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Phytochemical Screening and Evaluation of in-vivo Analgesic Activity of *Pterocarpus indicus* Leaf

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Abstract

This study aimed to conduct phytochemical screening and evaluation of the in-vivo analgesic activity of methanol extract and its different fractions of *Pterocarpus indicus* leaves. The phytochemical screening was done by following the standard procedures. The phytochemical screening of methanol extract of *P. indicus* leaves indicates the presence of carbohydrates, alkaloids, cardiac glycosides, tannins, saponins, fats, oils, terpenoids, flavonoids, and resins. The analgesic activity was evaluated by two methods: namely the acetic acid-induced writhing and the formalin-induced paw-licking method. In the acetic acid-induced writhing test, dichloromethane fraction (PIDCM) showed significant (p<0.001) and highest potential as a peripheral analgesic by giving 89.49% of inhibition at a dose of 400 mg/kg and 71.85% of inhibition at a dose of 200 mg/kg as compared to control and standard (91. 18%). At both doses (200 and 400 mg/kg), the crude methanol extract (PIME) and its n-hexane fraction (PINH) showed significant (p<0.001) peripheral analgesic activity as compared to the control. In the formalin-induced paw-licking test, PINH (400 mg/kg and 200 mg/kg dose; p<0.001) showed significant and effective peripheral analgesic activity with 87.54 and 78.39% of inhibition, respectively. The extract PINH (400 mg/kg dose; p<0.01) showed significant and moderate central analgesic activity by giving 33.34% of inhibition. PIDCM (400 mg/kg dose; p<0.001) showed an effective peripheral analgesic effect as compared to control with 74.53% of inhibition. This plant could be a potential source for the development of newer natural therapeutic analgesic entities.

Keywords: *Pterocarpus indicus*, phytochemicals, analgesic, acetic acid-induced, formalin-induced paw-licking.

Introduction

Medicinal plants have been utilized as the backbone of traditional medication for curing and preventing purposes since the inauguration of human civilization[1]. Nearly 50,000 metabolites have been uncovered and recognized from medicinal plants, which are the leading reservoir of natural products with diverse pharmacological actions[2]. Traditional medicines are supported and promoted by WHO despite the vast impression of modern medicine in the manufacturing of synthetic drugs. Due to their great reliability and lack of significant side effects, these medications are efficient treatments for a variety of disorders[3]. Besides, they are achieving popularity with greater consumer requirements, notably in developed countries over the last period. People spend a significant amount of money on home remedies since natural medicines are considered a balanced formulation for a wholesome life, which has become a strong commodity on the global market[4]. Ayurvedic, Chinese, and new phytomedicines are included in the world market as alternative treatments based on the diverse pharmacological activities of medicinal plants. Therefore, medicinal plants achieved the focus of attention in research for developing a novel drug to disclose the points behind their traditional utilization[5]. Pain is supposed to be an insipid emotional and sensory experience related to tangible and/ or possible tissue damage. It has been described in terms of such damage which is a

