Evaluation of antinociceptive and gastro protective effects of orally and inhaled administered *Thevetia peruviana* Pers. K. Schum essential oil

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ABSTRACT

In the present study, the antinociceptive and the gastroprotective effects of orally administered or inhaled *Thevetia peruviana* Pers. K. (Schum) (Oleander) volatile oil, and its principal constituents linalool and 1,8-cineole were evaluated in mice. Either when orally administered (100 µl/kg) or inhaled for 60 min, *T. peruviana* volatile oil significantly reduced the acetic acid-writhing response in a naloxone-sensitive manner. In the hot plate test, analgesic activity observed after oil inhalation was inhibited by naloxone and atropine sulphate pretreatment suggesting the involvement of opioidergic as well as cholinergic pathways. Regardless of the administration route and the experimental model used both linalool and 1,8-cineole did not produce significant analgesic response. Oral or inhalatory treatment with analgesic doses of volatile oil did not affect mice spontaneous locomotors activity. Concerning the gastric effects, Oleander oil, linalool and 1,8-cineole oral administration protected against acute ethanol-induced gastric ulcers but did not prevent indomethacin-induced lesions indicating no interference with arachidonic acid metabolic cascade. In conclusion, besides this gastroprotection, Oleander oil reveals an interesting analgesic activity mainly relevant after inhalation, at doses devoid of sedative side effect, suggesting the interest for potential application of this oil in aromatherapy.

Keywords: Antinociceptive activity, gastroprotection, essential oils, *Thevetia peruviana*, writhing test, locomotors activity, hot plate test.

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INTRODUCTION

Volatile oils obtained from different species of *Thevetia* (*T. peruviana*, *T. nerifolia*, *Nerium oleander*) are frequently used in aromatherapy and massage to obtain many clinical benefits traditionally ascribed to their antibacterial, antifungal, carminative, sedative and antidepressant actions. Several studies have been conducted in order to validate these applications. In clinical trials oleander volatile oil demonstrated to improve sleep (Graham et al., 2003) and to reduce anxiety (Dunn et al., 1995). Furthermore, in animal models, it has been proved to possess anticancer (Pathak et al., 2000) and HIV-1 Reverse Transcriptase and HIV-1 Integrase Inhibitory activity (Tewtrakul et al.,

2002). Volatile oil showed anti-inflammatory (Erdemoglu et al., 2003) effect on smooth muscle *in vitro* supporting its use as antibacterial, antitermite and analgesic agent.

In the recent years, the study of natural products, as Oleander volatile oils, continues to attract researcher's attention in order to detect possible clinical uses and in particular, the list of oleander biological activities is increasing. Indeed, we have recently highlighted for oleander volatile oil an interesting antiplatelet activity associated with a promising protective effect in an animal model of acute pulmonary thromboembolism (Ballabeni et al., 2004). These whole oil activities are shared by its major components, linalool and 1,8-cineole. Experimental

studies conducted with the terpenic alcohol linalool and 1,8-cineole revealed a significant anti-inflammatory activity in carrageenin-induced rat paw oedema (Erdemoglu et al., 2003). More recent investigations demonstrated that linalool prevents nociception in different experimental models of thermal, chemical and inflammatory pain (Begum et al., 1997) suggesting analgesic and anti-inflammatory potentials for linalool producing plant species.

Thus, in the current study we investigated the antinociceptive profile of the lavender essential oil, linalool and 1, 8-cineole, orally administered or inhaled in mice, in experimental models of chemical and thermal pain. Furthermore, based on the antiulcer activity previously reported for 1,8-cineole, we assayed also the so far unexplored gastroprotective action of oleander oil in ethanol- and indomethacin-induced gastric ulcers in rats.

