

Involvement of Opioid, Adenosine and 5-HT₃ Receptors in Antinociceptive Effects of an Ayurvedic Polyherbal Formulation

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Key Words

Acetic acid-induced writhing · Antinociceptive · Diclofenac sodium · Pethidine · Polyherbal formulation · Tail immersion test

Abstract

Objective: The present study was undertaken to evaluate the antinociceptive effects of an ayurvedic polyherbal formulation in rats and mice employing the tail immersion test and acetic acid-induced writhing test, respectively. **Materials and Methods:** With the tail immersion method, rats received two different doses (270 and 405 mg/kg BW, p.o.) of a formulation, pethidine (5.4 mg/kg BW, p.o.) as a reference standard and the combination of the higher dose of the formulation with naloxone (2 mg/kg, i.p.), an opioid receptor antagonist, and caffeine (16 mg/kg, i.p.), used as an adenosine receptor antagonist. In the acetic acid-induced writhing test, mice received two different doses (390 and 585 mg/kg, BW, p.o.) of formulation, diclofenac sodium (15 mg/kg, BW, p.o.) as a reference standard and the combination of the higher dose of the polyherbal formulation with ondansetron (0.5 mg/kg, i.p.), a serotonin receptor antagonist. **Results:** The polyherbal formulation (405 mg/kg) exhibited a significant ($p < 0.01$) antinociceptive effect using the tail immersion method. In the acetic acid-induced writhing test, the formulation showed significant ($p < 0.01$) dose-dependent activity. The antinociceptive effect of the polyherbal formu-

lation apparently involved an opiate-like mechanism, since its antinociceptive action was attenuated by naloxone pretreatment. In addition, antinociceptive activity was attenuated by caffeine and reversed by ondansetron pretreatment. **Conclusion:** Our data suggest that the polyherbal formulation possessed centrally and peripherally mediated antinociceptive properties. The activity could be mediated through opioid, adenosine, and serotonin receptors and via inhibition of cyclo-oxygenase- and/or lipoxigenase-dependent pathways.

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Introduction

From time immemorial, plants have served as the primary source of medicines and food, and they have continued to provide mankind with new and novel therapeutic remedies to date. Over the last four decades, there has been a remarkably steady resurgence of interest in the study and use of medicinal plants. This current renewed global interest in the study and use of medicinal plants has led to the characterization and identification of novel lead molecules, and the isolation of active chemical compounds of potential therapeutic value. This revival of interest in plant-derived drugs is mainly due to the widespread belief that 'natural medicines' are safe and more dependable than costly synthetic pharmaceutical drugs,

many of which are toxic and possess adverse side effects. Powders, extracts, decoctions or infusions of plants are being used in the traditional systems of medicine in many parts of the world, especially in rural communities, for the control, management and/or treatment of a variety of human and animal ailments [1]. The current worldwide trend towards utilization of plant-derived natural remedies has, therefore, created a dire need for accurate and up-to-date information on the properties and uses, efficacy, safety and quality of medicinal plant products.

The ayurvedic polyherbal formulation under investigation consists of an extract of 11 plants – namely 3 myrobalanes (*Terminalia belerica*, *Terminalia chebula*, *Emblica officinalis*), and *Tinospora cordifolia*, *Alpinia galanga*, *Withania somnifera*, *Asparagus racemosus*, *Glycyrrhiza glabra*, *Piper longum*, *Commiphora mukul*, and *Curcuma longa*. Extracts of some of the plants contained in the polyherbal formulation have been used as antiallergics, antiulcer agents, antioxidants, cytotoxic or apoptotic agents, for gouty arthritis, as anti-inflammatories, to promote wound healing, for protection against radiation oxidative damage, as well as for antimicrobial, analgesic, immunomodulatory, antidiabetic, antibacterial and antidepressant effects, among others [2–10]. The present study was focused on investigating the antinociceptive activity of the polyherbal formulation and to determine the possible involvement of opioid, adenosine and serotonin (5-HT₃) receptors in its activity.

