

## Anti-Inflammatory, Antinociceptive and CNS Depressant Activities of the Methanolic Extract of *Phyllanthus reticulatus* Leaves

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**Abstract:** Objectives- The study was carried out to assess the anti-inflammatory, analgesic and CNS depressant activity of the methanolic extract of leaves of *Phyllanthus reticulatus* (MEPR). Methodology- Anti-inflammatory effects of MEPR was done by carrageenan induced inflammatory method at the dose of 100 and 200 mg/kg b.wt., i.p. A significant ( $p < 0.05$ ) inhibition of paw oedema at 2, 3 and 4 hours as compared to reference drug diclofenac were observed. The acetic acid-induced writhing model and formalin test method determined the analgesic activity of the MEPR in mice. The extract showed 64.628% inhibition whereas 66.446% inhibition was observed for diclofenac sodium against pain in the late phase of the formalin induced test. The CNS depressant activity was evaluated by observing the reduction of locomotor and exploratory activities in the open field and hole cross tests at dose of 250 and 500 mg/kg body weight. The results of the statistical analysis showed that the plant extract have significant ( $p < 0.05$ ) dose dependent CNS depressant. In conclusion this study recommend that methanolic extract of leaves of *Phyllanthus reticulatus* has anti-inflammatory, analgesic and CNS depressant properties.

**Key words:** *Phyllanthus reticulatus* • Analgesic • Writhing Response • Anti-Inflammatory • Carrageenan • CNS Depressant

### INTRODUCTION

*Phyllanthus reticulatus* (*P. reticulatus*) (Family: Euphorbiaceae) is usually a dense deciduous shrub or small tree with a distinct smell that is emitted by the minute flowers when they open towards the early evening. This is one of the fascinating characteristic smells of Africa. *P. reticulatus* is a many branched shrub, sometimes partially scrambling, usually 1-5 m high, or a small twiggy tree that grows up to 8 m in height. The bark is light reddish-brown or grey-brown with hairy stems when young, which become smooth with age. The leaves alternate along slender branches. They are up to 25 cm long and appear as leaflets of large pinnate leaves. The leaves are thinly textured, usually hairless. They have a noticeable reddish net-veining which is more visible above than below [1].

The plant grows throughout tropical areas of India, Bangladesh, China and the Malay Islands [2]. Natural products are widely used to treat a plethora of acute and chronic diseases ranging from the common cold to complex human diseases all over the world. Literature survey reveals that the whole plant is astringent, sweet, cooling, diuretic, alternant, stomachic, constipating and attenuant. It is reported to be useful in vitiated condition of *pitta*, burning sensation, strangury, gastropathy, ulemorrhagia, ophthalmodynia, sores, burns, suppuration, diarrhea, skin eruption and obesity [3-5].

The previous phytochemical investigations showed that the *P. reticulatus* contain lupeol, lupeol acetate and stigmasterol(steroid) [6]. Dichloromethane extract of leaves of the plant have been reported the presence of three compounds, (5R\*,6R\*)-4,6-dimethoxycarbonyl-5-[2, 3, 4 -trihydroxy-6 (methoxycarbonyl) phenyl]-5,6-dihydro-