MATERIALS AND METHODS

Plant material and essential oil extraction

The flowers of *T. peruviana* were collected from the fields of Jaipur Dist., Rajasthan, India and were collected in the month of July to August 2010 in morning time. The plant was authenticated by a botanist in Department of Botany, University of Rajasthan, Jaipur. A voucher specimen (RUBL20856) has been kept in herbarium in Department of Botany, University of Rajasthan, Jaipur. Organoleptic examination refers to evaluation by means of organ of sense and includes the macroscopic appearance of the drug, its odor and tastes, occasionally the sound or 'snap' of its fracture and the feel of the drug to the touch.

The methods used to extract fragrant compounds today are based on the ancient principles of maceration, expression and steam distillation. An "absolute" is an extract obtained by extraction with volatile solvents or by Enfleurage. It is considered the purest perfume material, retaining most of the plant's aromatic constituents. Many modern techniques stem from those of ancient cultures.

Essential oil analysis

Essential oil samples were analyzed using a "Thermo TRACE GC ULTRA (GC)" gas chromatograph directly coupled to the mass spectrometer system (Thermo DSQ II – MS, Ionization for MS: Electron impact Ionization). Column - TR-5 Phase type: 5% Phenyl Methylpolysiloxane (Dimensions: 30 m Length × 0.25 mm ID × 0.25 μm film thickness) was used under the following conditions: oven temperature program from 60°C (5 min) to 250°C at 10°C/min and the final temperature kept for 5 min; injector temperature, 250°C; flow rate 1 ml/min; Used oven run time – Yes; No. of Ramps – 1; Post run temp. – Off; Hold time 10 min.; Enable cryogenics- off; Equilibration time – 0.50 min. For the MS Method; Acquisition time – GC run time; Cal Gas – off; Reagent gas – off; Aquire profile – No; Acq. Thresold – 0; Source temp. – 200°C; Segment – 1; Detector gain – 100000; Chrom filter width – off; Reagent gas flow – off; Scan event – 1; Scan rate – 500.00; Mass defect – 0.00; Scan mode – Full scan; First mass – 50.0; Last mass – 1000.0; Ionization energy 70eV. The constituents of essential oils were identified in comparison with Library calculated in relation to the retention time of a series of alkanes (C - C) (Derwich et al., 2010). The oil composition is reported in Table 1.

Animals

Experiments were performed on Swiss mice (20 to 30 g) of either gender, or female Wistar rats (150 to 200 g). All animals were fasted but had free access to water 18 h before the experiments.

Drugs

The following substances were used: linalool, 1,8-cineole, atropine sulfate, naloxone hydrochloride, mecamylamine hydrochloride, methylcellulose, acetic acid and ethanol, indomethacinate meglumine.

Oral treatment

Groups of 6 animals received by gavage Oleander oil (100 mg/kg), linalool (33 mg/kg) and 1,8-cineole (36 mg/kg) in a final volume of 1 ml/100 g body weight 1 h before the experiments. Linalool and 1,8-cineole were administered at doses chosen in accordance to the percentages of these constituents in the natural oil as detected by gas chromatography. Control animals received vehicle alone (0.1% methylcellulose). A 1% aqueous emulsion of Oleander oil in methylcellulose was prepared immediately before use.

Inhalation

When inhaled, 200 μl of Oleander oil, linalool or 1, 8 cineole, contained in a 10 ml glass baker, were positioned at the bottom of plastic cages covered with plastic film in order to saturate the ambient. At saturation, the concentration of the oils in the cage was 2.4 μl/L. Mice introduced into the cage were allowed to inhale oil vapours for controlled time periods (15, 30 and 60 min) prior to performing the final experiments. Control animals were caged in the same conditions but in the absence of the tested oils. All experiments were conducted between 9.00 and 16.00.

Acetic acid writhing test

The writhing test was performed according to Koster's method (Koster et al., 1959), briefly, concluded the inhalation time or passed 1 h from the oral administration of the oils under study or the vehicle, mice were intraperitoneally injected with 0.2 ml of 0.6% acetic acid. After treatment with the algogen agent, mice were placed in observational chambers and the number of writhes of each mouse was counted over a period of 30 min. Different sets of mice were pre-treated with the opioid antagonist naloxone (5 mg/kg i.p.), the muscarinic antagonist atropine (5 mg/kg i.p.) and the nicotinic antagonist mecamylamine (1 mg/kg i.p.) 10 min before the tested oils or vehicle challenge.