Materials and Methods

Test Substance

The substance tested was an ayurvedic polyherbal formulation consisting of an 11-plant extract; a gift sample (coded RD/M.ph.AA-01) was obtained from Pentacare Ayurpharma, Bangalore, India.

Drugs and Chemicals

Diclofenac sodium (Karnataka Antibiotics Pvt. Ltd.), pethidine sulfate (Astra Zeneca), naloxone hydrochloride (Troika), ondansetron (Cipla), caffeine (Thomas Baker), and acetic acid (Nice Chemicals) were used for the study. The agonists (diclofenac sodium, pethidine and polyherbal formulation) were suspended in 0.5% carboxymethyl cellulose (CMC), then administered perorally (p.o.) through intragastric tube and antagonists (ondansetron, naloxone and caffeine) were administered through intraperitoneal (i.p.) route.

Experimental Animals

Female Swiss albino mice weighing 20–30 g and female Wistar albino rats weighing 150–200 g were procured from the Indian Institutes of Sciences, Bangalore, India. Animals were kept in standard cages, provided with a standard diet supplied by Pranav

Agro Industries Ltd., Sangli, India and drinking water ad libitum. Animal housing conditions were: 25 ± 2°C with relative humidity of 65 ± 10% under 12-hour light/dark cycles. The study protocol was approved by the Institutional Animal Ethics Committee (Ref. No. IAEC/PP/03/2008-09).

Preliminary Phytochemical Screening

Phytochemical screening [11] of the polyherbal formulation was performed to detect the presence of different classes of constituents such as alkaloids, carbohydrates, flavonoids, glycosides, tannins, steroids, amino acids and oxalic acid, using the following reagents and chemicals: alkaloids with Mayer and Dragendorff's reagent, carbohydrates with Molisch's reagent, flavonoids with NaCl and HCl, glycosides with modified Borntrager's reagent, tannins with ferric chloride and lead acetate, steroids with Liebermann-Burchard's reagent, amino acids with ninhydrin reagent, oxalic acid with lead acetate.

Acetic Acid-Induced Writhing Test in Mice

The writhing test was carried out in five groups of mice (n = 6) and treatments were as follows: group 1 received vehicle (0.5% w/v CMC p.o.). Groups 2 and 3 received two different doses of the polyherbal formulation (390 and 585 mg/kg p.o., respectively) suspended in 0.5% w/v CMC. Dose selection was based on the extrapolation from the dose of the polyherbal formulation previously used in humans [12]. Group 4 received diclofenac sodium (15 mg/kg) as a reference standard also suspended in 0.5% CMC and administered p.o. The p.o. administrations were through intragastric tube [13]. In an attempt to investigate the participation of the serotonergic system in the antinociceptive activity of the polyherbal formulation, animals in group 5 were pretreated with the 5-HT₃ antagonist ondansetron (0.5 mg/kg, i.p.) [14] 30 min prior to the administration of the polyherbal formulation (585 mg/kg, p.o.). After 30 min, a 0.6% (v/v) solution of acetic acid (10 ml/kg) was injected i.p.. Each mouse was placed in a transparent observation cage and abdominal constrictions resulting from injection of acetic acid that occurred between 0 and 30 min after challenge were cumulatively counted. Results were expressed as percent inhibition of antinociception.

Tail Immersion Test in Rats

Rats were divided into seven groups of 6 animals each. The lower 5-cm portion of the tail was immersed in a beaker of water maintained at 55 ± 2°C. The time in seconds for tail withdrawal from the water was taken as the reaction time, with a cutoff time of immersion set at 10 s. Test groups were given the polyherbal formulation (270 and 405 mg/kg, p.o.), pethidine sulfate (5.4 mg/kg, p.o.) [12] as reference standard and caffeine (16 mg/kg, i.p.). In an attempt to investigate the participation of the opioid and adenosine system in the antinociceptive activity of the formulation, two separate groups of rats were pretreated with the non-selective opioid receptor antagonist naloxone (2 mg/kg, i.p.) [15], and the adenosine receptor antagonist caffeine (16 mg/kg, i.p.) [16] 30 min prior to the administration of the polyherbal formulation (405 mg/kg, p.o.). Reaction time for the test groups was taken at intervals of 30, 60, 120, 180 and 240 min after a latency period of 30 min following the administration of the polyherbal formulation and drugs. Reaction time was measured and calculated as percentage inhibition.