2H-pyran-2-one along with 3,4,3-tri-O-methylellagic acid and methyl gallate. The first compound reportedly demonstrated weak insecticidal activity against *Spodoptera frugiperda* [7]. *Reticulatus* contains Phytosterol (sitosterol), descendants of friedelin, olean and lupan type (butelin and glochidonol). It contains polyphenols, flavonoid glycosides, tannic acid, friedelin, epifriedelinol, betulin, taraxerone, betasitosterol, glochidonol, octacosanol, taraxeryl acetate and 21- $\alpha$ -hydroxyfriedelin-3-one [12]. The tannin of *P. reticulatus* are partly responsible for its medicinal and dyeing properties. A number of triterpenoids have been isolated from the stems and leaves, including sitosterol, friedelin and betulinic acid. Phytochemical experiments of the leaves revealed the presence of terpenoid glycosides, protein, carbohydrates but absence of alkaloids and steroids [8]. The biological investigation on *P. reticulatus* exhibited antinociceptive and antihyperglycemic [9], hypocholesterolemic [10], antibacterial [2]. The fruit is an astringent to the bowels and is used in the inflammation. The leaves are employed as a diuretic and cooling medicine [11, 12]. Moreover the leaf juice is a remedy for spongy and bleeding gums [13]. Ethanol extract of the plant has been revealed hepatoprotective activity against carbon tetrachloride-induced liver damages in rats [14]. Earlier reports also indicate the hypolipidemic action of methanolic and alcoholic extract of the *P. reticulatus* in animals [15, 16]. Recent studies also reveal the analgesic and anti-inflammatory properties of *P. reticulatus* extract (petroleum ether, ethyl acetate and methanol) in acetic-induced writhing and carrageenan induced rat paw edema models respectively [17]. Besides these 150 and 300 mg/kg dose of the petroleum ether, ethyl acetate and methanol extracts of *Phyllanthus reticulatus* possess significant analgesic and anti-inflammatory properties [18]. Considering the literature review and presence of constituents of the plant, our purpose of the study was to evaluate the anti-inflammatory, analgesic and CNS depressant activity at different doses of methanolic extract of leaves of *Phyllanthus reticulatus*.

## MATERIALS AND METHODS

**Plant Material:** For this present investigation, the fresh leaves of *Phyllanthus reticulatus* were collected from the area of Bandorban of Bangladesh and were identified by the experts of Bangladesh National Herbarium, Dhaka, where a voucher specimen has been retained. The collected plant parts were dried for one week and pulverized into a coarse powder with the help of a suitable

grinder. The powder was stored in an airtight container and kept in a cool, dark and dry place until analysis commenced.

**Preparation of the Extract:** About 150 gm of powdered material was taken in a clean, flat bottomed glass container and soaked in 200 ml of 85% methanol. The container with its contents was sealed and kept for a period of 7 days accompanying occasional shaking and stirring. The whole mixture then underwent a coarse filtration by a piece of clean, white cotton material. Then it was filtered through Whatman filter paper (Bibby RE200, Sterilin Ltd., UK). The filtrate (methanol extract) obtained was evaporated using rotary evaporator. It rendered a gummy concentrate of reddish black color. The gummy concentrate was designated as crude extract of methanol. The extract was transferred to a closed container for further use and protection.

**Animals:** In the present study, 16 albino mice (male), which weighed between 20-25g were used. The animals were obtained from International Centre for Diarrheal Disease Research, Bangladesh (ICDDRDB). All animals were kept under ambient temperature with 12h light followed by a 12h dark cycle. The animals were acclimatized for one week prior to actual experiments. The study was conducted following approval by the Institutional Animal Ethical Committee of University of Development Alternative, Dhaka, Bangladesh.

**Acute Toxicity:** The 50% lethal dose ( $LD_{50}$ ) of the extract of *Phyllanthus reticulatus* in mice was estimated by the up and down method (Bruce, 1985). Doses were adjusted up or down by a constant multiplicative factor (1.5) depending on the previous outcome.

**Drugs and Chemicals:** Indomethacin, aspirin, glibenclamide, loperamide and glucose were obtained from Square Pharmaceuticals Ltd., Bangladesh, Acetic acid was collected from Merck, Germany. Normal saline water (0.9%) NaCl was brought from Beximco Infusion Ltd. Bangladesh. BDH chemicals Ltd provided Tween 80. Formalin, Castor oil and all other chemicals were of analytical grade.

### Anti - Inflammatory Activity

**Carrageenan-Induced Paw Oedema:** The mice were divided into five groups each containing 5 mice. Acute inflammation was induced by injecting 0.1 ml of (1%) carrageenan into plantar surface of mice hind paw [19].

The methanolic leaf extract (100 and 200 mg/kg) of *P. reticulatus*, normal saline (1ml/kg) and diclofenac sodium at dose of (100 mg/kg/i.p.) as reference agent were administered 30 min before carrageenan injection. The paw volume was measured at 0, 1, 2, 3, 4 and 5hrs using a Vernier caliper to determine the diameter of oedema. The difference between the readings at time 0 h and different time interval was taken as the thickness of oedema.