Hot plate test

In the hot plate test, animals were divided in seven groups containing six animals each. one group serves as negative control group was pretreated with distilled water in the appropriate volume three groups were pre-treated with naloxone (5 mg/kg i.p.), atropine (5 mg/kg i.p.) and mecamylamine (1 mg/kg i.p.) followed by oleander oils after 10 min. One group was treated with oleander oil only and rest two groups were pretreated with linalool and 1,8–cineole as standard.

The hot plate test was performed according to the method described by Eddy and Leimbach (1953). Mice were individually placed on the 55°C hot plate and the time between the placement

Table 1. Chemical composition and retention index of the constituents of the volatile oil of *Thevetia peruviana* Pers. K. Schum.

Compounds	KI	%
α-Pinene	943	0.20
Camphene	967	0.09
β-Pinene	973	0.19
β-Myrcene	985	1.70
Hexyl acetate	1018	0.12
1,8-cineole	1035	34.23
Camphor	1054	7.42
Ocimene	1063	0.92
Cis-carviol dihydro	1103	8.98
1-Ally l4-methoxybenzene	1234	5.84
Hexyl butanoate	1294	0.73
Linlool	1322	23.07
Linalyl acetate	1327	3.04
Geranyl acetate	1367	0.80
β-Criophyllene	1423	0.62
D-glucitol	1487	1.09
1,1-Dihydropropanol	1501	0.03
Caryophyllene oxide	1535	0.21
Farnesene	1587	0.73
α-Bisabolol	1676	0.30
Total Identified		90.35%

and the occurrence of anterior paw licking, shacking or jumping was recorded as Latency Time(s). In order to exclude hypo- or hypersensitive mice, two hours before the final experiment all the animals were tested and those with latency time shorter than 10 s or longer than 18 s were eliminated from the study. Basal Latency Time (T_0) was measured before the administration of drugs or vehicle. Forward Latency Times (T_1) were measured starting 1 h after oral treatment or after 15, 30 and 60 min of exposure to oils vapour with intervals of 15, 30 and 60 min. Different groups of mice were pre-treated with naloxone (5 mg/kg i.p.), atropine (5 mg/kg i.p.) and mecamylamine (1 mg/kg i.p.) 10 min before the tested oils or vehicle administration. Time of 30 s was arbitrarily chosen as cut-off time (T_2). Results were expressed as percentage of analgesic effect as follows: % MPE (Percent maximal possible effect) = (T_1-T_0)/(T_2-T_0) × 100 (latency time after treatment-basal latency time)/(time of cut off – basal latency time).

Locomotor activity

For the locomotor activity, the animals were divided into four groups containing seven animals each. One group was pretreated with saline water from oral rout of administration; one group was pretreated with Oleander oil from inhalation route, one group was considered as control without any treatment and one group was pretreated with linalool as standard.

Locomotor activity was measured by means of an activity cage. Passed one hour from oral administration of oleander oil or vehicle or at the end of inhalation period times, mice were placed singularly into the activity cage and locomotor activity was recorded every 5 min for 90 min. All experiments were conducted from 9.00 to 15.00.

Acute gastrointestinal ulcerogenicity

Acute gastrointestinal ulcerogenicity was assessed following Rainsford's method (Rainsford, 1982). Briefly, rats were treated orally with oleander oil 100 mg/kg. After 5 h, animals were sacrificed by CO_2 inhalation; the stomachs were removed, fixed in 4% formaldehyde solution and processed for microscopic analysis using an image analyzer system. The total damaged area (mm^2) and the number of gastric ulcers were counted for each stomach by an observer unaware of the treatment given to the animals.

