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Prasarani Sandhan, a Polyherbal Preparation, Shows Anti-nociceptive and Anti-inflammatory Activities

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Authors' contributions

The work was carried out by the equal contribution of all of the authors. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: Prasarani Sandhan (PRS) is an Ayurvedic formulation approved by the "National formulary of Ayurvedic Medicine 2011", of Bangladesh. It is traditionally used in arthritic pain, lumbago and sciatia. Sparse scientific evidence is available to support the efficacy of this preparation. Hence, we planned to document scientific evidences of the pharmacological activity of this preparation.
Study Design: Our present study aims to elucidate the probable anti-nociceptive and anti-inflammatory mechanisms of PRS.
Place and Duration of the study: The experiments were performed at the pharmacology lab of North South University during the period of October 2010 to July 2011.
Methodology: Two thermal anti-nociceptive models were used, the hot-plate test and tail immersion test, to find out the possible role of the central nervous system in its action. Three *in-vivo* analgesic and anti-inflammatory models, carrageenan induced paw edema, acetic-acid writhing, and formalin induced paw lick tests, were carried out to test its potential anti-inflammatory and peripheral analgesic properties.
Result: The study of PRS (20mL/kg and 40mL/kg) showed no involvement of the CNS in anti-nociceptive activity of PRS. Carrageenan induced paw edema and acetic acid writhing tests both gave significant results (P=.05), indicating possible peripheral analgesic and anti-inflammatory action. Formalin induced paw-licking test (with and without naloxone co-administration), a differentiator of nurogenic pain (CNS modulated)

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and inflammatory pain (peripheral nociception), showed that PRS had significant effect in suppressing inflammatory pain (P=.05) but not neurogenic pain.
Conclusion: Compiling the results of the experiments, it can be reported that PRS has anti-inflammatory and peripheral analgesic action.

Keywords: Prasarani sandhan; peripheral analgesic; anti-inflammatory; hot plate.

1. INTRODUCTION

World health Organization (WHO) has stated that up to 80% of the population in many Asian and African countries depend on traditional and complimentary drugs to meet their medical necessities [1]. It is also an extremely attractive business for many drug vendors which often results in misleading claims being made and confusion in the mind of consumers. Few countries have a proper national policies or documentations regulating these claims [1]. Persistent continuation of a regimen with one of these drugs which do not have any pharmacological activity, in reality, would seriously aggravate the morbidity of the patients. For these reasons and others, there has been a demand for ensuring the safety and efficacy of some of these traditional/herbal medicines. Due to the gradual increase in use of such medicines, it is no longer prudent to rely solely on traditional beliefs and empirical evidences; hence, explaining their mechanism of action would be helpful both to the patients and the caregivers [2]. Under the status quo, these products are often sold under hyperbolic and outrageous claims without much scientific evidences [3]. In this paper, we analyzed the analgesic and anti-inflammatory property of *Prasarani Sandhan* (coded as: PRS), a commonly available herbal product licensed under the Directorate General of Drug Administration (DGDA) of Bangladesh.

Pain has been defined by The International Association for the Study of Pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage [9]. This process enables an individual to take protective measures, by providing with rapid awareness about threatening or potentially threatening injury [5]. However, if the painful sensation remains after removal of the detectable stimulus, it calls for a regimen for pain management [6]. New drugs originating from natural products have received significant scientific coverage, and many phytochemical compounds have been seen to possess anti-nociceptive activity [7].

PRS is included in the Bangladesh National Formulary of Ayurvedic Medicine 2011 (2nd Ed.) [13]. It is primarily indicated in rheumatoid arthritis, lumbago (low-back pain) and sciatica (pain which may arise from compression and/or irritation of one of five spinal nerve roots which give rise to each sciatic nerve) [8]. Pain relief was chosen to be the most desirable objective of treatment by a number of patients [9]. For this purpose, the non-narcotic analgesics are usually the drugs of choice [10]. This is primarily due to the adverse effects these drugs tend to possess including drowsiness, dependence etc. Narcotic analgesics are also considered to have a high abuse potential. They are generally not used in chronic pain management as widespread use inevitably would increase its proliferation.

PRS is prepared by the method of preparing asava, which is known as sandhana kalpana in Ayurveda. General Methods used in the Extraction of Medicinal Plants in asava are infusion and decoction [13]. A general description of preparing asava is presented below.

