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Antinociceptive and gastro-protective effect of the ethanolic extract of the flowering top of *Anthocephalus Cadamba Roxb*

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SUMMARY

The effect of alcoholic extract of *Anthocephalus (A.) Cadamba Roxb.* was evaluated in experimental models of pain and ulcer. Hot tail flick test, hot plate test and acetic acid induced writhing test were employed for evaluating the peripheral as well as central analgesic mechanism exerted by the extracts. Gastroprotective activity was examined by HCl and ethanol induced gastric damage test. Test group received crude extract 500 mg/kg showed maximum time needed for the response against thermal stimuli (6.26 ± 0.439 s) which is comparable to diclofenac sodium (6.56 ± 0.381 s) in hot tail flick method. These experimental results also followed the experimental results of hot plate test where crude extract 500 mg/kg showed maximum time needed for the response against thermal stimuli (4.74 ± 0.234 s) which is comparable to diclofenac sodium (5.58 ± 0.585 s). The crude extract at 500 and 250 mg/kg showed significant reduction in acetic acid induced writhing in mice with a maximum effect of 68.026% reduction at 500 mg/kg dose which is comparable to standard diclofenac sodium (79.93%). In gastroprotective study the extract of *A. Cadamba* (250 and 500 mg/kg) significantly inhibited ulceration induced by both HCl and ethanol dose dependently. Results of the study suggest that the extract possesses both analgesic and gastroprotective activity on mice.

Key words: *Anthocephalus Cadamba*; Nociceptive pain; NSAID; Diclofenac; Gastric protection

INTRODUCTION

The use of natural products is growing in the world especially in developing countries. Medicinal herbs have been used as a form of therapy for the relief of pain throughout history (Almeida *et al.*, 2001). The treatment of rheumatic disorder is an area in which the practitioners of traditional medicine enjoy patronage and success (Akah and Nwambie, 1994). Taking into account the most important analgesic prototypes (e.g. salicylic acid and morphine) were originally derived from the plant sources, the study of plant species traditionally used as pain killers should still be seen as a fruitful research strategy in the search of new analgesic and anti-inflammatory drugs. Recent studies found that different substances from plant sources not only afford gastroprotection but also accelerate ulcer healing. They may also possess anti-inflammatory action by suppressing the neutrophil/cytokine cascade in gastrointestinal tract (GIT) (Zayachkivska

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et al., 2005), promoting tissue repair through expression of various growth factors (Kim *et al*., 2004), exhibiting antioxidant activity (Kim *et al*., 2004), scavenging reactive oxygen species (ROS) (Liu *et al*., 2002; Pastrada-Bonilla *et al*., 2003) showing anti-nucleolytic, cytochome P450 2F1 inhibitory acitivity, anti-necrotic and anti-carcinogenic activities (Bagchi *et al*., 2002). *Anthocephalus (A.) Cadamba Roxb*. syn. *Anthocephalus chinensis* (Family-Rubiaceae) locally known as Kadam is widely distributed throughout Bangladesh, Nepal, eastward to India (Assam Province and Chotanagapur district at Bihar Province), Myanmar, Sri Lanka, the Philippines, Indonesia, and Papua New Guinea (Sahua *et al*., 2000). It is a large, fast growing species with spreading branches. The tree can reach up to 20 - 30 m in height. In the dry season, the tree sheds its leaves. The tree flowers in May through July. The flowers are yellow in color (Handa *et al*., 1984a). Plant parts are used as a folk medicine in the treatment of fever and anemia, as antidiuretic, and for improvement of semen quality (Umachigi *et al*., 2007). The leaves are recommended as a gargle in cases of stomatitis (Slkar *et al*., 1996). Some scientific studies have been carried out to reveal its antimalarial (Sianne and Fanie, 2002) and antihepatotoxic activities (Kapil *et al*., 1995). *A. Cadamba* is ethnomedicinally widely used in the form of paste by tribe in Western Ghats for treating skin diseases. Antibacterial, wound healing and antioxidant properties has also been reported in the recent years (Umachigi *et al*., 2007). There is little phytochemical information on *A. Cadamba*. The major constituents of bark are triterpenes, terpernoid glycosides, saponins, indole alkaloids cadambine, 3 a-dihydrocadambine, cadamine, isocadamine and isodihydrocadambine (Handa *et al*., 1984b; Sahua *et al*., 2000).