### Analgesic Activity

**Acetic Acid-Induced Writhing Method:** The analgesic activity of the samples was also studied using acetic acid-induced writhing model in mice [20]. Test samples (100 and 200 mg/kg body weight), vehicle (1% tween 80 in water) and indomethacin (10mg/kg) were administered orally 30 min before intraperitoneal administration of 0.7% acetic acid. Then the mice were observed for specific contraction of body referred to as 'writhing' for the next 20 min [19]. Full writhing was not always accomplished by the animal, because sometimes the animals started to give writhing but they did not complete it. This incomplete writhing was considered as half writhing. Accordingly, two half-writhing were taken as one full writhing. The number of writhes in each treated group was compared to that of a control group while indomethacin (10mg/kg) was used as a reference substance (positive control).

The percent inhibition (% analgesic activity) was calculated by inhibition % =  $\{(A-B)/A\} \times 100$

where, A= Average number of writhing of the control group; B= Average number of writhing of the test group.  
Formalin Test:

The antinociceptive activity of the drugs was determined using the formalin test described by Winter *et al.* [19]. Control group received 5% formalin. 20  $\mu$ l of 5% formalin was injected into the dorsal surface of the right hind paw 60 min after administration of MEPR (100 and 200 mg/kg, p.o.) and Indomethacin (10mg/kg, p.o.). The mice were observed for 30 min after the injection of formalin and the amount of time spent licking the injected hind paw was recorded. The first 5 min post formalin injection is referred to as the early phase and the period between 15 and 30 min as the late phase. The total time spent licking or biting the injured paw (pain behavior) was measured with a stop watch.

### CNS Depressant Activity

**Hole Cross Test:** The method was carried out as described by Takagi *et al.* [21]. A steel partition was fixed in the middle of a cage having a size of 30×20×14 cm. A hole of 3 cm diameter was made at a height of 7.5 cm in the center of the cage. Twenty animals were divided into four groups with four rats in each group. Group I animals received vehicle (1% Tween 80 in water, 10 ml kg<sup>-1</sup> p.o.), animals of Group II received diazepam at 1 mg kg<sup>-1</sup> body weight (p.o.) while animals of groups III and IV were treated with 250 and 500 mg kg<sup>-1</sup> body weight (p.o.) of the MEPR. The number of passages of mice through the hole from one chamber to other was counted for a period of 3 min on 0, 30, 60, 90 and 120 min after oral administration of test drugs.

**Open Field Test:** The animals were treated as discussed above. The experiment was carried out according to the methods described by Gupta *et al.* [22]. The floor of an open field of half square meter was divided into a series of squares each alternatively colored black and white. The apparatus had 40 cm height a wall. The number of squares visited by the animals was counted for 3 min, on 0, 30, 60, 90 and 120 min after oral administration of test drugs.

## DISCUSSION

Carrageenan -induced paw edema has been commonly used as an experimental animal model for acute inflammation and is believed to be biphasic in which the early phase (1-2) of the carrageenan model is mainly mediated by histamine, serotonin and increased synthesis of prostaglandins in the damaged tissue surroundings and the late phase is sustained by prostaglandin release and mediated by bradikinin, leukotrienes, polymorphonuclear cells and prostaglandins produced by tissue macrophase [23, 24]. In the acute inflammation model, the petroleum ether and methanolic extracts of leaf of *Phyllanthus reticulatus* in doses of 150 and 300 mg/Kg, p.o. produced dose dependent inhibition of paw edema [25]. As the crude methanol extract of *P. reticulatus* exhibited significant and sustained inhibition of paw edema from 1 to 4h, the possible mechanism of the observed anti-inflammatory activity might be its ability to reduce the release of histamine, serotonin or kinin like substances or biosynthesis of prostaglandins which is consistent with the test of analgesic activity.