In decoction process, the crude drug is boiled in a specified volume of water for a defined time; it is then cooled and strained or filtered. This procedure is suitable for extracting water-soluble, heat-stable constituents. This process is typically used in preparation of Ayurvedic extracts called “quath” or “kawath”. The starting ratio of crude drug to water is fixed, e.g. 1:4 or 1:16. The volume is then brought down to one-fourth its original volume by boiling during the extraction procedure. Then, the concentrated extract is filtered and used as such or processed further.

Fresh infusions are prepared by macerating the crude drug for a short period of time with cold or boiling water. These are dilute solutions of the readily soluble constituents of crude drugs. The basic equipment required for preparation of asava is an earthen pot sufficiently large and glazed, porcelain jar of suitable size; a lid to close the vessel, a cloth ribbon to seal the vessel; a paddle like stirrer; a clean cloth of fine and strong texture for filtering, vessel to keep the juices or boil the drugs. The major components are divided into 4 types according to their specific role in the process. These include: the main herbs from which the extract or decoction is taken out. They yield drugs, which are pharmacologically and therapeutically much important in the given medicine and the name of the medicine is derived from these herbs denoting their importance. The flavouring agents used in asava not only contributing to the flavour of the medicine but having their own pharmacological action too. The fermentation initiator provides inoculum for the fermentation to start. The medium of sugars is required for fermentation.

Medicinal substances such as roots, leaves or barks, etc. are cut into pieces, and powdered or decoction. The basic drugs from which the extract is to be prepared are first cleaned and rinsed in water to get rid of dirt. In the case of fresh plants, they are cleaned, pulverized and pressed for collection of juice. If the drug is dry and to be used in the preparation of *asava*, it is coarsely crushed and added to water to which the prescribed quantities of honey, *jaggery* /or sugar are added. The water used should be clean, clear and potable. When the extracts are obtained, the sugar (cane sugar), *jaggery*/or honey are added and completely dissolved. The sugar, *jaggery* and honey should be pure. The *jaggery* to be added should be very old (*prapurana*) because fresh *jiggery* aggravates *kapha* and suppresses the power of digestion. The flavouring agents are coarsely powdered and added to the sweetened extract. Very fine powder of the flavouring agent is undesirable as it causes sedimentation in the prepared medicine and its filtration is difficult. In *asava*s, the *avapa* (drugs which are added in powder form at the end) should be one in tenth in quantity and honey should be three fourth in quantity of *jaggery.* The earthen pot or jar intended for fermenting the medicine is tested for weak spots and cracks and similarly a lid is also chosen. It should be prepared of the soft mud collected from the silt in the bank of river or lake. It should be greasy, thick, light and smooth. It should be free from holes or cracks and homogenous. Echo should come out from inside of this jar. Its circumference in the middle should be 42 *angulas* (1 *angula* = ¾ inches) and its height should be 43 *angulas*. Its wall should be one *angula* in thickness and compact. In shape (pot shape), it should be like the fruit of *bakula* (*Mimusops elengi* L.). The pot should be perfectly dry before ghee is smeared and if it be moist, ghee will not stick, penetrate and block the pores.

When the pot or the jar is ready, the sweetened and flavoured drug extract is poured into pot, up to three fourth of the capacity. The unfilled space provides room for the fermenting liquid when it rises up due to frothing and evolving of a large amount of gases. Otherwise, the medium may damage the container and flow out. Then, the inoculum has to be added to initiate fermentation.

The process of fermentation necessitates the presence of fermenting microorganisms, yeasts. In the preparation of alcoholic medicaments in the Ayurvedic Systems, the inoculum of yeasts comes from the dhataki flowers, which contain the wild species of yeast. These flowers are nectariferous and highly tanniferous. The flowers contain the yeast spores in the dry nectariferous region. The presence of tannin in flowers favours suitable environment for yeast growth. The flowers are added and the contents are stirred well to distribute the inoculum of yeast. Apart from the fire flame flowers (dhataki), if other ingredients like honey and resins (gum) are added they also contain wild yeasts. When fire flame flowers are not used in some preparations, the inoculum of yeasts is done either from the mahua (Madhuca longifolia Macbr.) flowers, honey or resins initiating the process of fermentation. The yeasts multiply rapidly by division in a short time. Finally, the vessel should be closed and sealed. Sealing is done by winding around a long ribbon of cloth smeared with clay on one surface. While sealing, the blank surface of the ribbon should line the rim of the vessel and lid, the clay side should be external. After sealing, the vessel is placed in a dark place without much circulation of air. It may be kept in a grain store buried in a heap of grain or into a pit in the soil. Soft packing of straw should be provided around the vessel to prevent breakage by any force.