As a part of an investigation on natural bioactive substance from local medicinal plants of Bangladesh, in this paper, we report a study of the antinociceptive and gastroprotective activity of Anthocephalus cadamba flowering tops.

MATERIALS AND METHODS

Plant material

The flowering tops of Anthocephalus cadamba (Roxb.) Miq. were collected from Siddeswari campus, Stamford University, Bangladesh in June 2007, and identified by Bangladesh National Herbarium, Mirpur, Dhaka (Accession No.-32497).

Extraction

Dried ground coarse powder of flowering tops (200 mg) was extracted with 95% ethanol in a Soxhlet apparatus at an elevated temperature (45°C). The extract was concentrated by evaporation under reduced pressure at 40°C using Buchi rotary evaporator to have gummy concentrate of greenish color extract (yield appx. 6.32%).

Test samples and standards

Crude extract of *A. Cadamba* was prepared in distilled water and was administered at the dose of 250 and 500 mg/kg body weight per orally. Diclofenac sodium (100 mg/kg) was used as standard in antinociceptive tests. HCl and ethanol were used to produce damage to gastric mucosa in gastroprotective test. Gastric administration of all drugs was accomplished via oral gavages by feeding needle.

Animal

Male Swiss albino mice (20 - 25 g) were used in this investigation. Albino mice were obtained from the Animal house of the International Centre for Diarrhoeal Disease and Research, Bangladesh (ICDDR, B). Animals were maintained under standard environmental conditions and had free access to feed and water ad libitum. Experiments on animals were performed based on animal ethics guidelines (Zimmermann, 1983) and approved by Institutional Animal Research Ethics Committee. Albino mice (n = 5, per group) were used for anti-nociceptive activity screening and divided into four different groups. First group served as control animals they were treated with distilled water.

Second group of animals were treated with standard drugs. The standard drugs diclofenac sodium 100 mg/kg (anti-nociceptive activity) was used. The next two groups of animal were treated with the alcoholic crude extract at two different doses (250 and 500 mg/kg). In ethanol and HCl induced mucosal damage study similar mice (n = 5, per group) were divided into six groups. First two groups serve as control groups, next two groups were treated with 250 mg/kg extract and the last two groups with 500 mg/kg extract. One hour after administration of ethanol and HCl, each animal was killed by ether anesthesia and the stomach was removed and inflated by injection of 10 ml 1.5% formalin to fix the inner and outer layers of the gastric walls. Subsequently, the stomach was incised along the greater curvature; the lengths and numbers of gastric lesions were measured and were expressed as a lesion index.

Writhing test

The 'acetic acid' test method used in this study was adopted from those described in detail earlier by Hendershot and Forsaith, (1959), Koster *et al*. (1959), Williamson *et al*. (1996), Zakaria *et al*. (2001) and Silva *et al*. (2003). Experimental animals were randomly selected and divided into four groups denoted as group-I, group-II, group-III, group-IV, consisting of 5 mice in each group. Each group received a particular treatment i.e. control, positive control and the two doses of the extract. Group-I is served as the control and received only distilled water and tween-80. Group-II was received diclofenac sodium (100 mg/kg, i.p), administered 15 min prior to counting of writhings, the standard drug for comparison of potencies. The last two groups i.e. Group-III and Group-IV were administered the crude extract suspensions orally. 30 min interval was given to ensure proper absorption of the administered substances. Then each group was treated with intraperitoneally administered 0.2 ml of a 0.6% acetic acid solution (Koster *et al*., 1959). The number of writhes (i.e., abdominal contractions and stretches) that occurred within the first 20 min following acetic acid administration were counted and recorded. The recorded numbers of acetic acid-induced writhes that occurred in the positive control and test group i.e. crude extracts treated mice were compared with those in the control group mice.

Hot plate test

The hot plate test was carried out according to the method described by Woolfe and Mac Donald (1944). Mice were placed on a hot plate maintained at 55°C ± 1°C. Latency of nociceptive response such as licking of a hind limb or jumping was measured. Starting 30 min after oral administration of the test agents except diclofenac- sodium (15 min after administration), the nociceptive response was measured every 30 min over a 90 min period. Diclofenac sodium was injected intraperitoneally. The cut-off time was 15 s. Only the mice that showed nociceptive responses within 10 s were used for the experiments.