fundamental feature of inflammation and may differ in severity, quality, and endurance[6]. Nonsteroidal anti-inflammatory drugs (NSAIDs), antiepileptic agents, opioids, amine reuptake inhibitors, and steroidal drugs are the currently used analgesics in pain management[7]. But these agents exhibit lower efficacy and show multiple adverse effects, including liver damage, gastrointestinal bleeding, renal failure, manic depression, dormant diabetes, hypertension, osteoporosis, dizziness, thromboembolism, irregular menstruation, and stomach ulcers[8]. Newer and safer analgesic drugs need to be discovered to overcome the shortcomings of available analgesics. A wide variety of pharmacologically bioactive compounds is present in medicinal plants from which novel and effective analgesic drugs could be developed with higher potency, efficacy, and safety[9]. Plants belonging to the *Pterocarpus* genus have extensive folklore medicinal benefits and are affluently found in numerous Asian countries, including Indonesia, Thailand, China, South Korea, Malaysia, Myanmar, India, and Taiwan[10]. *P. santalinus* leaf, wood, fruit, and bark are utilized for diarrhea, fever, inflammatory diseases, chronic dysentery, blood vomiting, worm infection, and gonorrhea[11]. Various parts of *P. marsupium* are used for treating skin eruption, diabetes, anal disorders, leukoderma, urinary discharge, asthma, and bronchitis[12]. *P. erinaceus* is employed to relieve pain, headache, nose bleeds, and infectious diseases[13]. Pharmacological investigations have revealed the anti-hyperglycaemic and anti-cataract effects of *P. marsupium*, the anti-diarrheal activity of *P. santalinoides*, anti-ulcerogenic and anti-photo aging effects of *P. santalinus*, anti-inflammatory and analgesic actions of *P. erinaceus*, the anti-oxidant activity of *P. soyauxii and P. osun*. Some species of *Pterocarpus* such as *P. santalinoides, P. indicus, P. erinaceus, P. santalinus, and P. angolensis* exhibited strong antimicrobial activity[14]. *Pterocarpus indicus* (*P. indicus*) is a tall deciduous tree from the Fabaceae family, and it was found growing in secondary and coastal forested areas. It was traditionally utilized for diarrhea, headache, menstrual pain, rheumatoid arthritis, fever, ulcers, diabetes, tuberculosis, and gonorrhea treatment[15]. Earlier investigation indicated that its methanol extracts are an abundant resource of active ingredients, including pterocarpans, triterpenoids, flavonoids, chalcones, epicatechins, arylcoumarins, lignans, sesquiterpenoids, fatty acids, and sterols[14]. However, the *Pterocarpus* genus has proven to have considerable traditional implications. This directs our present research interest to explore the crude methanol extract of *P. indicus* leaves and its different fractions for the investigation of phytochemicals and analgesic activity.

Materials and Methods

Solvents and chemicals

Solvents and chemicals used in the experiments were maintained at the analytical and laboratory grade. Methanol (99.93%), n-hexane (95%), dichloromethane (99.5%), tween-80, diclofenac sodium (50 mg/kg), and normal saline were used. Analytical grade reagents like Mayer's reagent, Wagner's reagent, Fehling's solution A and B, ferric chloride, sulphuric acid, copper sulfate, sodium hydroxide, hydrochloric acid, zinc dust, Glacial acetic acid, acetone, etc. were used during phytochemical screening.

Plant collection and authentication

The leaves of *P. indicus* were collected from the hilly region of the University of Chittagong, Chittagong, Bangladesh, in October 2019. After that, the plant sample was identified and then authenticated by (taxonomist) Department of Botany, University of Chittagong.

Preparation of extract

The leaves of *P. indicus* were thoroughly washed and then sun-dried for one week before grinding. Then the leaves were grounded into a coarse powder using a high-capacity grinding machine in the Phytochemical Research Laboratory Biological faculty, University of Chittagong. Approximately 700 gm. of powdered

sample was taken in a tidy, round-bottomed flask which was soaked in 5 L methanol for 15 days. The entire mixture was filtered using Whitman filter paper No 1 and the volume of found filtrate was reduced using a rotary evaporator at 60 ℃ and low pressure. The weight of crude methanol extract was found of 17 gm. The percent yield extract is 2.43%.

Solvent-solvent partitioning

Solvent-solvent partitioning was performed, followed by the protocol proposed by Kupchan[16] and modified by Van Wagenen[17,18]. Four different solvents used to partition were Petroleum ether, n-Hexane, chloroform, and carbon tetrachloride.

Phytochemical screening

Qualitative phytochemical screening was performed to detect the presence of phytoconstituents like alkaloids, terpenoids, saponins, flavonoids, steroids, glycosides, cardiac glycosides, proteins, carbohydrates, resins, fat, oils, etc., by following standard test procedures[19].

Experimental animals

Male and female Swiss albino mice weighing about 25-35 gm. were used for pharmacological investigation. These test animals were purchased from the Animal Resource Faculty, International Centre for Diarrhoeal Disease and Research, Bangladesh (ICDDRB), Mohakhali, Dhaka. The mice were afforded water ad libitum and standard pellet diets (ingredients include wheat flour, Skim milk powder, Roasted Bengal gram flour, Salt mixture, Casein, Refined groundnut oil, and Vitamin mixture) throughout the study. The mice were accustomed to the laboratory environment before the commencement of the experiment. All mice were handled according to the guidelines of the Institutional Ethics Committee (IEC).

