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Antiinflammatory, antinociceptive and antioxidant activities of *Phyllanthus acidus* L. extracts

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ABSTRACT

Objective: To evaluate analgesic, anti-inflammatory and in vitro antioxidant potential and determine total phenolic, total flavonoid content of leaves extracts of *Phyllanthus acidus*, a folk medicinal plant of India. **Methods:** Anti-inflammatory activity was evaluated using carrageenan induced paw oedema, cotton pellet induced granuloma, membrane stabilizing activity method. Analgesic activity of the extracts was estimated against acetic acid induced writhing, tail immersion method, formalin test. Free radical scavenging and antioxidant potential of the extracts of *Phyllanthus acidus* leaves was performed using several in vitro and ex vivo assay models. Total phenolic and total flavonoid contents of the extracts were determined using standard chemical methods. **Results:** The extracts exhibited significant anti-inflammatory and analgesic activities at dose dependent manner. Methanol extract at a dose of 500 mg/kg showed superior activity which was comparable with the standard drugs. Ethyl acetate extract showed moderate activity while petroleum ether extract showed least activity. Total phenolic and total flavonoid content in methanol extract were 73.08±0.682 mg GAE/g and 61.28±0.062 mg QE/g respectively. The extracts possess significant antioxidant activity, methanol extract showed highest IC50 value. The contents of flavonoids and phenolic compounds could be correlated with the antioxidant, analgesic and anti-inflammatory activities observed for *Phyllanthus acidus* leaves. **Conclusion:** Our findings suggest that *Phyllanthus acidus* contains potential antioxidant, analgesic and anti-inflammatory compounds which could be tested as drug candidates against oxidative stress, pain and inflammation related pathological diseases.

1. Introduction

Phyllanthus acidus Skeels belonging to the family Phyllanthaceae is distributed commonly through out India, and often used by ethnic peoples of north-east India in folk medicine. P acidus have been used traditionally in the treatment of several pain, inflammatory and oxidative stress related disorders such as rheumatism, bronchitis, asthma, respiratory disorder, hepatic disease, diabetes and gonorrhoea. The plant is also important to improve eyesight, memory and to cure cough, psoriasis, skin disorders, sudorific[1,2,3]. Fruits of the plant used as astringent, root and seed are useful as cathartic, leaf and root use as antidote to viper venom[4]. The leaf of the plant found effective in hypertension[5]. Leaf, bark and roots are used to treat fever traditionally[6] The latex of the plant credited with purgative and emetic activity[7].

Methanolic extract of fruit and leaves was reported to possess antimicrobial activity[8]. In vitro screening of petroleum ether extract of fruits showed cytotoxic, antibacterial and antioxidant activity[9]. Different parts of *P. acidus* have been reported for several biological activities, fruits and leaves of the plant showed promising hepatoprotective activity[10]. Phyllanthosols A and B were isolated from roots, which were proposed as promising antitumor activity[11]. Adenosine, kaempferol, and hypogallic acid were found in leaves which showed airway chloride secretion, a potential treatment in cystic fibrosis[12]. Phyllanthus genius is reach in different secondary metabolite like alkaloids, tannins, flavonoids, lignans, phenolics and terpenes[13]. Several species of phyllanthus have shown antinociceptive activity in mice[14].

In this study, *P. acidus* leaves extracts were assessed for analgesic, antiinflamatory and antioxidant activity

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to find credence of the traditional usage of *P. acidus* in antiinflamatory and oxidative stress related disorder. In addition, we measured the content of total phenolic compounds and flavonoids, in the extracts in order to correlate them with the assayed activities.

2. Materials and methods

2.1. Plant materials

The leaves of *Phyllanthus acidus* was collected in February 2011 from Tripura, India. The plant identified as *Phyllanthus acidus* L. Skeels was confirmed by Dr. B. K. Datta, Department of Botany, Tripura University, Tripura, India. A voucher specimen was deposited in Plant Taxonomy & Biodiversity Laboratory, Tripura University for further reference.

2.2. Drugs and chemicals

Indomethacin capsule, diclofenac sodium, aspirin was obtained from Ranbaxy Laboratories Limited (India). Acetic acid, 2,2-diphenyl-picrylhydrazyl (DPPH), phenazine methosulfate (PMS), and nitro blue tetrazolium (NBT), Folin-Ciocalteau reagent, ferrozine, butylated hydroxyanisole (BHA), ascorbic acid were purchased from Sigma Aldrich (Bangalore, India). Trichloro acetic acid (TCA), thio barbituric acid (TBA), 2-deoxy-ribose, and quercetin, α-tocopherol, and gallic acid were procured from SD Fine Ltd. Mumbai. Linoleic acid, All other chemicals used in the study were obtained commercially and were of analytical grade.