Tail flick test

Mice were treated with distilled water, diclofenac sodium (100 mg/kg) and two doses of the crude extract (250 and 500 mg/kg). Antinociceptive effect of the test substances was determined by the hot tail-flick method described by Sewell and Spencer (1976). Basal reaction time of animals to radiant heat was recorded by placing the tip (last 1 - 2 cm) of the tail on the radiant heat source. The tail withdrawal from the heat (flicking response) is taken as the end point. The animals, which showed flicking response within 3 - 5 s, were selected for the study. A cut off period of 15 s is observed to avoid damage to the tail. The measurements of withdrawal time using the tail flick apparatus was conducted at 30 and 60 min after administration of drugs.

HCl and ethanol-induced mucosal membrane lesions

Gastric mucosal lesions were induced by the method

of Mizui and Doteuchi (1983). The mice were divided into groups of 5 animals. After 24 h fasting, the extracts and drugs were administered orally to the mice. 30 min thereafter, each mouse received 0.2 ml of 1 N HCl or absolute ethanol by oral administration. 60 min after administration of the necrotizing agent, each animal was killed by ether euthanasia, and the stomach was excised, inflated by injecting 2 ml of normal saline and then fixed for 30 min in 5% formalin solution. After opening along the greater curvature, HCl induced gastric damage was observed in the gastric mucosa as elongated black-red lines parallel to the long axis of the stomach of the mice. The lesion index was determined as the sum or length of erosion per mouse (Sun *et al.*, 1991). Ethanol induced lesion was assessed and scored for severity according to, (0) absence of lesion, (1) superficial 1 - 5 hemorrhagic points, (2) superficial 6 - 10 hemorrhagic points, (3) submucosal hemorrhagic lesions with small erosions (4) severe hemorrhagic lesion and some invasive lesions.

Statistical analysis

Results were expressed as mean ± S.E.M. and were analyzed for statistically significant difference using one-way ANOVA, followed by the Bonferroni post-hoc test. P values < 0.05 were considered significant.

RESULTS

Analgesic activity was determined by evaluating the response against a series of experimental animal model like hot plate test; hot tail flick test, writhing effect which was produced by administration of the acetic acid by the test drug i.e. crude extracts in comparison with a standard drug might prove efficacy of the desired action.

Acetic acid-induced writhing test

Dose dependent antinoceceptive effect was noted with the extract at the tested dose levels (Table 1). Maximum percentage of inhibition of writhing response exhibited by the extract at 500 mg/kg was 68.026%, while the same at 250 mg/kg showed 40.133% reduction in acetic acid induced writhing response respectively, which was comparable to that of standard diclofenac sodium (100 mg/kg) that caused 79.93% pain inhibition.

Hot plate test

Two doses of extracts of *A. Cadamba* increased the reaction time in a dose-dependent manner to the thermal stimulus which is presented in the Table 2. The highest nociceptive inhibition of thermal stimulus was exhibited at a higher dose of the extracts 500 mg/kg of crude extract which has maximum time needed for the response against thermal stimuli (4.74 ± 0.225), which is comparable to diclofenac sodium (5.58 ± 0.585).

Tail immersion/hot tail-flick

Table 3 shows the results of the Tail immersion/ hot tail-flick test results. Two doses of extracts of *A. Cadamba* increased the reaction time in a dose-dependent manner to the thermal stimulus. The highest nociceptive inhibition of thermal stimulus was exhibited at a higher dose of the extracts 500

Table 1. Effect of *A. Cadamba* ethanolic extract on acetic acid induced writhing in mice

Treatment	Dose (mg/kg)[a]	Route of administration	Writhings[b]	% of writhing	% of writhing Inhibition
Control (Distilled water)	10 ml/kg	p.o.	29.9 ± 1.303	100	0%
Diclofenac-Na	100	i.p.	6.1 ± 0.314*	20.06	79.93%
A. Cadamba Extract	250	p.o.	17.9 ± 0.758*	59.86	40.133%
	500	p.o	9.56 ± 0.733*	31.97	68.026%

[a]Administered 30 min before 0.2 ml of 0.6% acetic acid administration (10 ml/kg, i.p.). [b]Counted for 20 min, starting 5 min after acetic acid administration; *P < 0.05 vs. control, Student's t-test; values are mean ± S.E.M. (n = 5).