Experimental design

In the evaluation of analgesic activity, fourteen groups of mice were selected and used in both methods; the acetic acid-induced and formalin-induced methods. Each group comprises five mice. Here, Group, I and Group II were treated as control and standard, respectively. Other groups were treated for the methanol extract of *P. indicus* and its different fractions. In the acetic acid-induced and formalin-induced methods, diclofenac sodium (50 mg/kg) and morphine sulfate (10 mg/kg) were used, respectively.

Acute Toxicity Studies

Acute toxicity studies were performed according to guidelines prescribed by the Organization for Economic Co-operation and Development (OECD). The LD_{50} for each of the extracts was ascertained and one-tenth of the lethal dose (LD_{50}) was considered the effectual therapeutic dose for exploring different pharmacological activities. The LD_{50} was calculated using the following equation[20]: $LD50 = LD100 - \sum(a \times b)/n$.

Evaluation of the analgesic activity

Acetic acid-induced writhing method

The acetic acid-induced writhing method was carried out to identify the peripheral analgesic effect of the investigated extract. The test was performed followed by the procedure described by Koster and modified by Dambisya & Lee[21]. Acetic acid (0.7% v/v; 10 ml/kg, i.p.) was given intraperitoneally to all groups of mice 15 min and 30 min after administration of standard and extract, respectively, to create pain sensation featured by abdominal constriction (writhes). Peripheral analgesic activity of the extract was assessed by carefully measuring the number of writhing for 15 minutes after a latency period of 5 minutes. The percentage inhibition of abdominal writhing is calculated as follows:

$$\%\ \text{of pain Inhibition} = \frac{\text{mean writhing count (control} - \text{treated)}}{\text{mean writhing count(control)}} \times 100$$

Formalin induced paw-licking method

Formalin-induced paw licking test, a persistent pain model was performed to determine the central analgesic action of the *P. indicus* leaves extract[22]. In this method, freshly prepared 1% formalin (0.02 ml) was given to all groups of overnight fasted mice into their right-hand

paw by sub-planter injection after 1 hour of oral administration of control, standard, and investigated extracts. The time that the mice passed in licking the injected paw was observed and recorded as it indicated pain. The reaction of mice at first 5 min denotes the early or neurogenic phase and later 15-30 expresses the late or inflammatory phase of post formalin injection. The percent of pain inhibition was evaluated by the following formula.

$$\% \text{ of pain inhibition} = \frac{\text{Reaction time(control)} - \text{Reaction time(treatment)}}{\text{Reaction time (control)}} \times 10$$

Statistical Analysis

All the data were expressed as Mean ± SEM (Standard error of the mean). The results were analyzed statistically by one-way ANOVA followed by post hoc Dunnett's "t" test using statistical software Statistical Package for Social Science (SPSS, Version 25.0, IBM Corporation, NY). Significance was considered at $^{***}p<0.001$, $^{**}p<0.01$, and $^{*}p<0.05$ compared with the control.

Results and Discussion

Evaluation of phytochemical screening

The preliminary phytochemical evaluation of methanol extract confirmed the presence of alkaloids, carbohydrates, saponins, flavonoids, fats and oils, terpenoids, resin, and cardiac glycoside as presented in Table 1.

Evaluation of analgesic activity

Acetic acid-induced method

The acetic acid-induced writhing method is a model of visceral pain[23], which employs dilute acetic acid to

Table 1: Phytochemical screening of methanol extract of *P. indicus*

Phytochemicals	Test	Result
Alkaloids	Mayer's test	+++
	Wagner's test	+
Carbohydrate	Molisch's test	++
	Fehling's test	++
Glycosides	Sodium hydroxide reagent test	_
Saponins	Foam test	+
Tannins	Ferric chloride test	++
Phenols	Ferric chloride test	_
Flavonoids (Flavones)	Alkaline reagent test	+
	Zinc-Hydrochloric acid test	++
Proteins	Biuret test	_
Fixed oils and fats	Copper sulfate test	+
Cardiac glycosides	Keller-Kiliani test	+
Terpenoids	Liebermann-Buchard's test	+++
Steroids	Liebermann-Buchard's test	_
Resins	Acetone test	++
Anthraquinones	Hydroxyantraquinone test	_

'+' =presence, '++'= more presence, '+++'=very much presence, '-'=Absence

cause a writhing reflex in mice. This method was employed to assess peripheral analgesic activity[24, 25].