Table 1. Evaluation of antinociceptive activity of the polyherbal formulation using the acetic acid writhing test in mice

Treatment and dose	Writhes	Change in pain perception
Control	16.83 ± 1.13	–
Polyherbal formulation (390 mg/kg)	3.66 ± 0.66 ^{a*}	77.74% ↓
Polyherbal formulation (585 mg/kg)	2.33 ± 0.84 ^{a*}	85.70% ↓
Diclofenac sodium (15 mg/kg)	1.5 ± 0.42 ^{a*}	90.79% ↓
Ondansetron (0.5 mg/kg) + polyherbal formulation (585 mg/kg)	21.16 ± 7.5 ^{b**}	25.72% ↑

Values are expressed as mean ± SEM. Number of animals: n = 6. ** p < 0.01 = very significant; * p < 0.05 = significant.

^a Compared with the control group. ^b Compared with the polyherbal formulation (585 mg/kg) group.

Statistical Analysis

Results were expressed as mean ± standard error mean (SEM). The statistical significance was assessed using one-way analysis of variance followed by Dunnett's comparison test using GraphPad InStat 3.06. Differences were considered as statistically significant when p < 0.05 and p < 0.01.

Results

Acetic Acid-Induced Writhing Test in Mice

Analgesic effects induced by various doses of the polyherbal formulation on the writhing test in mice are shown in table 1. The polyherbal formulation administered orally at two different doses (390 and 585 mg/kg) exhibited significant (p < 0.05) dose-dependent inhibition of control writhes to the extent of 77.74 and 85.70%, respectively. Maximal inhibition of the writhing response (85.70%) by the formulation (585 mg/kg) was slightly lower compared to inhibition by diclofenac sodium (90.79%) at a dose of 15 mg/kg. Administration of the 5-HT₃ receptor antagonist ondansetron (0.5 mg/kg) prior to the polyherbal formulation (585 mg/kg) resulted in an enhancement (rather than inhibition) of the antinociceptive effect of the formulation.

Tail Immersion Test in Rats

A significant reduction of the painful sensation by various doses of the polyherbal formulation on the tail immersion in rats is shown in table 2. The polyherbal formulation administered orally at two different doses (270 and 405 mg/kg) exhibited dose-dependent activity. At a dose of 405 mg/kg the polyherbal formulation showed

significant (p < 0.01) antinociceptive activity where as the 270-mg/kg dose exhibited statistically nonsignificant antinociceptive activity when compared to the control group. The percentage increases in reaction time after the administration of the polyherbal formulation (405 mg/kg) were 64.81, 78.14, 101.38, 67.12 and 27.31% at 30, 60, 120, 180, and 240 min, respectively (fig. 1). The antinociceptive effect of the polyherbal formulation (405 mg/kg) was significantly (p < 0.01) inhibited by prior administration of naloxone (2 mg/kg) when compared with the formulation (405 mg/kg) alone. The treatment of rats with caffeine only resulted in antinociceptive activity up to 1 h of reaction time. The administration of caffeine (16 mg/kg) to rats decreased the maximum antinociceptive effect of the formulation (405 mg/kg) to 46.4%. However, this was not statistically significant when compared with the formulation (405 mg/kg) alone (table 2, fig. 1).