2.3. Preparation of extracts

Air dried fine leaf powder extracted using methanol, ethyl acetate, petroleum ether separately for 14 h and concentrated yield was 17.7%, 16.2%, 10.6% respectively, which were then stored at 4 ℃ until the time of use.

2.4. Animals

Wistar rats and albino mice used to different analgesic, anti-inflammatory and lipid peroxidation inhibition assay. Animals were housed under standard environmental conditions (24±1 ℃) with 12 h light - 12 h dark cycles. The study was approved by the Institutional Animal Ethical Committee (Reg. no: 1305/ac/09/CPCSEA).

2.5. Animal groups

Healthy adult rats or mice were divided into 8 groups each consists 6 animals for each analgesic and anti-inflammatory test in following manner

Group I (Control) : Sodium CMC solution (0.5%).
Group II (Standard): Indomethacin (10 mg/kg, *p.o.*) or diclofenac sodium (10 mg/kg, *p.o.*)
Group III & IV: Methanol extract of *P. acidus* (250 mg/kg, 500 mg/kg *p.o.*).
Group V & VI: Ethyl acetate extract of *P. acidus* (250 mg/kg, 500mg/kg *p.o.*).
Group VII & VIII: Petroleum ether extract of *P. acidus* (250 mg/kg, 500 mg/kg *p.o.*).

2.6. Nociceptive tests

2.6.1. Writhing reflex induced by acetic acid in mice

The antinociceptive activities of *P. acidus* extracts were determined in mice using the writhing test[15]. The each mouse injected with acetic acid (0.6%, v/v, 10 ml/kg), and the intensity of nociceptive behaviour was quantified by counting the total number of writhes over a period of 25 min. The extracts, indomethacin, and vehicle were administered orally 1 h prior to acetic acid injection. The percentage analgesic activity was calculated as follows:

Percentage analgesic activity = [(Nc - Nt)/ Nc] × 100%

Where Nc is the average number of stretches of the control group, and Nt is the average number of stretches of the test drug group.

2.6.2. Tail immersion test

Tail immersion test was performed by immersing extreme 3 cm of the albino mouse tail in a hot water at a temperature of 55±0.5 ℃[16]. Within few second each mouse was reacted by withdrawing the tail, and the reaction time was recorded with a stopwatch. The drugs were given orally to the respective groups. The experiment was repeated at 0, 0.5, 1, 2, 3, 5 h following the administration of extracts and standard drug.

2.6.3. Formalin-induced licking response in mice

One hour after oral administration of vehicle, test samples and diclofenac sodium (10 mg/kg), 25 μL of 1% formalin in saline was injected subcutaneously into the subplantar region of right hind paw. The mice were immediately placed in a clear jar after and the time spent on licking the injected paw was recorded. The first period (early phase) was recorded at 0-5 min and the second period (late phase) was recorded at 10-35 min[17].

2.7. Anti-inflammatory activity

2.7.1. Carrageenan-induced paw oedema in rats

Carrageenan induced paw inflammation in rats was produced by injecting carrageenan (1% solution, 0.1 mL) subcutaneously into the plantar surface of left hind paw of each rat after administration of respective drug treatment to each group. The volume of the rat paws was measured with a plethismometer before and 1, 2, 3, 4, and 5 h after [18].

2.7.2. Granuloma formation induced by cotton pellet in rats

A sterilized cotton pellet of 30 mg was put subcutaneously into the interscapular area of anaesthetized rats. Male rats were anesthetized using 25 mg/kg of pentobarbitone sodium. Under sterilized conditions, cotton pellets of 30 mg were

implanted subcutaneously in the interscapular area. The extract solution, indomethacin (5 mg/kg, p.o.), and vehicle water were administered once daily for 5 consecutive days. On the 5th day, animals were killed via cervical dislocation after 1h of drug treatment. The cotton pellets with the granuloma tissue around them were dissected out carefully. The weights of these pellets (wet and dry) were measured and the anti-proliferative effects of extracts and indomethacin were determined by comparing with control group[19].

2.7.3. Membrane stabilizing activity

The blood was collected from rats under mild anaesthesia and mixed with heparin to prevent clotting. The blood centrifuged was washed three times using 0.9% saline. The 10 mM sodium phosphate buffer (pH 7.4) was used to reconstitute 40% v/v suspension of erythrocyte and used for this test. The heat induced haemolysis and hypotonic solution induced haemolysis assay was performed to access membrane stabilizing activity as per the standard procedures[20].

2.8. Estimation of total phenolic component and total flavonoid content

The total phenolic content was determined using Foline-Ciocalteu reagent method and expressed as mg gallic acid equivalents (GAE)/g dry extract[21]. The total flavonoid content of extracts was determined following a colorimetric method and values were expressed as mg quercetin equivalent (QE)/ g dry extract[22].