Protection against acute indometacin- and ethanol-induced gastric lesions

For acute gastrointestinal ulcerogenicity the rats were randomly assigned to 5 groups of 6 animals each one. One group was considered as vehicle without any pre-treatment and rest three groups were pre-treated with oleander oil (100 mg/kg), linalool (33 mg/kg), 1,8-cineole (36 mg/kg) and linalool (33 mg/kg) plus linalyl acetate (36 mg/kg) on ethanol-induced gastric ulcers.

Oleander oil, linalool and 1,8-cineole were tested as potential gastro protective drugs in two different models of acute gastric ulcers. For this purpose, rats were randomly assigned to 5 groups of 6 animals each one. To evaluate the ability of oils to protect against NSAIDs-induced gastric ulcers, animals were treated simultaneously with indomethacin (40 mg/kg i.p.) and oils per os. The animals were killed 5 h later. The protection against ethanol-induced gastric lesions was tested administering orally 1 ml of 90% ethanol to animals which 1 h previously had been treated orally with essential oils. The animals were killed 1 h later. Stomachs were then removed and processed as described before.

Statistical analysis

All data are expressed as mean ± S.E.M (n = 6 observations per group). Results were analyzed statistically using Student's t-test for
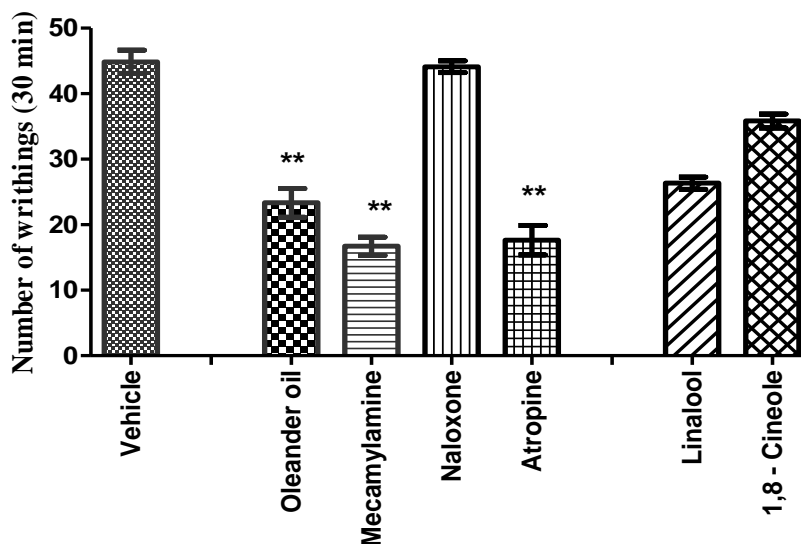
Figure 1. Effect of oral administration of oleander oil (100 mg/kg), linalool (33 mg/kg) and 1,8-cineole (36 mg/kg), on the acetic acid-induced writhings and effect of pretreatment of animals with naloxone (5 mg/kg ip), atropine (5 mg/kg ip) and mecamylamine (1 mg/kg ip) on oleander antinociceptive effect. The number of writhings was counted for 30 min following acetic acid injection. The vertical bars indicate the standard error of the mean. The number of mice used for each group was 6. **P<0.01 compared to vehicle-treated mice.

unpaired data. P values less than 0.05 or 0.01 were considered as indicative of significance or high significance respectively.

RESULTS

Table 1 show the composition and the relative abundance of the constituents identified in the oleander volatile oil. The major components are linalool (23.07%) and 1, 8-cineole (33.23%).

Acetic acid writhing test

Administration of oleander oil 100 mg/kg significantly reduced the writhing response to acetic acid treatment to 51% over the control group (P = 0.0002) (Figure 1). This antinociceptive effect was significantly prevented by opioid antagonist naloxone pre-treatment but it was completely unaffected by either nicotinic antagonist mecamylamine or muscarinic antagonist atropine administered at doses by themselves unable to modify nociceptive response. When the effects of the two major components, linalool and 1,8-cineole, were separately considered, a modest antinociception was observed only with linalool oral administration. Inhalation of oleander volatile oil attenuated the writhing numbers in a time dependent manner producing a significant antinociception (61% reduction over control, P < 0.0001) (Figure 1) only after 60 min of exposure. In this case, the oleander oil antinociceptive effect was completely

prevented by the administration of all the three different antagonists. Linalool and 1, 8-cineole inhalation for 60 min caused only a partial reduction of writhing response (Figure 2).