Acetic acid causes pain by increasing PG2 and PG2α at organ cavity receptor sites, implying that carboxylic acid acts indirectly by promoting the release of endogenous mediators and eventually causes animals to writhe by activating chemosensitive nociceptors[23, 26] (Figure 2). Nonsteroidal anti-inflammatory medications work by inhibiting sensory neuron activation in response to inflammatory mediators[26]. In this method, pain feeling is produced by inducing a localized inflammatory response by releasing free arachidonic acid from tissue phospholipids via cyclooxygenase, prostaglandin production, and lipoxygenase products[27]. The increase in the prostaglandin levels, specifically PGE2 and PGE2α and the level of lipoxygenase products within the peritoneal cavity subsequently induce swelling and pain by the increased capillary permeability and by the release of endogenous chemicals that trigger pain in nerve endings, which eventually results in inflammatory pain[24, 27,28]. NSAIDs block the COX enzyme in peripheral tissues, affecting the transduction mechanism of important afferent nociceptors. The analgesic effect of the methanol extract and its different fractions of *P. indicus* leaf was comparable with the NSAID standard drug diclofenac sodium[27]. The percentage reduction in the number of abdominal constrictions indicates the extent of analgesia in acetic acid-induced models[23] (Figure 2). The agent that reduces twitching will produce analgesic effects, preferably via limiting prostaglandin synthesis, a peripheral mode of pain inhibition[23]. In the acetic acid writhing procedure, the methanol extract of *P. indicus* and its fractions showed dose-dependent peripheral analgesic activity as reported in Table 2. Among them, the fractions PINH and PIDCM at the doses of 200 and 400 mg/kg, showed more significant analgesic activity by reducing the number of writhing. The fraction PIDCM showed the highest percentage of inhibition of writhing (89.49%) at the dose of 400 mg/kg. On the

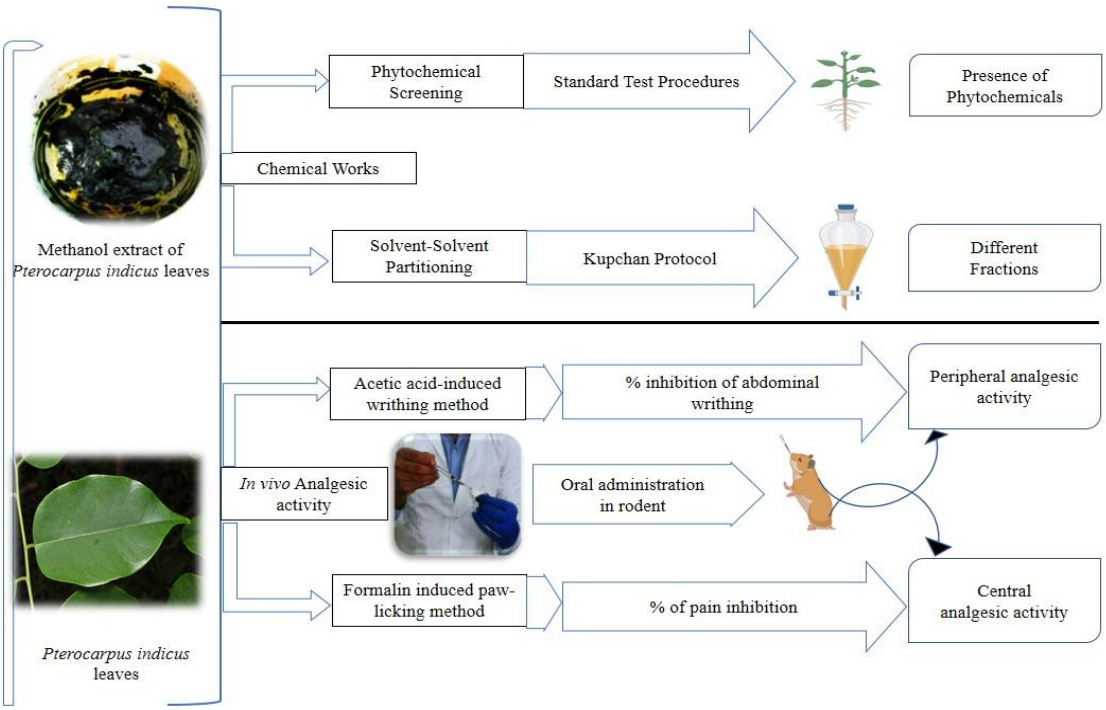

Figure 1. Summarized graphical representation of methods involved in the investigation methanol extract of *P. Indicus* leaves on phytochemical screening and *In vivo* analgesic activity.