During autumn and summer seasons, fermentation takes place in 6 days. In winter, it takes 10 days. During rainy season and spring, fermentation takes place in 8 days. The fermentation vessel is left undisturbed for a month and then opened. The medicine is filtered and taken for use. If the filtered medicine shows further sedimentation, it is allowed to stand for few more days and again filtered to separate the sediment. In the usual practice, 7-10 days are enough in the hot tropical climate and the long period of 30 days is allowed in cool temperature climate when biological activity is at its low. In old practices, performing fermentation in a heap of whole grain of that season was indicated. A crude match-box method is applied to check whether fermentation has occurred. This method depends upon the release of carbon dioxide during the process.

Fermentation processes help in rupturing of cells of the herbs and expose its contents to the bacteria and enzymes for transformation. Fermentation also creates active transport system with dissolved constituents from the herbal material. There are claims that yeast cell walls naturally bind heavy metals and pesticide residues and act as natural cleaning system, making self-fermentation of herbal products safer than powder decoctions [13].

Methanol and hexane extracts of *Paederia foetida*, the major constituent of PRS, were previously shown to possess peripheral antinociceptive activity in acetic acid writhing test only. [11]. An investigation on the anti-inflammatory activity of the butanol fraction of a methanol extract (BMEL) of the defatted leaves of *Paederia foetida* produced a significant inhibition of granulation tissue formation in cotton-pellet implanted rats [12]. The marketed formulation of PRS is a complex mixture of eight constituents as stated in Table 1.

Five experimental models were chosen to test the analgesic and anti-inflammatory properties of this formulation.

Hot-plate test was performed to observe the central anti-nociceptive activity. Acetic-acid writhing test was performed to test for the peripheral anti-nociceptive activity. Reduction of paw inflammation induced by Carageenan in rats was measured to gauge the extent of anti-inflammatory activity. Tail-immersion test was performed to further confirm the possibility of existence of central anti-nociceptive action. Lastly, formalin induced Paw licking test was

carried out to specifically assay for central activity of the test sample, and it is a good model for both acute and chronic pain.

The experimental models were chosen very carefully in order to establish and to revalidate the modulation site of pain stimuli.

Table 1. Composition of Prasarani Sandhan

Sl.	Bengali name	Common name	Scientific name	Used part	Amount used
1.	Prasarani/ Gandhabhadule	Skunkvine	*Paederia foetida*	Root	2.44gm/5mL
2.	Rasun	Garlic	*Allium sativum*	Bulb	0.61gm/5mL
3.	Gur	Jaggery	NA	NA	0.30gm/5mL
4.	Pippali	Long Pepper	*Piper longum*	Seed	0.15gm/5mL
5.	Pippali	Long Pepper	*Piper longum*	Root	0.15gm/5mL
6.	Chabya	Blatt. and Hallb	*Piper chava*	Root	0.15gm/5mL
7.	Chitrak	Ceylon Leadworth	*Plambago zeylanica*	Root	0.15gm/5mL
8.	Ada	Ginger	*Zingibar officinale*	Rhizome	0.15gm/5mL

Source: Bangladesh National Formulary of Ayurvedic Medicine 2011 (2nd Ed.); p 146 [13]

2. MATERIALS AND METHODS

2.1 Reagents Used

All reagents and chemicals that were used in the experiments were of analytical grade. Distilled water, centrally circulated around the laboratory, was used for any dilution, washing, or control purpose. PRS were purchased from University Ayurvedic Research Centre, Jahangirnagar University, Savar, Bangladesh. Pharmaceutical grade Tramadol (Fig. 1), and Diclofenac Sodium (Fig. 2) were purchased from Square Pharmaceuticals Bangladesh Ltd. Ketamine for anesthesia was purchased from Advanced Chemical Industries (ACI) limited. All other reagents were purchased from Sigma Aldrich (USA) unless mentioned otherwise.

2.2 Dose and Route of Administration

0.9% NaCl was administered to the animals Per Oral (p.o.) at a volume that would not cause any additional psychological or physiological stress to the animals. For experimental purpose 20mL/kg and 40mL/kg doses of PRS were used.

2.3 Maintainence and Use of Test Animals

Healthy Swiss Albino mice (5-6 weeks old, only males) weighing 20-25g and Sprague-Dawley rats weighing 130-160g were procured from Jahangir Nagar University Animal House. All test subjects were kept in the air-conditioned animal house of the Pharmacy Department North South University at a temperature of 25±2ºC with a 12h light/dark cycle. The rats were kept in white plastic cages of dimension 30×20×13 cm. Soft sterilized wood shavings were used as bedding. The test subjects were provided with standard rat pellet diet and filtered drinking water ad libitum. This study was approved by an ethics committee of

North South University which gave its consent in absolute accordance with the recommendations of the international Association for the study of Pain [13].