Hot plate test

Oral administration of oleander oil 100 mg/kg failed to prolong latency time compared with controls in mice hot plate test. On the other hand, inhalation of oleander oil produced an inhibition of the hotplate response proportional to the time of exposure to oil vapors, yielding a significant delay (P < 0.01) in reaction time after 60 min inhalation. This analgesic activity peaked at the suspension of inhalation and progressively diminished disappearing at 60 min. This oleander oil antinociceptive effect was significantly prevented by pretreatment with naloxone, atropine and mecamylamine, administered at doses by themselves unable to modify nociceptive response (Table 2). No analgesia was accounted after 60 min inhalation of linalool and 1, 8-cineole.

Effect on locomotors activity

No significant alterations of locomotors activity were observed in mice after treatment with oleander oil either orally administered at the dose of 100 mg/kg (2347 ± 567 counts in 90 min) or inhaled for 60 minutes (2767 ± 756 counts in 90 min) with respect to vehicle-treated animals
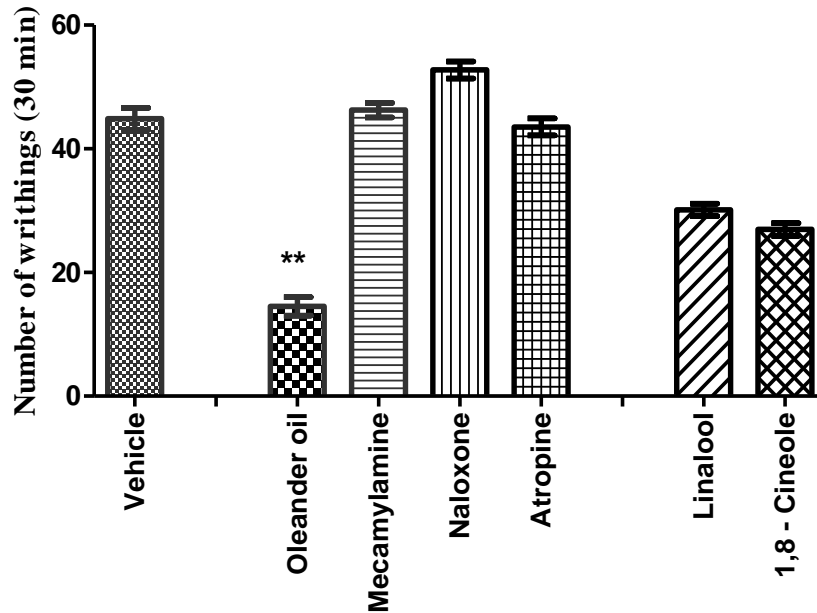
Figure 2. Effect of oleander oil, linalool and 1,8-cineole inhaled for 60 min on the acetic acid-induced writhings and effect of pretreatment with naloxone (5 mg/kg ip), atropine (5 mg/kg ip) and mecamylamine (1 mg/kg ip) on antinociceptive effect produced by oleander oil. The number of writhings was counted for 30 min following acetic acid injection. The vertical bars indicate the standard error of the mean. The number of mice used for each group was 6. **$P<0.01$ compared to vehicle-treated mice.

Table 2. Time dependence of analgesic effect of inhaled oleander oil, linalool and 1,8-cineole in hot plate test in mice in the absence and in the presence of pretreatment with naloxone (5 mg/kg/ip), atropine (5 mg/kg/ip) and mecamylamine (1 mg/kg/ip).