Table 2: Analgesic activity of methanol extract and its different fractions of *P. indicus*

Group	Number of writhing (mean ± SEM)	Inhibition of Writhing (%)
Control	47.60 ± 1.61	-
Standard	4.20 ± 0.86***	91.18
PIME 200	33.80 ± 2.66***	28.99
PIME 400	19.70 ± 2.18***	58.61
PINH 200	14.50 ± 1.34***	69.54
PINH 400	9.00 ± 1.17***	81.09
PIDCM 200	13.40 ± 1.62***	71.85
PIDCM 400	5.00 ± 0.65***	89.49

PIME= Methanol Extract of *P. indicus*, PINH= n-hexane fraction, PIDCM= Dichloromethane fraction. Results were expressed as mean ± SEM; Results were significant as $^{*}p<0.05$, $^{**}p<0.01$, and $^{*}p<0.001$ compared to the control.

other hand, PINH exhibited the highest percentage of inhibition of writhing (81.09%) at the dose of 400 mg/kg. Both the activity could be compared to standard drugs. The methanol extract (PIME) showed the lowest percentage of inhibition of writhing in both doses. The sequence of the investigated extract as an analgesic agent with higher to lower potential will be; PIDCM>PINH> PIME. The methanol extract of *P. indicus* and its fractions showed dose-dependent significant peripheral analgesic activity in the experimental animals.

Formalin induced paw-licking method

The formalin test is a simple way to induce and quantify pain in rats. Pain intensity is deduced from objective behavioral categories that are distinct and consistently expressed by individual animals. The observations can then be simply turned into numerical values in this method[29]. The formalin assay is the most widely used chemical nociception test that includes peripheral inflammation and central sensitization[28] (Figure 2). This method has two phases: an early transient phase which

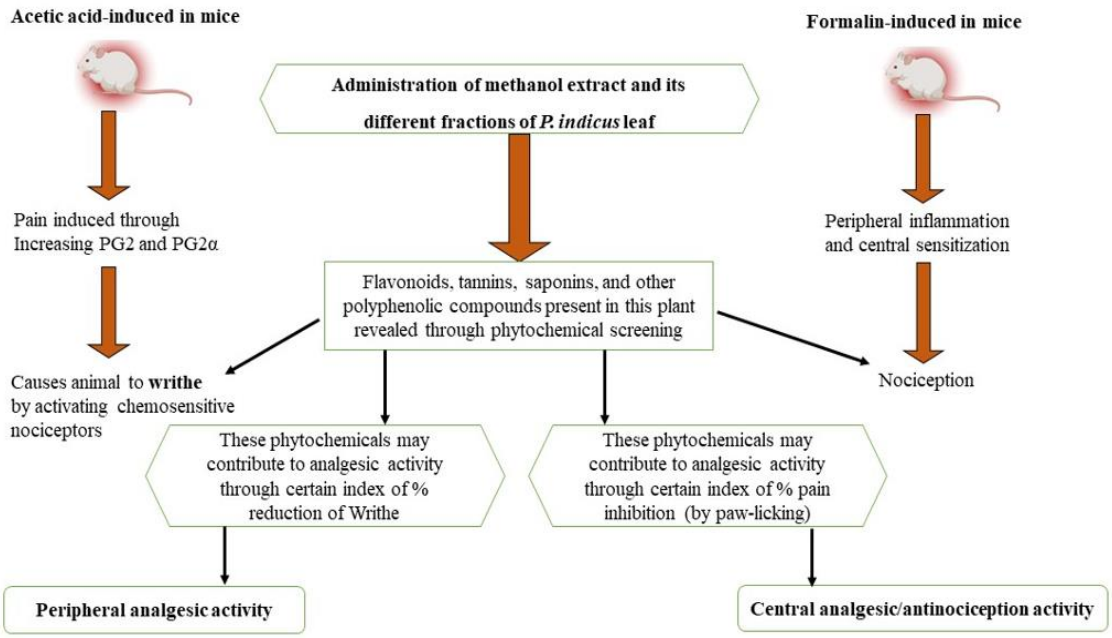

Figure 2. The mechanism related to analgesic activity exhibited by methanol extract of *P. indicus* and its different fractions.