2.9. Antioxidant activity

2.9.1. DPPH radical scavenging assay

Free radical scavenging capacity of *P. acidus* leaf extracts was determined in terms of hydrogen donating or radical scavenging ability of extracts, using the stable DPPH radical[23]. The scavenging activity of extracts was calculated based on the percentage of DPPH radical scavenged using the equation (1)

% inhibition = [(Acontrol − Asample)/Acontrol] × 100

Where, Asample is the absorbance of a sample solution and Acontrol is the absorbance of the control solution (containing all reagents except the test sample).

2.9.2. Hydrogen peroxide scavenging activity

Scavenging activity of hydrogen peroxide by the plant extract was determined by the method of Bozin *et al*.[24]. Gallic acid was used as standard, percentage inhibition of extracts and standard compounds were calculated using the equation 1.

2.9.3. Superoxide anion-scavenging activity

The superoxide anion radical scavenging activity of extracts and BHA was performed according to a NTB reduction method[25]. In this assay, non enzymatic phenazine methosulfate − nicotinamide adenine dinucleotide (PMS/ NADH) system generates superoxide radicals, which reduce nitro blue tetrazolium (NBT) to a purple formazan. The scavenging activity was calculated by the equation (1).

2.9.4. Hydroxyl radical-scavenging activity

The effect of extracts on hydroxyl radicals was assayed by using the colorimetric deoxyribose method[26]. In this method, 2-deoxyribose is degraded on exposure to hydroxyl radicals generated from the Fe^{3+}/ascorbic acid/ EDTA/ H_2O_2 system, and degraded to malondialdehyde (MDA). Percentage scavenging activity of extracts and standard quercetin was measured to find IC50 value.

2.9.5. Nitric oxide radical scavenging assay

The method of Yen *et al*. [27] was adopted to determine the nitric oxide radical scavenging activity of extracts of *P. acidus*. Briefly, 4.0 mL extract solution at different concentrations mixed with 1.0 mL of 25 mM sodium nitroprusside solution and incubated at 37 ℃ for 2 h. Two millilitre of incubated solution was mixed with 1.2 mL Griess reagent and absorbance was measured at 570 nm. The experiment was performed (in triplicate) and percentage scavenging activity was calculated using the Equation (1).

2.9.6. Ferric thiocyanate method

The antioxidant activity of extracts and standard α-tocopherol was determined according to the ferric thiocyanate method[28]. All data on total antioxidant activity are the average of triplicate experiments. The inhibition percentage of lipid peroxidation in linoleic acid emulsion was determined by equation (1).

2.9.7. Reducing power ability

Reducing power was measured as per the method performed by Pal *et al*. [29]. The reducing capacity of ascorbic acid (25-400 μg/mL) was also determined. The test was run in triplicate and averaged. Increased absorbance indicated increased reducing power of extracts.

2.9.8. Ferrous ions (Fe2+) chelating activity

The extracts and chelating standard α-tocopherol were assessed for their ability to compete with ferrozine for iron (II) ions in free solution. The chelating ability of ferrous ions by the *P. acidus* leaves extracts was estimated by the method of Gulcin[30]. The inhibition percentage of ferrozine-Fe^{2+} complex formation was estimated by using the equation (1).

2.9.9. Lipid peroxidation assay

Inhibition of lipid peroxidation was assayed by using brain tissue of mice according to the method described by Tai *et al*.[31]. The formation of MDA, a cytotoxic product of lipid peroxidation reaction is widely used to remark the oxidation, which was measured at 532 nm spectrophotometrically.

2.10. Statistical analysis

Data are given as mean±SEM (for invivo experiments n =6, for invitro experiment *n* =3), statistical comparisons were made using one way ANOVA followed by Turkey Multiple

range test. $P < 0.05$ was considered as significant. For in vitro antioxidant experiments the concentration of the extract needed to produce a 50% effect (IC50) was calculated graphically.

3. Results

3.1. Analgesic activity of P. acidus leaves

Methanol, ethyl acetate and petroleum ether extract at a dose of 500 mg/kg demonstrated a significant analgesic effect against acetic acid induced writhing, inhibiting pain by 85.12%, 59.99%, and 26.81% as compared to the control respectively (Table 1). Indomethacin at 5 mg/kg had 83.84% ($p<0.001$) inhibition of writhing response. Lower dose of petroleum ether extract did not produced significant inhibition of pain response.

After a latency period of 0.5 h following oral administration of the extracts at a dose of 250 and 500 mg/kg, reduction of painful sensation was observed against tail immersion test and the effect was dose dependent. The significant inhibition was of painful reaction was observed 1 h after drug administration. The analgesic effects of the extracts became pronounced between 1 and 3 h post-dosing and but activity decreased after 5 h. Higher dose of methanol extract had similar activity to that of morphine between 1-3 h. Standard drug morphine produced significant activity up to 5th h after drug administration (Table 1). Results represent significant activity of extracts though the duration of analgesic activity was less than the standard.