Treatment	Time after the suspension of inhalation			
	0 min	15 min	30 min	60 min
Vehicle	-2.7 ± 6.8	-3.6 ± 9.7	-2.0 ± 8.9	-1.8 ± 7.2
Oleander oil	38.7 ± 13.7**	32.4 ± 10.2*	16.9 ± 7.8	-6.5 ± 7.2
Oleander oil + Naloxone	1.6 ± 8.2	-26.8 ± 7.7	-14.9 ± 5.5	-11.3 ± 8.3
Oleander oil + Atropine	-16.4 ± 6.3	-11.2 ± 8.8	-29.2 ± 11.2	18.9 ± 8.6
Oleander oil + Mecamylamine	-10.3 ± 4.3	-20.3 ± 9.9	-24.5 ± 7.4	24.8 ± 8.4
Linalool	-4.9 ± 5.9	18.8 ± 8.6	-6.4 ± 5.4	-12.5 ± 5.8
1, 8 - cineole	4.2 ± 5.9	2.3 ± 7.1	-3.5 ± 7.2	17.3 ± 7.2

Results are expressed as mean ± S.E.M of percent maximal possible effect. * $P < 0.05$ compared to vehicle-treated mice. ** $P < 0.01$ compared to vehicle-treated mice.

(2012 ± 497 counts in 90 min).

Gastrolesivity and gastro protection

Acute oral treatment with oleander oil (100 mg/kg os) did not produce any damage on rat gastric mucosa. Regarding the gastro protection, neither oleander oil oral administration nor oil inhalation protected against indomethacin-induced gastric ulcers being the number and the area of gastric lesions observed in oleander treated rats comparable to vehicle-treated animals (ulcer number 13.7 ± 4.6 and 17.9 ± 4.5 respectively and injured area 8.4 ± 4.7 mm^2 and 6.7 ± 2.5 mm^2 respectively).

A significant prevention of acute ethanol-induced gastric lesions was elicited by oleander oil oral administration (100 mg/kg) as it reduced the total injured area by an 87% (P = 0.003) compared with control. A lower but still significant gastro protection was obtained with the administration of linalool (33 mg/kg os) and
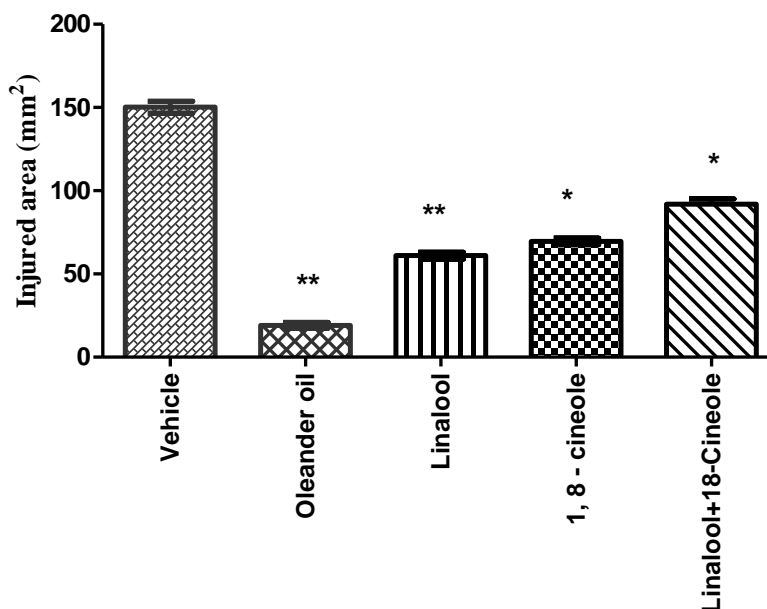
Figure 3. Effect of oral administration of oleander oil (100 mg/kg), linalool (33 mg/kg), 1,8-cineole (36 mg/kg) and linalool (33 mg/kg) plus 1,8-cineole (36 mg.kg) on ethanol-induced gastric ulcers. Results are expressed as total injured area. Vertical bars indicate the standard error of the mean. The number of rats used for each group was 6. *P<0.05 and **P<0.01 compared to vehicle-treated rats.