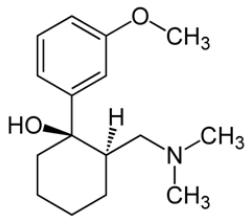

Fig. 1. Chemical structure of Tramadol

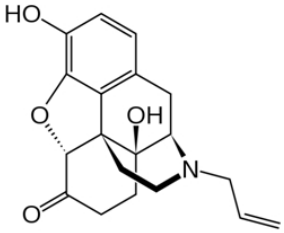

Fig. 2. Chemical structure of Naloxone

2.4 Grouping and Drug Administration

The animals were randomly divided into several groups of 8 mice or rats per group for the planned analgesic and anti-inflammatory tests. The animals were marked on their tail using a black marker to properly identify them before and after the experiments. Control groups were treated with 0.9% NaCl p.o. at a volume that would not cause any additional psychological or physiological stress to the animals. Positive controls were treated with Tramadol or Diclofenac Na. Treatment groups were treated with two doses (20mL/Kg and 40mL/Kg) of PRS (p.o.).

2.5 Determination of CNS Modulation in Analgesic Activity

2.5.1 Hot plate test

The hot plate test was performed on the test subjects in a slightly modified version from the one described earlier [14]. The animals were placed on hot plate apparatus (Model-35100, manufacturer-UGO Basile of Italy) maintained at a temperature of 54±0.5˚C for a maximum time of 20s per exposure to prevent blister formation and skin damage, both of which might affect the results. The mice were screened for initial nociceptive effect; only those which showed an initial response (jumping or paw-licking) within 8-13 seconds were retained to carry out the experiment.

The control group was administered with 0.9% NaCl. The treatment groups were treated with PRS (20mL/Kg and 40mL/kg, p.o.) and Tramadol (10mg/kg p.o.). Naloxone (5mg/kg i.p.) was administered with PRS (20mL/Kg & 40mL/Kg) and Tramadol to three different groups, other than the treatment groups, to ensure that the observed results were not caused due to activity of endogenous opioids. It would also reconfirm the role of opioid agonism of the

PRS, if any, as Naloxone is an antagonist of opioid receptor and would inhibit any activity, if shown, by PRS. Reaction time was recorded as *latency period*, when the animals licked their fore and hind paws and jumped, at 0, 30, 60, 120, 180, 240 and 300 minutes after the treatment.

2.5.2 Tail immersion test

The tail immersion test was performed according to the procedures used by Wang et al. [19], with minor modifications. Briefly, the lower two-third of mouse's tail was immersed in a constant temperature water bath at 50±0.2ºC. The reaction time, i.e. the amount of time it takes the animal to withdraw its tail, was measured at 0, 30, 60, 90, and 120 min after drug treatment. PRS (20mL/Kg & 40mL/Kg p.o.), Tramadol (10mg/Kg p.o.), and 0.9% NaCl (p.o.) were administered to treatment groups. Naloxone (5mg/kg i.p.) was administered with PRS (20mL/Kg & 40mL/Kg) and Tramadol to three different groups, other than the treatment groups, to ensure that the observed results were not caused due to activity of endogenous opioids. It would also reconfirm the role of opioid agonism of the PRS, if any, as Naloxone is an antagonist of opioid receptor and would inhibit any activity, if shown, by PRS. To avert any sort of tissue injury, the cut-off time for tail immersion was fixed at 20s.

2.6 Determination of Peripheral Analgesia

2.6.1 Acetic-acid induced writhing test

The experimental mice were randomly assigned to four groups; each group had eight mice. The test was carried out using a modified method from the procedure perviously described [15]. PRS at two doses (20mL/kg and 40mL/kg) were administered p.o. to treatment groups. Positive control group was administered with Diclofenac sodium (10mg/kg p.o.) and 0.9% NaCl was administered to the control group. 45 minutes after drug treatment, the mice were given 0.7% v/v acetic acid (0.15mL/10mL i.p.) to induce writhing. The total number of writhing occurring between 15 and 20 min after acetic acid injection was recorded for each of the control and experimental groups.