In first phase of formalin induced pain model, methanol, ethyl acetate and petroleum ether at 500 mg/kg produced 69.28, 60.33, 47.28% inhibition of pain response, while at second phase the inhibition was 67.10, 62.89, 53.49% respectively. We found that *P. acidus* produced antinociceptive activity both in the early and late phases of formalin test (Figure 1). Results showed that methanol extract at 500 mg/kg produced better effect, therefore extract exert its analgesic effect through both peripheral and central mechanism.

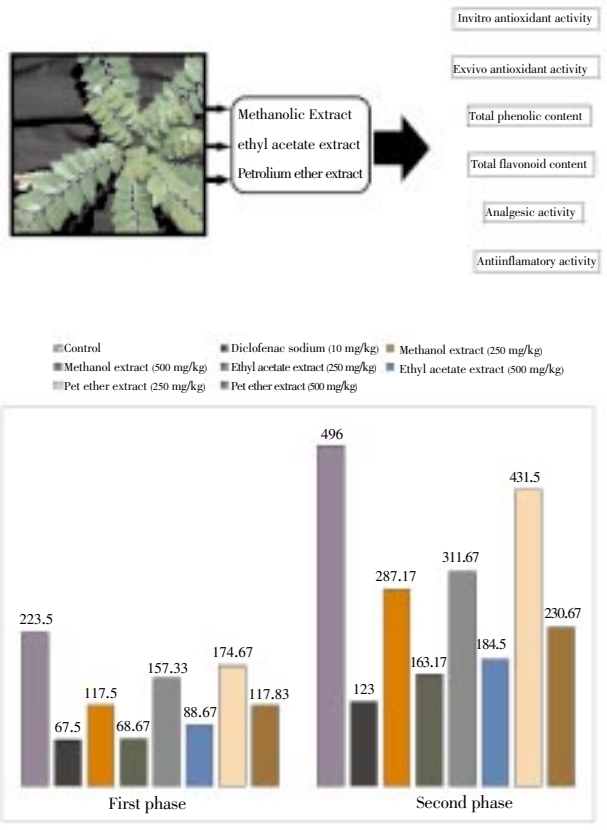

Figure 1. Effects of extracts from P acidus leaves and diclofenac sodium (10 mg/kg) on the formalin-induced licking response in mice. Values are mean±S.E.M. (n=6). *P<0.05, **P<0.01, ***P<0.001 significantly different from control group (ANOVA followed by Tukey's test).

3.2. Anti-inflammatory activity leaves of P. acidus

In the present study, an attempt has been made to evaluate the anti-inflammatory activity of *P. acidus* leaves by using carrageenan induced paw oedema, cotton pellet granuloma

Table 1
Effects of the leaves extracts of P. acidus on acetic acid-induced writhing response and tail immersion test in mice.

Treatment	Dose (mg/ kg)	Writhing test		Tail immersion method Latency period (s)					
		Number of writhing0.5% CMC	Percentage inhibition (%)	0 min	30 min	1 h	2h	3h	5h
Control		39.17 ±2.82	–	1.67±0.33	2.17±0.31	1.5±0.34	1.67±0.21	2.00±0.26	1.67±0.33
Indomethacin	5	6.33 ±1.65***	83.84	–	–	–	–	–	–
Morphine	10	–	–	2.17±0.31	5.00 ±0.77***	10.33±0.61***	10.67 ±.67***	10.83±0.83***	10.33 ±.56***
Methanol extract	250	24.33 ±.69***	37.88	1.67 ±0.33	2.00±0.37	5.67±0.56*	5.17 ±0.75*	3.50±0.62	2.83±0.31
	500	5.83 ±1.30***	85.12	1.83 ±0.31	2.67 ±0.33	9.33 ±1.09***	10.33 ±.84***	10.33±.95***	3.67 ±0.56
Ethyl acetate extract	250	29. 67±1.84**	24.25	1.50 ±0.22	2.17 ±0.31	3.17 ±0.40	6.33 ±0.80**	3.67±0.84	2.83 ±0.31
	500	15.67±.20***	60.00	2.00 ±0.37	3.00 ±0.26	8.17 ±1.45***	9.00 ±0.63***	7.00±1.03**	3.50 ±0.42
Petroleum ether extract	250	37.17±1.08	5.11	1.50 ±0.22	2.17 ±0.48	4 .50 ±0.34	4.33 ±0.42	4.33±0.67	1.50 ±0.22
	500	28.67±0.88**	26.81	2.17 ±0.40	3.17 ±0.31	6.33 ±0.49**	5.83 ±0.65*	5.83 ±0.83*	3.33 ±0.49

Values are mean±S.E.M. (n=6).
*P<0.05, **P<0.01, ***P<0.001 significantly different from control group (ANOVA followed by Tukey's test).