Discussion

The ayurvedic polyherbal formulation significantly inhibited the mice's writhing response in the acetic acid-induced writhing test. It has been postulated that acetic acid, which was used to induce writhing, acts indirectly by releasing endogenous mediators that stimulate pain nerve endings. Increased levels of prostaglandin E₂ and prostaglandin F₂α as well as lipoxygenase, liberation of sympathetic nervous system mediators in the peritoneal fluid and the release of cytokines, such as tumor necrosis factor-α, interleukin-1β and interleukin-δ, by resident peritoneal macrophages and mast cells have been reported to be responsible for pain sensation caused by i.p. administration of acetic acid [17]. The polyherbal formulation exhibited dose-dependent antinociceptive activity against the acetic acid-induced writhing response. Diclofenac sodium also exhibited antinociceptive activity against the acetic acid-induced writhing response. Ku et al. [18] reported that diclofenac sodium is a compound with a generalized effect on the arachidonic acid cascade (cyclo-oxygenase and lipoxygenase pathway) that results in a reduction in prostaglandin, prostacyclin, and thromboxane formation. However, there is potential for additional inhibitory effects on the lipoxygenase pathway, i.e., reduced leukotriene formation, thereby resulting in a dual inhibitory effect on both cyclo-oxygenase and lipoxygenase pathways [18]. On the basis of our result, it can be postulated that the mode of action of the polyherbal preparation might involve a peripheral mechanism mediated via inhibition of lipoxygenase and/or cyclo-oxygen-

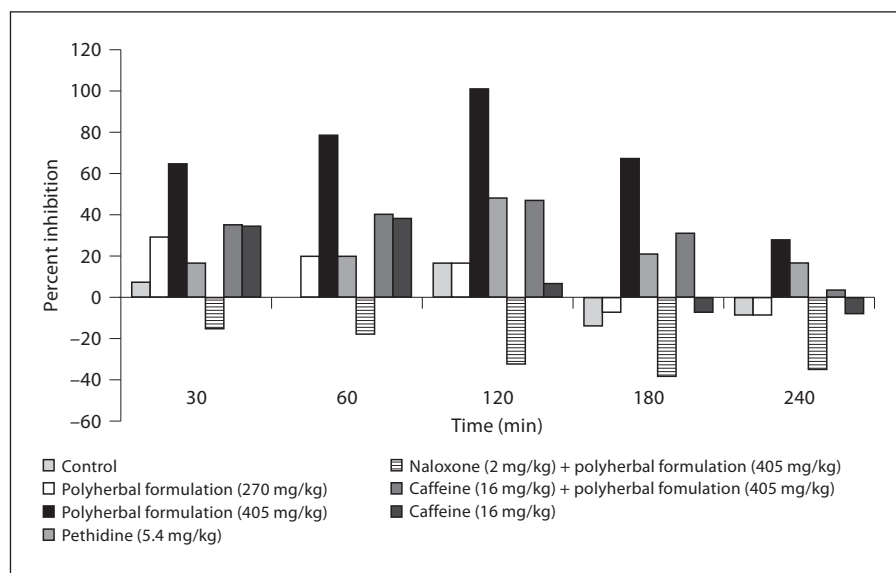


Fig. 1. Evaluation of antinociceptive activity of the polyherbal formulation using the tail immersion method in rats.

Table 2. Evaluation of the antinociceptive activity of the polyherbal formulation using the tail immersion method in rats

Treatment and dose	Time interval, min				
	30	60	120	180	240
Control	0.22 ± 0.16	0.25 ± 0.10	0.53 ± 0.25	-0.45 ± 0.28	-0.29 ± 0.31
Polyherbal formulation (270 mg/kg)	0.87 ± 0.41 ^a	0.58 ± 0.17 ^a	-0.21 ± 0.19 ^a	-0.24 ± 0.30 ^a	-0.27 ± 0.29 ^a
Polyherbal formulation (405 mg/kg)	1.40 ± 0.43 ^a	1.37 ± 0.25 ^{a*}	2.20 ± 0.38 ^{a**}	1.45 ± 0.26 ^{a**}	0.59 ± 0.36 ^a
Pethidine (5.4 mg/kg)	0.54 ± 0.54 ^a	0.70 ± 0.24 ^a	1.65 ± 0.49 ^a	0.71 ± 0.29 ^a	0.55 ± 0.54 ^a
Naloxone (2 mg/kg) + polyherbal formulation (405 mg/kg)	-0.57 ± 0.40 ^{b**}	-0.64 ± 0.33 ^{b**}	-1.18 ± 0.47 ^{b**}	-1.42 ± 0.50 ^{b**}	-1.29 ± 0.44 ^{b**}
Caffeine (16 mg/kg) + polyherbal formulation (405 mg/kg)	0.88 ± 0.36 ^b	1.00 ± 0.32 ^b	1.17 ± 0.16 ^b	0.87 ± 0.32 ^b	0.27 ± 0.31 ^b
Caffeine (16 mg/kg)	1.06 ± 0.14 ^a	1.18 ± 0.31 ^a	0.15 ± 0.26 ^a	-0.24 ± 0.25 ^a	-0.40 ± 0.19 ^a