2.6.2 Carrageenan induced paw edema test

Carrageenan induced paw edema test was carried out by following the method described previously [19]. Male or female Sprague-Dawley rats with a body weight between 130 and 160g were used. The animals were starved overnight. The control rats received 0.9% NaCl p.o. and the experimental rats received PRS (20mL/Kg and 40mL/kg p.o.). Thirty minutes later, the rats were given a subcutaneous injection of 0.05mL of 1% solution of carrageenan, a sulphated polysaccharide, into the plantar side of the left hind paw. The paw was marked with ink at the level of the lateral malleous and immersed in mercury up to this mark. The paw volume was measured plethysmographically at 0min, 30min, 1h, 2h, 3h, 4h, and 5h after carrageenan injection.

2.7 Dissociation between CNS and Peripheral Analgesic Activity

2.7.1 Formalin induced Paw-licking test

The experimental mice were randomly assigned to four groups; each group had eight mice. The formalin test was conducted based on the method of Tjølsen et al. [18]. For the formalin test, groups of mice were treated p.o. with NaCl (0.9% w/v) (for control), PRS at two doses

(20mL/kg, 40mL/kg; p.o.) (for treatment group), Tramadol (10mg/Kg p.o.), and Diclofenac Na (10mg/Kg p.o.) (both for positive control). After 60 min, each mouse was given 20µL of 5% formalin in 0.9% NaCl, using an injection, to the left paw (sub-plantar). These mice were individually placed in large (2L capacity) beakers for observation. The duration of paw licking was used as an index to measure the painful response during the nurogenic period at 0-5 min (initial phase) and the inflammatory period at 20-35 min (secondary phase) after formalin injection. Tramadol was used as the positive control drug for both nurogenic phase and inflammatory phases. Diclofenac Na was used as the positive control drug for the later inflammatory phase.

Naloxone (5mg/kg i.p.) was administered with PRS (20mL/Kg & 40mL/Kg) and Tramadol to three different groups, other than the treatment groups, to ensure that the observed results were not caused due to activity of endogenous opioids. It would also reconfirm the role of opioid agonism of the PRS, if any, as Naloxone is an antagonist of opioid receptor and would inhibit any activity, if shown, by PRS. Percentage inhibition was obtained by using this formula [17]:

$$\frac{T_o - T_t}{T_o} \times 100$$

T0 = mean licking time for the control group
Tt = mean licking time for the test group

2.8 Statistical Analysis

Results were expressed as mean ± SEM. All tests were done using SPSS Software Ver. 20. For hot plate test, ail immersion test, and carrageenan induced rat paw edema test, statistical significance was determined by repeated measures one-way analysis of variance (ANOVA) followed by post hoc dunnett test. Later, pair-wise comparison test along with bonferroni correction were done. For acetic acid induced writhing test and formalin test, statistical significance was determined by one-way analysis of variance (ANOVA) followed by post hoc dunnett test. Then Pair-wise comparison test along with bonferroni correction were done. The *P* values less than .05 were considered to be significant.

3. RESULT

3.1 Hot-plate Test

In the hot Plate Test, PRS treatment caused no significant increase in analgesia. The analgesic effects of PRS were quiet similar to that of the Control group but quite different from the effects of Tramadol. Tramadol gave significant analgesia with the highest antinociception of 130.6% at 4 h after drug treatment (Table 2). In the presence of Naloxone, a partial agonist of opioid receptor, the effect of Tramadol was reduced profoundly as shown in Table 2.

3.2 Tail Immersion Test

Table 3 shows that the analgesic effect of PRS (20mL/Kg & 40 ml/Kg) was also not significant in tail immersion test. PRS (20mL/Kg & 40 mL/Kg) failed to induce any “tail flick antinociceptive” index comparable to that of Tramadol, a centrally acting opioid analgesic agent. Tramadol exhibited powerful activity recorded at 30 min after treatment (571.69%, $p<.001$), as shown in Table 3.

Table 2. Effect of PRS on nociceptive responses in the hot plate test at different observation time.