Table 1: Anti-inflammatory activity of *P. Reticulatus* on carrageenan induced paw edema in mice

Group	Dose	Oedema diameter (mm) Inhibition (%)							
		1h	2hrs	3 hrs	4 hrs	1 hrs	2 hrs	3 hrs	4 hrs
Control		5.12±0.36	6.2±0.43	5.94±0.43	6.4±0.67				
Indomethacin	10mg/kg	4.63±0.55*	4.49±0.49*	4.34±0.61*	4.21±0.41*	9.76	27.54	26.97	34.22
MEPR	100mg/kg	5.08±0.39	4.82±0.46*	4.66±0.64*	4.49±0.57*	0.98	22.30	21.54	29.84
MEPR	200mg/kg	5.003±0.4	4.76±0.37*	4.60±0.38*	4.41±0.29*	2.39	23.31	22.55	31.093

Effects of methanolic extract of the leaves of *P. reticulatus* on carrageenan induced paw edema test. Values are mean ±SEM, (n = 4); \*p<0.05 as compared to vehicle control (One way ANOVA followed by Dunnet test). Group I animals received vehicle (1% Tween 80 in water), Group II received indomethacin 10 mg/kg body weight, Group III and IV were treated with 100 and 200 mg/kg body weight (p.o.) of the MEPR.

Table 2: Analgesic activity of *P. Reticulatus* on acetic acid induced writhing method in mice

Goup	Treatment	Dose, rout	No. Of Writhing	Percent of inhibition
Group-I (Control)	!% Tween 80 in water	0.1ml/10gm body weight	26.50±1.14	-
Group-II (Standard)	Indomethacin	10mg/kg,p.o	10.50±0.76*	60.38
Group-III (extract)	MEPR	100mg/kg,p.o	15.75±0.98*	40.57
Group-IV (extract)	MEPR	200mg/kg,p.o	12.50±1.14*	52.83

Values are mean ± SEM, (n = 4); \*p<0.05 as compared to vehicle control (One way ANOVA followed by Dunnet test). Group I animals received vehicle (1% Tween 80 in water), Group II received Indomethacin 10 mg/kg body weight, Group III and IV were treated with 100 and 200 mg/kg body weight (p.o.) of the MEPR

Table 3:Analgesic activity of the *P. Reticulatus* on hind paw licking in formalin test in mice.

Goup	Treatment	Dose,rout	Early phase (sec)	% of inhibition	Late phase (sec)	% of inhibition
Group-I (Control)	Distill water	0.1ml/10gm body weight	35.5±1.14		35.75±0.978	
Group-II (Standard)	Indomethacin	10mg/kg,p.o	10.50±0.75*	53.52	16.5±0.75*	53.85
Group-III (extract)	MEPR	100mg/kg,p.o	15.75±0.75*	19.72	24.25±0.97*	32.17
Group-IV (extract)	MEPR	200mg/kg,p.o	12.50±0.97*	35.92	24.25±0.97*	32.17

Values are mean ± SEM, (n = 5); \*p<0.05 as compared to vehicle control (One way ANOVA followed by Dunnet test). Group I animals received vehicle (1% Tween 80 in water), Group II received Indomethacin 10 mg/kg body weight, Group III and IV were treated with 100 and 200 mg/kg body weight (p.o.) of the MEPR.

Table 4: CNS depressant activity of *P. Reticulatus* on Hole cross test in mice

Group	Treatment	Dose (P.O)	No. of movement				
			0min	30min	60min	90min	120min
Group-I	1% tween 80 in water	10ml/kg	11.5±1.38	10.5±1.29	10.25±1.12	10±1.35	9.75±1.22
Group-II	Diazepam	1mg/kg	10.75±1.12	9.75±0.91	7±1.07*	6.5±1*	4.25±1.31*
Group-III	MEPR	250mg/kg	11.75±1.3.68	10.75±0.91	8.5±1.14	7.5±1.14*	5.25±1.12*
Group-IV	MEPR	500mg/kg	12±2.47	10.5±1.06	7.75±1.31	6.75±0.98*	4.5±1.14*

Values are mean ± SEM, (n = 4); \* p<0.05, Dunnet test as compared to vehicle control. Group I animals received vehicle (1% Tween 80 in water), Group II received diazepam 1 mg/kg body weight, Group III and Group IV were treated with 250 and 500 mg/kg body weight (p.o.) of the MEPR.

