Involvement of Opioid, Adenosine and 5-HT<sub>3</sub> Receptors in Antinociceptive Effects of an Ayurvedic Polyherbal Formulation

S.B. Chandrasekar<sup>a</sup> Kalyani Divakar<sup>a</sup> A.T. Pawar<sup>a</sup> Divakar Goli<sup>a</sup> R. Harisha<sup>b</sup>
M. Bhanumathy<sup>c</sup> G. Sushma<sup>c</sup> H.N. Shivaprasad<sup>c</sup>

<sup>a</sup>Department of Pharmacology, Acharya BM Reddy College of Pharmacy, Soldevanahalli, <sup>b</sup>Milind Institute of Pharmacy, Kurubarahalli, and <sup>c</sup>Natural Remedies Pvt. Ltd., Bangalore, India

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 formulation 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 lipoxygenase-dependent pathways.

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 myrobalans (Terminalia bellerica, 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, antifulcer agents, antioxidants, apopotic agents, for gouty arthritis, as anti-inflammatory, to promote wound healing, for protection against apoptotic agents, for gouty arthritis, as anti-inflammatories, antiulcer agents, antioxidants, cytotoxic or chemotherapeutic activity of the polyherbal formulation and to determine the possible involvement of opioid, adenosine and serotonin (5-HT_3) 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 Ayurveda, 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% w/v CMC and administered p.o. The p.o. administrations were through intragastric tube and administered p.o. The p.o. administrations were through intragastric tube and administered p.o.

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 Mann-Burchard’s reagent, amino acids with ninhydrin 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-HT_3 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.), naloxone hydrochloride (Troika), acetic acid (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 opioid and adenosine system in the antinociceptive activity of the polyherbal formulation, animals in group 5 were pretreated with the 5-HT_3 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.


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

<table>
<thead>
<tr>
<th>Treatment and dose</th>
<th>Writhes ± SEM</th>
<th>Change in pain perception</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.83 ± 1.13</td>
<td>-</td>
</tr>
<tr>
<td>Polyherbal formulation (390 mg/kg)</td>
<td>3.66 ± 0.66**</td>
<td>77.74% ↓</td>
</tr>
<tr>
<td>Polyherbal formulation (585 mg/kg)</td>
<td>2.33 ± 0.84**</td>
<td>85.70% ↓</td>
</tr>
<tr>
<td>Diclofenac sodium (15 mg/kg)</td>
<td>1.5 ± 0.42**</td>
<td>90.79% ↓</td>
</tr>
<tr>
<td>Ondansetron (0.5 mg/kg) + polyherbal formulation (585 mg/kg)</td>
<td>21.16 ± 7.5***</td>
<td>25.72% ↑</td>
</tr>
</tbody>
</table>

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

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-HT_3_ 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 E_2_ and prostaglandin F_2α_ 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-6, 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-oxygenase.
Antinociceptive Action of a Polyherbal Formulation

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, A2A, A2B, 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-

![Fig. 1. Evaluation of antinociceptive activity of the polyherbal formulation using the tail immersion method in rats.](image)

![Table 2. Evaluation of the antinociceptive activity of the polyherbal formulation using the tail immersion method in rats](table)

<table>
<thead>
<tr>
<th>Treatment and dose</th>
<th>Time interval, min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td>Control</td>
<td>0.22 ± 0.16</td>
</tr>
<tr>
<td>Polyherbal formulation (270 mg/kg)</td>
<td>0.87 ± 0.41*</td>
</tr>
<tr>
<td>Polyherbal formulation (405 mg/kg)</td>
<td>1.40 ± 0.43a</td>
</tr>
<tr>
<td>Pethidine (5.4 mg/kg)</td>
<td>0.54 ± 0.54a</td>
</tr>
<tr>
<td>Naloxone (2 mg/kg) + polyherbal formulation (405 mg/kg)</td>
<td>-0.57 ± 0.40b**</td>
</tr>
<tr>
<td>Caffeine (16 mg/kg) + polyherbal formulation (405 mg/kg)</td>
<td>0.88 ± 0.36b</td>
</tr>
<tr>
<td>Caffeine (16 mg/kg)</td>
<td>1.06 ± 0.14a</td>
</tr>
</tbody>
</table>

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.
significant antinociceptive activity 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.

References