Treatment Group	Dose	Latency Period (s)						
		0 min	60 min	90 min	120 min	180 min	240 min	300 min
Control	-	10.40±0.39	10.72±2.00	10.62±2.25	10.95±2.04	10.96±2.04	8.53±2.1	9.22±1.98
PRS	20mL/Kg	11.13±0.40	11.28±2.00	14.52±2.25	13.40±2.05	7.93±2.05	9.73±2.10	10.38±1.98
PRS	40mL/Kg	10.38±0.30	11.65±2.00	11.80±2.25	12.23±2.05	11.30±2.05	14.23±2.10	9.48±1.98
Tramadol	10mg/Kg	10.90±0.40	18.67±2.33*	19.83±2.25**	19.33±2.05**	19.13±2.08**	19.67±2.10**	19.78±1.98**
Co-treatment with Naloxone								
PRS+ Naloxone	20mL/Kg +1mg/Kg	9.63±0.46	8.20±1.63	8.73±1.79	9.55±1.59	9.18±1.68	9.13±1.72	9.30±1.57
PRS+ Naloxone	40mL/Kg +1mg/Kg	10.00±0.55	8.17±1.63	9.58±1.78	9.32±1.59	8.37±1.68	8.20±1.72	10.22±1.57
Tramadol+naloxone	10mg/Kg +1mg/Kg	8.32±0.51	14.85±1.63	14.48±1.78	14.87±1.59	15.97±1.68*	14.58±1.72*	14.05±1.57*

*Values are expressed as Mean±S.E.M. (n=8). Differences between groups are determined by One-Way Repeated Measures ANOVA followed by post hoc Dunnett test and then pair-wise comparison tests were done with Bonferroni correction. *p<0.05 and **p<0.01 compared to the control treated group.*

Table 3. Effect of PRS on nociceptive responses in the tail immersion test at different observation time

Treatment Group	Dose	Latency Period (s)				
		0 min	30 min	60 min	90 min	120 min
Control	-	7.33±1.81	3.25±2.21	4.92±2.91	3.75±2.01	3.83±2.59
PRS	20mL/Kg	3.12±1.81	5.00±2.21	7.75±2.91	7.67±2.01	10.17±2.60
PRS	40mL/Kg	3.30±1.81	4.10±2.21	7.40±2.91	4.58±2.01	9.32±2.58
Tramadol	10mg/Kg	3.83±1.81	21.83±2.21**	21.90±2.91**	20.33±2.01**	17.67±2.59**
Co-treatment with Naloxone						
PRS + Naloxone	20mL/Kg+1mg/Kg	4.50±1.81	7.17±2.20	6.50±2.90	7.50±2.00	8.33±2.7
PRS + Naloxone	40mL/Kg+1mg/Kg	7.25±1.81	7.00±2.21	7.70±2.95	8.16±2.00	6.17±2.60
Tramadol + Naloxone	10mg/Kg+1mg/Kg	5.00±1.80	13.17±2.30**	15.83±2.93*	5.33±2.03	7.17±2.53

*Values are expressed as Mean±S.E.M. (n=8). Differences between groups are determined by One-Way Repeated Measures ANOVA followed by post hoc Dunnett test and then pair-wise comparison tests were done with Bonferroni correction. *p<0.05 and **p<0.01 compared to the control treated group.*

3.3 Acetic Acid Induced Writhing Test

Intraperitoneal injection of 0.7% acetic acid given to the control group caused 16.83±0.87 writhes in a 5 minute interval. The treatment with PRS (20mL/Kg & 40mL/Kg) induced a significant decrease, compared to that of the control group, in the mean number of writhing (Table 4). The data showed that the analgesic effect of PRS was 54.11% (p=.05) inhibition observed in the 20mL/Kg group and 81.35% (p<.01) in the 40mL/Kg. The reference drug, Diclofenac Na, caused 89.74% (p<.01) reduction which is higher than that of PRS 40mL/Kg.

Table 4. Effect of PRS on nociceptive responses in the acetic acid induced writhing test

Treatment	Dose	Number of Writhungs (15-20 min)	Inhibition (%)
Control	-	16.83±0.87	-
PRS	20mL/Kg	7.67±0.72	54.11%*
PRS	40mL/Kg	3.17±1.40	81.35%**
Diclofenac Na	10mg/Kg	1.83±0.60	89.74%**

*Values are expressed as Mean±S.E.M. Differences between groups are determined by One-Way ANOVA followed by post hoc Dunnett test. *p<0.05 and p<0.01 compared to the control treated group.*

3.4 Formalin Induced Paw-licking Test

In the formalin induced paw-licking test, PRS (20mL/Kg & 40mL/Kg) treated mice groups showed significant (p=.05) anti-nociceptive activities in the later phase pain responses (52.79% and 67.37% respectively) compared to that of the control group. Both doses (20mL/Kg & 40mL/Kg) of PRS failed to induce any significant analgesic activity at early phase of the experiment as shown in the Table 5. Tramadol showed significant anti-nociceptive activities in both of the phases of the experiment (early phase 95.07% and later phase 89.64%). Diclofenac Na showed significant activity in the later phase (87.33%) but failed to show any activity in the early phase. In combination studies using Naloxone, an opioid antagonist, the analgesic activity of the Tramadol was diminished in both phases. The analgesic activity of Diclofenac Na was not diminished by the co-treatment with Naloxone. Co-treatment with naloxone also did not affect the analgesic activity of PRS 40mL/kg in the late phase of the experiment, suggesting that there might be no involvement of opioid receptor in the analgesic activity of PRS.