Table 2. Effect of *A. Cadamba* ethanolic extracts on hot plate test in mice

Treatment	Dose (mg/kg, p.o)[a]	Response Time (s)			
		0 min	30 min	60 min	90 min
Control (Distilled water)	10 ml/kg	1.78 ± 0.263	2.5 ± 0.187	2.9 ± 0.370	3.08 ± 0.451
Diclofenac-Na	100 (i. p.)	1.98 ± 0.198	4.72 ± 0.435*	4.94 ± 0.577*	5.58 ± 0.585*
A. Cadamba Extract	250	2.04 ± 0.288	3.88 ± 0.451*	4.06 ± 0.386*	4.2 ± 0.206*
	500	2.08 ± 0.163	4.8 ± 0.254*	4.7 ± 0.234*	4.74 ± 0.225*

[a]Beginning 30 min after oral administration of test agents (or 15 min after Diclofenac-Na.), the nociceptive response was measured every 30 min over a 90-min period. Each datum represents the latency of nociceptive responses (s) ± S.E.M. (n = 5). *P < 0.05 compared with the control group (Student's t-test)

Table 3. Effect of *A. Cadamba* ethanolic extracts on hot tail -flick test in mice

Treatment	Dose (mg/kg, p.o)[a]	Response Time (sec)		
		0 min (Latency)	30 min	60 min
Control (Distilled water)	10 ml/kg	2.2 ± 0.418	2.64 ± 0.749	2.6 ± 0.273
Diclofenac-Na	100 (i.p.)	4.2 ± 0.418*	4.69 ± 0.329*	6.56 ± 0.381*
A. Cadamba extracts	250	2.4 ± 0.447	4.9 ± 0.245*	4.934 ± 0.616*
	500	2.8 ± 0.418	5.26 ± 0.460**	6.26 ± 0.439*

[a]Beginning 30 min after oral administration of test agents (or 15 min after Diclofenac-Na.), the nociceptive response was measured every 30 min over a 60 min period. Each datum represents the latency of nociceptive responses (s) ± S.E.M. (n = 5) *P < 0.05 compared with the control group (Student's t-test)

Table 4. Ulcer protective effect of the ethanolic extract of *A. Cadamba*

Treatment	Dose (mg/kg)	Route of administration	Ulcer Score	
			HCl (mm)	Absolute ethanol
Control 1 N HCL or Absolute ethanol	0.2 ml	p.o.	15.6 ± 0.758	11.6 ± 0.908 (2.31, 100%)
A. Cadamba extracts	250	p.o.	10.8 ± 0.651*	6.00 ± 0.612* (1.2; 51.724%)
	500	p.o	5.2 ± 0.418*	4.2 ± 0.418* (0.84; 6.206%)

Data represents the mean ± S.E.M., *P < 0.05 compared with the control considered as statistically significant. Ulcer score was determined in case of HCl induced lesion as lengtn/mouse and score of ethanol induced lesion was calculated as the number of ulcer spot found per mouse.

mg/kg of crude extract (6.26 ± 0.439), which is comparable to that of diclofenac sodium (6.56 ± 0.381).

HCl or ethanol-induced mucosal membrane lesions

The extract of *A. Cadamba* (250 - 500 mg/kg) significantly (P < 0.05) inhibited ulceration induced by both HCl and ethanol dose dependently in mice. The results obtained were compared with the control group animals which received no treatment other than exposed to HCl or ethanol orally (Table 4 and Fig. 1).

DISCUSSION

The results demonstrate that the ethanol extract obtained from the flowering tops of *A. Cadamba* attenuated centrally acting pain mechanism as well as nociceptive responses to chemical stimuli in the acetic acid induced writhing in mice.