Table 2
Effects of the leaves extracts of P. acidus on carrageenin-induced rat paw edema and cotton pellet-induced granuloma in rats.

Treatment	Dose (mg/kg)	Carrageenin-induced rat paw edema						Cotton pellet-induced granuloma			
		Paw edema after carrageenin injection at h (cm)						Weight of granuloma (mg)		Inhibitory rate (%)	
		0	1	2	3	4	5	Wet	Dry	Wet	Dry
Control	–	0.254±0.003	0.375±0.008***	0.443±0.003***	0.488±0.003***	0.529±0.006***	0.452±0.003***	413.2±5.08***	78.5±3.02	–	–
Indomethacin	5	0.258±0.002	0.284±0.003***	0.298±0.005***	0.308±0.004***	0.286±0.006***	0.265±0.002***	267.7±6.22***	58.2±2.93***	35.21	25.86
Methanol extract	250	0.257±0.003	0.303±0.004***	0.323±0.003***	0.335±0.002***	0.315±0.001***	0.306±0.003***	329.7±4.33*	67.4±3.06**	20.21	14.14
	500	0.254±0.003	0.289±0.005***	0.304±0.004***	0.320±0.003***	0.299±0.002***	0.272±0.002***	271.8±4.89***	61.2±2.77***	34.22	22.04
Ethyl acetate extract	250	0.257±0.001	0.306±0.003***	0.324±0.004***	0.334±0.003***	0.319±0.003***	0.316±0.003***	366.7±6.13***	71.9±2.50	11.25	8.41
	500	0.255±0.003	0.287±0.003***	0.306±0.004***	0.321±0.003***	0.303±0.004***	0.301±0.004***	302.5±3.80**	68.7±2.89*	26.79	12.48
Petroleum ether extract	250	0.248±0.002	0.291±0.002***	0.320±0.003***	0.337±0.002***	0.318±0.006***	0.315±0.005***	384.8±4.12***	75.4±3.21	6.87	3.95
	500	0.251±0.001	0.289±0.005***	0.310±0.005***	0.320±0.004***	0.303±0.003***	0.299±0.003	340.4±5.03***	70.2±3.33*	17.62	10.57

Values are mean±S.E.M. (n=6).
*P<0.05, **P<0.01, ***P<0.001 significantly different from control group (ANOVA followed by Tukey's test).

model. The results are presented in Table 2. All the extracts showed reduction in carrageenan-induced paw oedema in dose dependent manner. Highest activity was produced at 5th h. Methanol extract (500 mg/kg) produced highest activity. After 5th h percentage inhibition by methanol extract (500 mg/kg) was 90.91 %, while indomethacin (5 mg/kg) produced 96.46 % inhibition. Extract also produced significant anti-inflammatory activity against cotton pellet induced inflammation. Lower dose of ethyl acetate and petroleum ether extract did not produce significant inhibition of granuloma formation. Inhibition rate for methanol extract (500 mg/kg) and indomethacin (5 mg/kg) were 34.22% and 35.21% for wet weight and 22.04% and 25.86% for dry weight respectively.

To confirm the membrane s-ilizing action of *P. acidus* leaves extract heat and hypotonic solution induced haemolysis of erythrocyte membrane assay were performed. Extracts were able to protect erythrocytes against haemolysis in a dose dependent manner and the protective effect of methanol extract was better then the NSAID, aspirin (Figure 2). The percentage inhibition of methanol extract at 100 and 200 μg/ml concentration were 78.13%, 86.09%, 53.58%, 58.49% against heat and hypotonic solution induced haemolysis respectively.

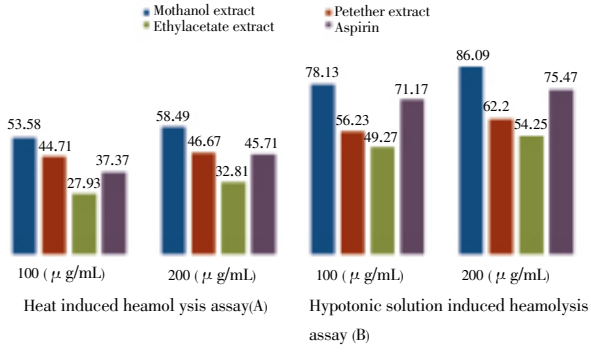

Figure 2. Membrane stabilizing activity of P acidus leaves extracts. (A) Heat induced haemolysis assay, (B) Hypotonic solution induced haemolysis assay. Values are mean±S.E.M. (n=6). *P<0.05, **P<0.01, ***P<0.001 significantly different from control group (ANOVA followed by Tukey's test).