3.5 Carrageenan Induced Paw Edema Test

The anti-inflammatory activity of PRS (20mL/Kg & 40mL/Kg) is presented in Table 6 showing the paw volume at different time intervals after induction of edema by carrageenan. The injection of carrageenan at rat paw created an edema that increased gradually. PRS 20mL/kg showed 21.43% and 32.81% reduction in the volume of the edematous paw at 4h and 5h after carrageenan injection respectively. Whereas, PRS 40mL/Kg showed significant (p=.05) anti-inflammatory activity starting from 2h after the injection of carrageenan to throughout the experiment time with a highest reduction of 36.72% (p<.01) (5h after the carrageenan injection). The reference drug, Diclofenac Na, showed significant anti-inflammatory activity starting from 2h after the carrageenan injection to throughout the experiment time with a highest reduction of 39.06% (5h after the carrageenan injection).

Table 5. Effect of PRS on nociceptive response in the formalin induced paw-licking Test

Treatment Group	Dose	Early Phase		Later Phase	
		Licking time (s)	Inhibition (%)	Licking time (s)	Inhibition (%)
Control	-	84.50±12.79	-	43.42±13.50	-
PRS	20mL/Kg	71.50±10.33	15.38	20.50±0.764*	52.79*
PRS	40mL/Kg	67.00±9.63	20.71	14.17±0.477*	67.37*
Tramadol	10mg/Kg	4.17±1.49**	95.07**	4.50±0.764**	89.64**
Diclofenac Na	10mg/Kg	62.50±8.65	26.04	5.50±1.88**	87.33**
Co-treatment with naloxone					
PRS + Naloxone	40mL/Kg+1mg/Kg	78.00±11.47	7.69	16.00±1.83*	63.15*
Tramadol + Naloxone	10mg/Kg+1mg/Kg	44.50±2.79	47.34	23.17±1.38	46.64
Diclofenac Na + Naloxone	10mg/Kg+1mg/Kg	71.83±5.87	15	4.83±1.08**	88.88**

*Values are expressed as Mean±S.E.M. Differences between groups are determined by One-Way ANOVA followed by post hoc Dunnett test. *p<0.05 and **p<0.01 compared to the control treated group.*

Table 6. Effect of PRS on anti-inflammatory responses in carrageenan induced rat paw edema rest at different observation time

Treatment Group	Dose	Volume of Paw (ml)						
		0 min	30 min	1 h	2h	3h	4h	5h
Control	-	0.77±0.01	0.95±0.08	1.01±0.07	1.16±0.11	1.17±0.08	1.26±0.12	1.28±0.13
PRS	20mL/Kg	0.73±0.04	0.99±0.03	0.90±0.02	0.97±0.14	0.97±0.09	0.99±0.06*	0.86±0.05*
PRS	40mL/Kg	0.79±0.04	1.03±0.06	0.88±0.03	0.91±0.10*	0.92±0.05*	0.90±0.07*	0.81±0.03*
Diclofenac Na	10mg/Kg	0.76±0.05	0.94±0.03	0.95±0.21	0.90±0.14*	0.89±0.11*	0.85±0.12*	0.78±0.09*

*Values are expressed as Mean ± S.E.M. (n=8). Differences between groups are determined by One-Way Repeated Measures ANOVA followed by post hoc Dunnett test and then pair-wise comparison tests were done with Bonferroni correction. *p<0.05 compared to the control treated group.*

4. DISCUSSION

PRS is an Ayurvedic formulation, and is approved by an authoritative compendial literature [13]. Some of the individual components of this concoction, eg: *Paederia foedita* have been subjected to isolated studies indicating probable anti-nociceptive and anti-inflammatory activity [11] [12]. However, we believed the studies were inconclusive, as too few *in-vivo* tests were employed in these studies to draw a reasonably meaningful conclusion. The present study aimed towards fully elucidating the probable mechanism of action of PRS by simultaneous application of five anti-nociceptive and anti-inflammatory *in-vivo* tests.