The writhing test is generally used for screening of antinociceptive effects (Hendershot and Forsaith, 1959; Koster *et al.*, 1959). With respect to the writhing test, the research group of Deraedt *et al.* (1980)

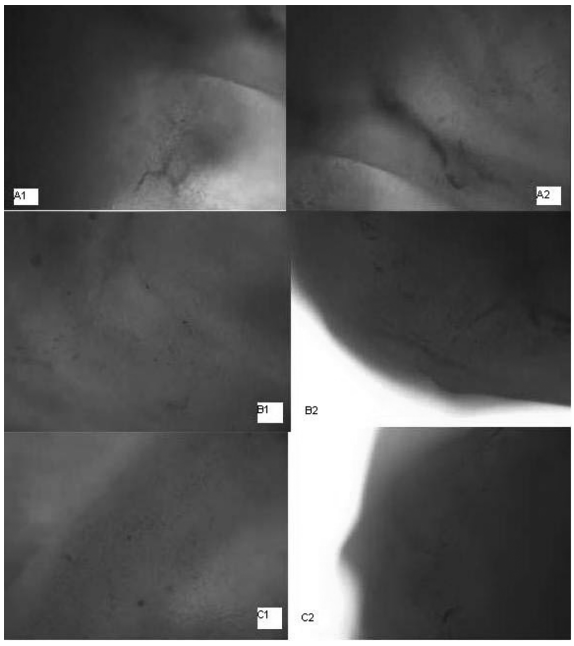

Fig. 1. Typical photomicrograph of the ulcer induced by ethanol and HCl. A1, A2- Untreated control group for HCl and ethanol; B1, B2- *A. Cadamba* extract (250 mg/kg) + 1 N HCl acid or Ethanol; C1, C2- *A. cadamba* extract (500 mg/kg) + 1 N HCl acid or ethanol. (Binocular microscope Axistar Plus with cannon A 610/620 photo documentation system, Magnification × 10).

described the quantification of prostaglandins by radioimmunoassay in the peritoneal exudates of rats, obtained after intraperitoneal injection of acetic acid. They found high levels of prostaglandins PGE2a and PGF2a during the first 30 min after acetic acid injection. Nevertheless, it was found that the intraperitoneal administration of acetic acid induces the liberation not only of prostaglandins, but also of the sympathetic nervous system mediators (Hokanson, 1978; Duarte *et al.*, 1988). The ethanol extracts of *A. Cadamba* showed significant inhibition on acetic acid-induced writhing response compared to reference drug diclofenac sodium (100 mg/kg) in mice. Diclofenac, a non-steroidal anti-inflammatory drug (NSAID), is commonly employed in the treatment and/or management of rheumatoid arthritis, osteo-arthritis and ankylosing spondylitis (Eddy and Leimback, 1953; Siraux, 1977) and for its anti-inflammatory and analgesic effects (Brooks *et al.*, 1980). Diclofenac reduces inflammation, swelling and arthritic pain by inhibiting prostaglandins synthesis and/or production (Skoutakis *et al.*, 1988; Todd and Sorkin, 1988; Small, 1989).

It is known that non-steroidal anti-inflammatory drugs usually do not increase the pain threshold in normal tissues, whereas local anesthetics and narcotics do (Ferreira *et al.*, 1978). Hence, the hot plate test and hot-tail flick test was undertaken to verify whether the ethanol extract of *A. Cadamba* have any central analgesic effect. Thermic painful stimuli are known to be selective to centrally but not peripherally acting analgesic drugs (Chau, 1989). Thermal induced nociception indicates narcotic involvement (Besra *et al.*, 1996) and are more sensitive to opioid μ receptors and non-thermal tests are to opioid κ receptors. (Abbott and Young, 1988) The centrally acting analgesics generally elevate the pain threshold of mice towards heat. Ethanolic extract of *A. Cadamba* significantly ($P < 0.05$) increased the reaction time of animals towards the thermal source in a dose-dependent manner. In hot plate test 500 mg/kg dose of ethanolic extract of *A. Cadamba* showed a pain inhibition by increasing the reaction time (licking of paw) from 2.08 ± 0.163 s to 4.74 ± 0.225 s after 90 min of the dose administered whereas the standard drug diclofenac showed a pain inhibition by increasing the reaction time (licking of paw) from 1.98 ± 0.198 s to 5.58 ± 0.585 s (Table 2). Ethanolic extract of *A. Cadamba* 250 mg/kg dose also significantly increase the reaction time.