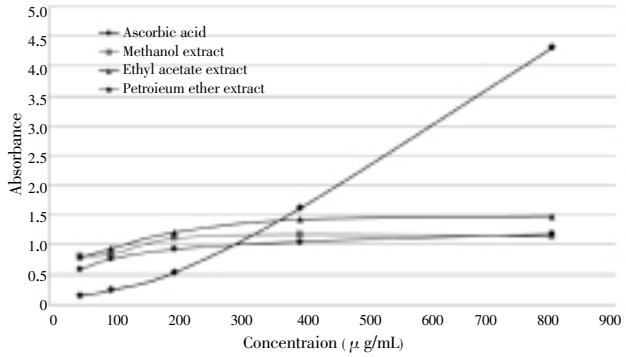

Figure 3: Reducing power ability of different leaves extracts from the P acidus at different concentrations. Results are of triplicate measurements.

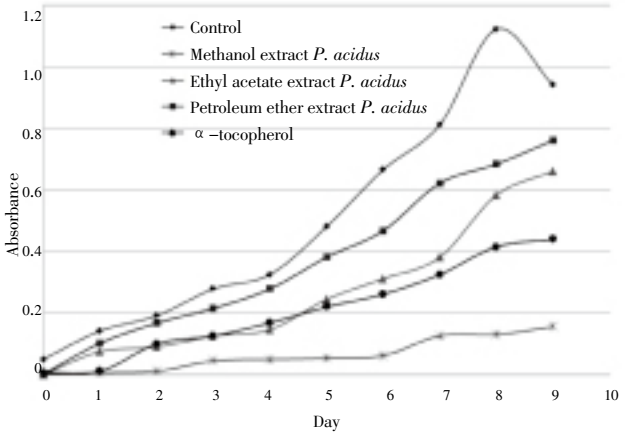

Figure 4. Antioxidant activity of P acidus leaves extracts by the FTC method. Results are of triplicate measurements.

3.3. Polyphenolic content of extracts of P. acidus leaves

The quantity of total phenolic content present in different extracts of *P. acidus* leaves was determined from gallic acid calibration curve ($Y = 0.0044x + 0.031$, $R^2 = 0.9995$), while calibration curve of quercetin, with the regression equation $Y = 0.0288x + 0.0058$, $R^2 = 0.9991$ was used to estimate total flavonoid content. Quantity of total phenolics and total flavonoids content among different extracts were

significantly varies (Table 3). The phenolic and flavonoid content in methanol extract were 73.08±0.682 GAE mg/g of dry material and 61.28±0.062 QE mg/g of dry material, which was highest compare to other extracts.

3.4. Free radical scavenging and antioxidant activity

Free radical scavenging and antioxidant activity of the extracts were evaluated by different invitro and exvivo model.

P. acidus leaves extracts possess a concentration-response relationship in DPPH radical scavenging activity. The ethyl acetate extract had highest DPPH scavenging effect compare to other extracts, with an IC_{50} value of 28.6±0.72 μg/mL. Standard antioxidant ascorbic acid showed high scavenging activity (IC_{50}=3.3±0.01 μg/mL). The IC_{50} value of methanol and petroleum ether extract was 86.0±1.03 and 117.4±1.33 μg/mL respectively (Table 4).

P. acidus leaves extracts are capable to inhibit the formation of blue NBT, which was the indication of superoxide anion scavenging activity of extracts at concentration of 20-160 μg/mL. The IC50 value of methanol, ethyl acetate and petroleum ether extracts was found 21.7±0.09, 71.8±0.39, 31.7±0.11 μg/mL, and BHA (standard drug) showed IC_{50} value 23.8±0.89 (Table 4).

The degradation of deoxyribose by Fe+3-ascorbic acid-EDTA-H2O2 system was significantly (P<0.01) decreased by all extracts of *P. acidus* tested in hydroxyl radical scavenging assay model. IC50 value was higher for methanol extract (17.2±0.13 μg/mL) than the other two extracts and standard drug (Table 4). At 80 μg/mL concentration methanol extract produced 86.34% scavenging activity for hydroxyl radical, while at same concentration quercetin produced 90.13% scavenging effect.

P. acidus leaves extracts significantly decreased the generation of NO radical in the assay system. Methanol extract exhibited superior NO scavenging activity with an IC50 of 13.0±0.06 μg/mL than the ethyl acetate (IC_{50}=49.8±0.19 μg/mL), petroleum ether extract (IC_{50}=100.0±0.28 μg/mL) and standard drug ascorbic acid (IC_{50}=23.0±0.08 μg/mL) (Table 4).

