-HT receptors (Abdel-Fattah *et al*., 1995). Harmaline can induce spasmolytic effects with muscarinic, histaminic and β-adreno-receptors (Aitadafoun *et al*., 1996); while the vasorelaxant activity of harmane and harmaline is due to its ability to induce endothelial cells to release NO, and the vascular smooth muscles to inhibit the contractions induced by $Ca2^+$ channels (Shi *et al*., 2001). Harmane and harmine had moderate antiproliferative activity toward human monocytes and is non-toxic to human cells, exerted strong activity against the intracellular amastigote of *Leishmania infantum* partly due to its capacity to inhibit leishmanial PKC activity (Di Giorgio *et al*., 2004). This beta-carboline alkaloid also has scavenging action on reactive oxygen species and inhibits thiol oxidation (Kim *et al*., 2001).

The analgesic and antiinflammatory activity of ON extract, as found in our study, may be due to either ursolic acid (fraction A), β-sitosterol (fraction

B) or harmaline (fraction C) alone or in combination, as ursolic acid isolated from *Melaleuca leucadendron* inhibit concavalin A induced histamine release (Tsuruga *et al*., 1991) while the ursolic acid of *Phillyrea latifolia* inhibit cyclooxygenase and 5-lipooxygenase of arachidonoate cascade (Diaz *et al*., 2000) and harmala alkaloid of *Peganum harmala* had antinociceptive activity (Monsef *et al*., 2004). In conclusion, the results suggest that the traditional medicament *Ophiorrhiza nicobarica* may offer some beneficial effects in the management of pain and inflammatory conditions. However, further studies are required to know the exact mechanism of analgesic and antiinflammatory activity of ON extract. The vernacular medicinal use of this plant to treat ailment related to inflammatory conditions may gives us a multiple advantage of their use in primary health care.

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