The drugs showed greater activity after 60 min of drug administration, in which ethanolic extract of *A. Cadamba* (500 mg/kg, p. o.) exhibited greater pain inhibition by increasing the reaction time (licking of tail from hot water) from 2.8 ± 0.418 s to 6.26 ± 0.439 s (Table 3) in Tail flick test. The results for the group-treated with the ethanol extracts of *A. Cadamba* significantly inhibit the central pain mechanism from those obtained for the negative control group. Thus the anti-nociceptive activity shown by crude extract of *A. Cadamba* in hot plate,

hot tail-flick and acetic acid induced writhing test indicate that alcoholic extracts of the plant might possess centrally and peripherally mediated anti-nociceptive properties.

Gastric mucosal layers play a role of a barrier that limits the exposure of the gastric mucosal cells to numerous injurious luminal agents and irritants of exogenous and endogenous origin (Zayachkivska *et al.*, 2005). Mucosal surface epithelium is a subject of attack by physical, chemical or microbiological agents acting from the gastric lumen, which are involved in multiple pathologies, such as gastritis, peptic ulcer or gastric cancer. Pretreatment with different substances could effectively prevent gastric mucosa from the development of erosions and ulcerations. This action is called gastro- or cyto-protection. There are various plant-originated "gastroprotectors" with different composition that have been used in clinical and folk medicine for many countries due to their beneficial effects on the mucosa of GIT. In China and Japan, polyphenol extracts such as Sopharadin extracts, containing flavonoids and its synthetic flavonoid derivative known as Solon are widely employed in peptic ulcer therapy and also as food additives and nutritional supplements, mainly because of their strong inhibition of prostaglandin (PG) metabolism and vasoconstrictive leukotriene inhibition (Zayachkivska *et al.*, 2005).

Inhibition of ethanol induced gastric lesion indicates a promising antiulcerogenic activity of the extract and suggested that an antisecretory or cytoprotective action may be involved (Morimoto *et al.*, 1991). It is possible that the inhibitory effects of extracts are due, at least partly, to the presence of terpenes (Alvarez *et al.*, 1999; Gonzales *et al.*, 2000). Terpenes were found to be associated with antiulcerogenic activity in other plants (Moehle *et al.*, 1985; Mahran *et al.*, 1992). Some triterpenes are known as antiulcer drugs and their action has been suggested to be due to the activation of cellular protection, reduction of mucosal prostaglandins metabolism-cytoprotective action, reduction of gastric vascular permeability and stimulation of mucus secretion (Lewis and Hanson, 1991; Sertié *et al.*, 2000). Flavonoids also have antiulcer and gastroprotective activities (Mizui and Doteuchi, 1983; Sun *et al.*, 1991; Sertié *et al.*, 2000). The aqueous extracts of Phoradendron crassifolium and Franserio artemisiodes that contain polyphenolic agents exerted cytoprotective activity in rats (Gonzales *et al.*, 2000). Two flavonoids have been isolated from A. graveolens seed, quercetin 3-O-beta-D-glucuronide and isoharmentin 3-O-beta-D-glucuronide, have antioxidant activity and could counteract with free radicals. This effect may help to prevent ulcer peptic (Moehle *et al.*, 1985, Mahran *et al.*, 1992). The dose dependent reduction in HCl or absolute ethanol induced ulceration by the ethanolic extract of *A. Cadamba* probably suggests the presence of some active ingredients possibly triterpenes which act through one or more ulcer protecting mechanisms.

In this investigation the extract showed both significant centrally and peripherally acting antinociceptive activity and gastroprotective activity. Though peripherally mediated antinociceptive activity is thought to be closely related to prostaglandin inhibition, triterpenes present in the extract at the same time protects the gastric mucosa probably by maintaining normal level of prostaglandin in stomach and stimulating mucus secretion i.e. the extract can be used as a novel analgesic that rather than damaging gastric mucosa, protects it.

The ability of the extracts to suppress abdominal writhes, increase pain threshold latency, suppression of the HCl or Ethanol induced inflammation confirms the analgesic and anti-ulcerative properties of the extract. These findings justify traditional use of this plant in the treatment of pain and other inflammatory conditions and validate its claim of being used for the said purpose in folklore medicine. It can be concluded that alcoholic extracts of *A. Cadamba* possesses analgesic properties, which are probably mediated via inhibition of prostaglandin

synthesis as well as central inhibitory mechanisms which may be of potential benefit for the management of pain where ulceration is a problem with NSAID therapy. Further research should be necessary for elucidating the active principle as well as toxicological studies.

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