The ability of extracts and positive control gallic acid to scavenge hydrogen peroxide is shown in Table 4. All extracts produced a dose-dependent hydrogen peroxide scavenging activity (IC_{50} was 230.0±3.03 μg/mL for methanol extract, 333.8±3.90 μg/mL for ethyl acetate extract and 297.6±3.32 μg/mL for petroleum ether extract), though IC_{50} value of gallic acid was 65.0±1.32 μg/mL. Moderate activity was exhibited by all extract when compared to gallic acid.

In reductive ability method, the extracts cause the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Figure 1 shows the dose-response curves for the reducing powers of the leaf extracts from *P. acidus*. The sequence for reducing power was ascorbic acid > ethyl acetate extract > methanol extract > petroleum ether extract. The reducing power of methanol, ethyl acetate and petroleum ether extracts increased from 0.804±0.003, 0.785±0.005 and 0.588±0.007 respectively at 50 μg/mL to 1.383±0.006, 1.479±0.006 and 1.180±0.007 at 800 μg/mL respectively. Result suggests that extracts had high to moderate levels of ferrous ion chelating activity in concentration dependent manner. The sequence for chelating power was α-tocopherol > methanol extract > petroleum ether extract > ethyl acetate extract. The IC_{50} value of iron chelating activity for the methanol, ethyl acetate, petroleum ether extract and α-tocopherol was 121.7±1.39, 178.3±2.01, 159.7±1.98 and 107.2±1.23 μg/mL (Table 4).

The total antioxidant activity of the extracts was determined by the FTC method. The effects of various solvent extracts of *P. acidus* leaves in preventing the linoleic acid peroxidation are shown in Figure 2. On 9th day, the formation of peroxides was stopped because of non-availability of linoleic acid. But at the presence of extracts the oxidation of linoleic acid was slow, which clearly showed that all extracts exhibited significant (P<0.05) antioxidant activity. Methanol extract produced better activity than standard, while ethyl acetate

Table 3

Total phenolic and total flavonoid content in P. acidus leaves extracts

Extract	Total phenolic content±SEM (SD)(GAE mg/g of dry material)	Total phenolic content±SEM (SD)(QE mg/g of dry material)
Methanol	73.08±0.682 (1.181)	61.28±0.062 (0.107)
Ethyl acetate	45.87±0.789 (1.366)	37.13±0.091 (0.158)
Petroleum ether	25.25±0.577 (1.000)	26.04±0.082 (0.142)

Values are expressed as the mean±SEM, n =3. GAE, gallic acid equivalent; QE, quercetin equivalent.

Table 4

Antioxidant activities of the extracts of P. acidus leaves by using different in vitro models

Screening method	Positive control	IC_{50} value			
		Methanol extract	Ethyl acetate extract	Pet ether extract	Standard
DPPH Scavenging activity	Ascorbic acid	86.0±1.03	28.6±0.72	117.4±1.33	3.30±0.01
Superoxide radical scavenging activity	BHA	21.7±0.09	71.8±0.39	31.7±0.011	23.8±0.89
Nitric oxide radical scavenging activity	Ascorbic acid	13.0±0.06	49.8±0.19	100±0.28	23.0±0.08
Metal chelating activity	α-tocopherol	121.7±1.39	178.3±2.01	159.7±1.98	107.0±1.23
Lipid peroxidation assay	Rutin	58.9±0.77	115.8±1.01	138.5±1.31	75.9±0.92
Hydrogen peroxide scavenging activity	Gallic acid	230.1±3.03	333.8±3.90	297.6±3.32	65.0±1.32
Hydroxyl radical	Quercetin	17.2± 0.13	30.3 ± 0.22	55.3 ± 0.26	20.8 ± 0.78

Values are expressed as the mean±SEM, n =3 with P < 0.01 compared to positive control group.

and petroleum ether extract showed moderate antioxidant activity.

Malondialdehyde (MDA), a cytotoxic product generated during lipid peroxidation and used to observed the oxidation. Table 4 showed that methanol extract possess better inhibition of MDA formation in liver tissue homogenates than other extracts and rutin. IC50 value of rutin, methanol, ethyl acetate and petroleum ether extract was 75.9±0.92, 58.9 ±0.77, 115.8±1.01, and 138.5±1.31 μg/mL respectively. These data suggested that the crude extracts of this plant leaf significantly ($P < 0.005$) inhibited the formation of MDA in liver tissue.

4. Discussion

Tail immersion method was selected to investigate central analgesic activity. The drugs acting against tail immersion induced pain attributed their actions through mu (μ) opioid receptors rather than kappa (κ) and delta (δ) receptors[16]. Acetic acid induced writhing response was selected to find peripheral analgesic effect of extract [15, 32]. Acetic acid responsible for increase in the peritoneal fluids of PGE2 and PGF2α, serotonin, and histamine[15]. Acetic acid induced writhing test presents a good sensitivity but poor specificity. Therefore to get specific result and to avoid misinterpretation of the results, the formalin test was carried out which has two distinctive phases that can possibly indicate different types of pain. The early phase reflects centrally mediated pain, which was a result of direct stimulation of nociceptors; the late phase pain is caused by local inflammation with a release of inflammatory and hyperalgesic mediators. Therefore this model is useful not only to screen the analgesic substances, but also for elucidating the mechanism of analgesia[32,33]. Results showed that methanol extract produced better activity and the effect of extract may medicated through both peripheral and central mechanism.