Table 5: CNS depressant activity of *P. Reticulatus* on open field test in mice

Group	Treatment	Dose(P.O)	No. of movement				
			0min	30min	60min	90min	120min
Group-I	1% tween 80 in water	10ml/kg	99.5±2.12	120±1.76	92.5±1.92	100±2.18	90.5±2.05
Group-II	Diazepam	1mg/kg	90±1.72*	88±1.11*	70±2.06*	65±1.91*	52±1.78*
Group-III	MEPR	250mg/kg	108±1.91*	95±2.03*	80±2.17*	75±1.57*	60±2.1*
Group-IV	MEPR	500mg/kg	90±1.98*	85±1.46*	78±1.72*	60±1.98*	45±1.61*

Values are mean ± SEM, (n = 4); \* p<0.05, Dunnet test as compared to vehicle control. Group I animals received vehicle (1% Tween 80 in water), Group II received diazepam 1 mg/kg body weight, Group III and Group IV were treated with 100 and 200 mg/kg body weight (p.o.) of the MEPR.

Acetic acid induces pain by enhancing levels of PGE<sub>2</sub> and PGF<sub>2α</sub> [26] at the receptors of peritoneal cavity [27, 28], which mean the acetic acid acts indirectly by increasing the release of endogenous mediators, leading to stimulation of the nociceptive neurons which are sensitive to most of the non-steroidal anti-inflammatory drugs. In this study, *P. reticulatus* exhibited dose dependent and significant inhibition of acetic-induced writhes in mice, which suggest the taking part of peripheral mechanisms of analgesia.

The formalin test is another important model of analgesic which is better related with clinical pain [29, 30]. This method elucidate central and peripheral activities. Formalin-induced nociception is biphasic in which first phase involves direct stimulation of sensory nerve fibres representing neuropathic pain and second phase involves inflammatory pain mediated by prostaglandin, serotonin, histamine, bradikinin and cytokines such as IL-1β, IL-6, TNF-α, eicosanoids and NO [31-36] *P. reticulatus* showed inhibition of second phase of formalin induced nociception in mice is higher than the standard Indomethacin. The MEPR was found to show analgesic effect by reducing hypernociception induced by bradikinin and cytokines (TNF-α, IL-1β) and the release of IL-1β and PGE<sub>2</sub> in paw skin induced by polysaccharide [37]. Moreover the lupeol acetate (Triterpenes) inhibits NO production, iNOS and COX2 expression may take part in the antinociceptive and anti-inflammatory effects of *Balanophoraspicata* [38]. Since the literature review showed the presence of lupeol acetate in *P. reticulatus*. So, it is strongly revealed that the antinociceptive and anti-inflammatory effects of *P. reticulatus* may be in a large part due to lupeol acetate. Hence, Inhibition of licking response of the MEPR in the early phase and the late phase signifying the analgesic effect of the extract on the formalin test.

Locomotor activity considered as an increase in alertness and decrease in locomotor activity indicated sedative effect [39]. Extracts of *P. reticulatus* decreased locomotor activity indicates its CNS depressant activity. Gamma-amino-butyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system. Different anxiolytic, muscle relaxant, sedative-hypnotic drugs are elucidation their action through GABA (A), therefore it is possible that extracts of *P. reticulatus* may acts by potentiating GABAergic inhibition in the CNS via membrane hyperpolarization which leads to a decrease in the firing rate of critical neurons in the brain or may be due to direct activation of GABA receptor by the extracts

[40]. Many research presented that plant containing flavonoids, saponins and tannins are useful in many CNS disorders [41]. Earlier investigation on phytoconstituents and plants suggests that many flavonoids and neuroactive steroids were found to be ligands for the GABA(A) receptors in the central nervous system; which led to the assume that they can act as benzodiazepine like molecules [39]. So might be this phytoconstituents are responsible for *P. reticulatus* CNS depressant activity.

## CONCLUSION

On the basis of results obtained from the present study, it can be concluded that the plant extract possesses remarkable anti-inflammatory, antinociceptive and CNS depressant activities. Present work was a preliminary effort which will require further detailed investigation including characterization of active compounds and requires preformulation studies for development of a potential dosage form.

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