1,8-cineole (36 mg/kg os) which diminished the hemorrhagic erosion areas of about 54% (P = 0.003) and 47% (P = 0.013) respectively (Figure 3). Co-administration of the two components did not enhance the gastro protection produced by the oils singularly administered. Essential oil inhalation for 60 min failed to protect gastric mucosa from necrotizing action of ethanol.

DISCUSSION

In the current study we demonstrate that oral treatment with whole oleander oil produces significant antinociception and gastroprotective activity in animal models. This pharmacological activity could derive from the contribution of various active principles composing the whole oil such as linalool, myrcene and linalyl acetate, previously proved to possess antinociceptive activity (Peana et al., 2002; Rao et al., 1990). Higher analgesic efficacy was exhibited by oleander oil when administered through inhalatory route being the nociceptive responses to chemical (writhing test) and thermal (hot plate test) stimuli significantly reduced. At variance with oleander oil, linalool and 1,8–cineole produce only scarce or no analgesic effect in the two pain models here adopted. The different dosage applied in this study with respect to previous investigations (Peana et al., 2002) can account for the lack of antinociceptive activity of linalool both in writhing and hot plate tests. The

absence of any modification of spontaneous locomotor activity after oral/inhaled administration of antinociceptive doses of whole oil let us to rule out the occurrence of sedative effect confounding analgesia studies. It must be pointed out that in literature the sedative effect of oleander oil upon inhalation in mice has been clearly described (Buchbauer et al., 1993).

However present findings are not conflicting with previous data describing the sedative effect of oleander oil since in this work we studied locomotor activity by exposing mice to oleander oil lower air concentration than that proved to produce sedative serum oils levels after inhalation (Buchbauer et al., 1993; Letizia et al., 2003). As for the mechanism underlying oleander oil analgesic action it is noteworthy that opioidergic neurotransmission seems to be primary involved in oral induced analgesia since only naloxone pretreatment prevents oleander effect in writhing test. Also cholinergic system appears to play a significant role in oleander oil analgesia displayed after inhalation since also the blockade of muscarinic and nicotinic receptors prevented antinociception. The involvement of cholinergic transmission could be ascribed to some component terpenes of the oil in addition to linalool, since potent *in vitro* anticholinesterase activity was reported for terpenoids such as 1,8-cineole, a constituent of various essential oils (Savelev et al., 2003).

As concerns antiulcer activity, interestingly, linalool as well as 1,8-cineole demonstrate to contribute to the gastroprotective effect of oleander oil which, orally

administered, caused a dramatic reduction of ethanol-induced gastric injury in rats. The involvement also of additional active principles, such as the gastroprotective agent 1, 8-cineole (Santos and Rao, 2001), cannot be ruled out since the antiulcer effect of the co-administration of linalool and 1,8-cineole is lower than that of whole oil.

The lack of protective effect against gastric mucosal damage caused by indomethacin led us to hypothesize that gastroprotection afforded by oleander oil cannot be attributed to interference with arachidonic acid metabolic cascade. Actually, we have already described an interesting ability of oleander oil to prevent experimental thrombus formation with an ASA-unlike mechanism of action (Ballabeni et al., 2004). The amelioration of gastric microcirculation could be the mechanism underlying the oleander gastroprotection against ethanol injury which is known to be dependent on microvasculature engulfment in the gastric mucosa (Oates and Hakkinen, 1988).

In conclusion, the results of this study reveal a remarkable analgesic and gastroprotective activities of oral oleander oil at doses 100 to 400 fold lower than those proved to be acutely toxic for the main components of the phytocomplex (Letizia et al., 2003). Furthermore, the effectiveness of oil inhalation in controlling chemical and thermal pain without evidence of central adverse effects supports the interest for potential application of oleander essential oil in aromatherapy.

ACKNOWLEDGEMENTS

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