Values are expressed as mean ± SEM. Number of animals: n = 6. ** p < 0.01 = very significant; * p < 0.05 = significant.

^a Compared with the control group. ^b Compared with the polyherbal formulation (405 mg/kg) group.

ase activities. In addition, our results also showed that pretreatment with a 5-HT₃ receptor antagonist, ondansetron, actually reversed the antinociceptive effect of the polyherbal formulation. These findings clearly suggest that the antinociceptive effect of the polyherbal formulation is mediated by activation of the serotonergic system.

In the immersion test for the evaluation of a possible central antinociceptive effect of the polyherbal formulation, the reaction time was increased in animals treated with the polyherbal formulation. Pethidine acts as an opiate agonist and has a pharmacological effect similar to morphine. Pethidine alters the perception of pain in the spinal cord and central nervous system but has no effect on afferent nerve endings [19]. The μ -receptor has generally been regarded as the receptor type associated with

analgesia and has been shown to be involved in regulating thermal pain [20]. The effectiveness of analgesic agents in the tail immersion pain model is highly correlated with the relief of pain in humans [21]. Our results showed that the antinociceptive action of the polyherbal formulation was attenuated by naloxone, an opioid receptor antagonist. Caffeine is widely consumed due to its mild psychostimulant properties; it is a nonselective inhibitor of adenosine A₁, A_{2A}, A_{2B}, with little affinity for A₃ receptors. At peripheral nerve terminals in rodents, adenosine A₁ receptor activation produces antinociception by decreasing, while adenosine A₂ receptor activation produces pronociceptive or pain-enhancing properties by increasing cyclic adenosine monophosphate levels in sensory nerve terminals. Adenosine A₃ receptor acti-

vation produces pain behavior due to the release of histamine and 5-HT from mast cells and subsequent actions on sensory nerve terminals [22, 23]. The pretreatment of animals with caffeine, an adenosine receptor antagonist, altered the antinociceptive response and it seemed that adenosine receptors may be involved in the antinociceptive effect. Our findings suggest that the antinociceptive effect of polyherbal formulation is most likely mediated through opioid and adenosine receptors.

Preliminary phytochemical screening carried out on the polyherbal formulation showed the presence of numerous constituents such as alkaloids, carbohydrates, flavonoids, glycosides, tannins, steroids, amino acids and oxalic acid. Since several flavonoids and tannins isolated from medicinal plants have been shown to possess significant antinociceptive activity [24], it is possible that the antinociceptive effects observed with the polyherbal formulation in the present study may be attributed to its flavonoid and tannin components.

Conclusion

The proposed mechanisms of antinociceptive activity based on the pain models used in the present study showed that they were likely mediated peripherally and centrally on the nervous system. In addition, the antinociceptive effect of the polyherbal formulation was completely blocked by naloxone, partially blocked by caffeine in the tail immersion test and completely blocked by ondansetron in the writhing test. Hence the polyherbal formulation may be acting through opioid, adenosine, 5-HT₃ receptors, via inhibition of lipooxygenase and/or cyclo-oxygenase pathways and other inflammatory mediators. The presence of flavonoids and tannins in the polyherbal formulation might be responsible for the observed activity. Further research in this area is warranted.

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