Table 3: Analgesic activity of methanol extract and its different fractions of *P. indicus*

Group	0-5 min (early phase)		15-30 min (late phase)	
	paw licking time (sec)	Inhibition of pain (%)	paw licking time (sec)	Inhibition of pain (%)
Control	53.6 ± 3.29	-	71.73 ± 3.63	-
Standard	8.57 ± 0.53***	84.01	4.71 ± 0.51***	93.43
PIME 200	47.53 ± 3.53	11.32	29.72 ± 3.60***	58.57
PIME 400	33.21 ± 2.86**	30.04	24.76 ± 3.18***	65.48
PINH 200	51.34 ± 4.16	4.22	15.5 ± 2.52**	78.39
PINH 400	35.73 ± 3.89**	33.4	8.94 ± 0.93***	87.54
PIDCM 200	51.59 ± 3.57	3.75	43.65 ± 2.25***	39.14
PIDCM 400	43.38 ± 4.25	19.07	18.27 ± 3.42***	74.53

PIME= Methanol Extract of *P. indicus*, PINH= n-hexane fraction, PIDCM= Dichloromethane fraction. Results were expressed as mean ± SEM; Results were significant as $^{*}p<0.05$, $^{**}p<0.01$, and $^{*}p<0.001$ compared to control.

commences immediately after formalin injection in the mice paw and lasts for about 5 minutes and reflects direct stimulation of nociceptors and C nerve fiber activity, and a late tonic phase (inflammatory), which lasts from the 15th to the 30th minutes after injection and may be connected with the inflammatory mediators' release; nevertheless, the role of sensitization of central pain pathways should also be recognized[24,30]. This method is mostly caused by neurogenic inflammation, which is followed by the involvement of kinins and leukocytes with their pro-inflammatory substances, including prostaglandins. It has also been shown that formalin-induced acute inflammation comes from cell damage, which promotes the synthesis of endogenous mediators[28]. Opioid analgesics, which frequently act centrally, block both stages of formalin assay with roughly equal severity[30]. Morphine, a very active opioid analgesic with a central effect, was chosen as the reference standard, inhibiting pain both in the early and late phase of the formalin assay[29,30], whereas Control did not suppress pain at all. Compared to control and standard, ME showed a mild and significant anti-nociceptive effect during the early phase, but it gave a moderate analgesic effect during the late phase. So, it could not be effective as a centrally acting analgesic, while it could have moderate effects as a peripherally acting analgesic. Although NHF inhibited pain very mildly during the early phase at both doses, it inhibited the pain excellently in the late phase, which was significant and indicated the highly active potential of NHF to be a peripherally acting anti-nociceptive agent. It exhibited the highest percentage of inhibition of pain in the late phase. DCMF gave a very mild anti-nociceptive effect during the early phase at both doses which significantly increased to a higher percentage of inhibition of pain during the late phase and was discerned to be a moderately peripherally acting anti-nociceptive agent at the high dose. The analgesic activity of crude methanol extract and its different solvent fractions of the plant can be sequenced as NHF> DCMF> ME based on the percentage of inhibition of pain in comparison to the reference standard. The extract's mechanism of action may be related to lipoxygenase and/or cyclooxygenase of the arachidonic acid cascade, as well as opioid receptors[28]. Flavonoids, tannins, saponins, and other polyphenolic compounds are reported to have analgesic and anti-inflammatory properties[24, 28, 30]. The presence of any of these phytochemicals in the investigated extracts might contribute to their analgesic activity[28] (Figure 2). The results obtained in the formalin-induced paw licking method are tabulated in Table 3. The mechanism of analgesic activity by both these methods are explained in Figure 2.

Limitations of the study

This study was limited to leaf extract of *P. indicus.* The same study needs to be conducted on other parts (stems, roots, etc.) of this plant is suggested. Besides this study only reveals preliminary phytochemical screening and *in vivo* analgesic activity in mice. Other tests are recommended to conduct on this plant.

Conclusion

The current investigation revealed the presence of various phytochemicals. The methanol extract and different fractions of *P. indicus* showed significant and dose-dependent analgesic effects. More advanced research is required to be sure of the present findings of the study.

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