Two well known models of thermal nociception, hot-plate test and tail immersion test were employed to double check on possible involvement of spinal, supra-spinal pathways, and μ-opiate receptor agonism in regulation (CNS modulation) of pain response by PRS. Our findings demonstrated no activity of PRS (20mL/kg and 40mL/kg) in either model. Tramadol, however, gave rapid analgesic effect which neared peak within the first 30 min of its administration, which is typical for μ-opioid receptor agonists [6]. Hence, probable involvement of the central nervous system, in this case, could be ruled out.

To reinforce the above findings, we employed the formalin induced paw-licking test. This test is capable of discerning between nurogenic pain (early phase, acute, non-inflammatory and CNS modulated) and inflammatory (chronic and peripheral pain) [20-22]. The neurogenic pain (first phase) is caused by direct chemical stimulation of nociceptive afferent fibers (predominantly C fibers) which can be suppressed by opiate like morphine [23]. The inflammatory pain (second phase) is caused by the release of inflammatory mediators like histamine, prostaglandins, bradykinin, serotonin in the peripheral tissues [22], and from functional changes in the spinal dorsal horn [24]. Our results indicated that Tramadol was effective in preventing both the neurogenic and inflammatory phases of formalin-induced nociception, while Diclofenac sodium predominantly suppressed the inflammatory phase pain. Hence, our findings corroborated the reports suggesting that the most centrally acting drugs have pain inhibitory effect on both the phases, while peripherally acting drugs, such as steroids and NSAIDs, may cause only slight pain inhibition in the early phase [22] [25] [26]. Our results showed that PRS had no effect on neurogenic pain suppression (first phase) but had effective anti-nociceptive effect in the peripheral inflammatory (second phase) pain. Co-treatment with naloxone, a partial μ-opiate receptor agonist, partially blocked the activity of Tramadol in both the phases while that of PRS and Diclofenac Sodium remained unaffected. Hence, we have definitive evidence to conclude that PRS has no CNS modulated pain suppression activity; however, probably has significant peripheral analgesic and anti-inflammatory effect.

To further ascertain its anti-inflammatory activity, we performed two tests in tandem: the Acetic Acid induced writhing test and carrageenan induced paw edema test. Carrageenan induced edema is commonly used as an experimental model for acute inflammation, and is proven to be biphasic [27]. The early phase (1-2 hours) of the carrageenan model is chiefly mediated by serotonin and histamine release and increased synthesis of prostaglandins in the damaged paw tissues. These induce inflammation and paw swelling. The later phase is sustained by prostaglandin release and is also mediated by bradykinin, leukotrienes, poly-morphonuclear cells, and prostaglandins produced by tissue macrophages [28]. PRS showed, in a dose dependent manner, significant peripheral analgesic activity at the end of the early phase (2h) and throughout the later phase indicating its possible ability to hinder endogenous synthesis or release of inflammatory mediators such as prostaglandins, histamine, serotonin, bradykinin and leukotrienes.

The acetic acid induced writhing test was carried out to confirm the peripheral analgesic activity of PRS. The acetic acid used in this test increased the prostaglandin level (mainly PGE_2) in the peritoneal fluid of the mice [29]. Prostaglandins induce abdominal constriction by activating and sensitizing the peripheral chemo-sensitive nociceptors [30] which are mostly responsible for causing inflammatory pain [31]. NSAIDs, like diclofenac sodium, impart their effects via inhibitory action of prostaglandin synthesis resulting in peripheral analgesia. In our study, PRS significantly attenuated the writhing in mice in response to IP acetic acid administration, albeit to a lesser extent compared to the highly potent diclofenac sodium. Hence, the analgesic and anti-inflammatory action of PRS can be attributed to reduction of peripheral nociception by inhibition of prostaglandin release.

5. CONCLUSION

In summary, our present study has successfully elucidated the likely mechanism of anti-nociceptive and anti-inflammatory effect of PRS. We have drawn a sound conclusion that PRS does not have any CNS modulated effect in pain inhibition, based on three different *in-vivo* models. Its peripheral analgesic activity has been also repeatedly confirmed by three *in-vivo* models. Through this study, it is apparent that the mechanism of action of PRS is similar to that of the commonly used NSAIDs. Hence, its traditional use in arthritis, sciatia, and lumbago held the test of time, not by its mere placebo effect but by some potent analgesic and anti-inflammatory molecules hidden in this age old Ayurvedic concoction.

CONSENT

Not applicable.

ETHICAL APROVAL

All authors hereby declare that “Principles of laboratory animal care (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws were applicable. All experiments have been examined an approved by the appropriate ethics committee of North south University.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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