The carrageenan induced paw oedema is frequently used as an experimental model of acute inflammation, while cotton pellet granuloma model was used to measure granuloma formation in the proliferative phase or in chronic inflammation[19,34]. The carrageenan induced paw oedema shown biphasic response; first phase is mediated by release of histamine and serotonin while the second or delayed phase is related to neutrophil infiltration and release of other neutrophil derived mediators, eicosanoid release, and production of free radicals [18, 35]. The release of kinin like substances (e.g. bradykinin) involved in oedema produced in between early and late phase, which later induces the biosynthesis of prostaglandin and other autacoids, which are responsible for formation of the inflammatory exudates [18, 36]. Extracts specifically methanol extracts found effective in both models and results were almost similar to that of standard. Therefore chemical constituents present in extracts might be effective against both acute and chronic inflammation.

A protective effect on heat and hypotonic solution induced erythrocyte lysis is considered as a biochemical index of anti-inflammatory activity[18,20]. Since there is a close resemblance of the RBC membrane system to the lysosomal membrane system, defence against hypotonicity or heat induced RBC lysis is often seen as an indication of stabilization of lysosomal membranes, and used as a index of anti-inflammatory activity[18,37]. Further, plants with membrane stabilizing properties can interfere with the early phase of inflammatory mediator release, specifically by mediating the release of phospholipase A2 that stimulates the inflammatory mediator release[18,39]. Therefore, the membrane stabilizing effect mediated by *P. acidus* leaves extract may contribute to the anti-inflammatory activity of extracts.

The DPPH radical scavenging assay is based on the decolourization of DPPH by the antioxidants compound present in extracts[40]. Therefore, DPPH radical scavenging effect of extract might be attributed to a direct role in trapping free radicals by donating hydrogen atom. Superoxide anion radical is considered as a one of the strongest reactive oxygen species among the free radicals, responsible for generation of active reactive species like hydrogen peroxide and singlet oxygen. These radicals can liberate highly reactive hydroxyl radical through Fenton-chemistry, thereby initiate lipid peroxidation[21,30]. Hydroxyl radical is the most reactive radical responsible for enormous biological toxicity and cell damage through lipid peroxidation [41]. NO has been associated with a number of physiological processes in the human and plays an important role in respiratory, immune, neuromuscular functions. NO act as an atypical neural modulator which is involved in neurotransmitter release, neuronal excitability and learning and memory. It also participates in diverse pathological condition including muscle diseases, inflammatory bowel disease, sepsis and septic shock, primary headaches, HIV-associated dementia, multiple sclerosis and stroke[42]. Strong scavenging of superoxide, hydroxyl and nitric oxide radical will be helpful in protecting free radical induced diseases.

Hydrogen peroxide can be formed in vivo by different enzymes like superoxide dismutase. Inactivation of deferent enzymes usually by oxidation of essential thiol groups was caused by H_2O_2. However, H_2O_2 can be highly toxic in presence of Fe2+ or Cu^{2+}, and can initiate hydroxyl radical generation through Fenton reaction[25,41,43,44]. Extract significantly inhibit generation of hydrogen peroxide. The reducing power ability of a compound may afford key sign about the antioxidant ability of the compound. Reducing power abilty of compound be linked with the presence of reductones[25,28,45]. Therefore, in this study, the antioxidant activity of the extracts may be related to its reductive activity.

The ferrous state of iron is highly reactive and can induce lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals via the Fenton reaction very quickly[45,46]. Ferrozine can quantitatively form complexes with ferrous state of ion, but chelating agents

can inhibit this complex formation[25,41,47]. All extracts and standard antioxidant α-tocopherol interfered with ferrous and ferrozine complex the formation effectively. FTC method was used to evaluate the ability of extrcts/compound to scavenge peroxyl radicals during polyunsaturated fatty acids (PUFA) oxidation was measured. A red-colored complex which has a maximum absorbance at 500 nm was formed during the reaction between ferric ion and thiocyanate[28,48]. Extracts showed significant total antioxidant capacity tested against FTC method.

Lipids such as free and ester forms of polyunsaturated fatty acids and cholesterol are more vulnerable to the attack of reactive species. Lipid peroxidation can induce disruption membrane transport proteins and deactivation of membrane-associated enzymes, generates potentially toxic products, damage to genomic and mtDNA, and ultimately may lead to unstable cytological conditions such as apoptosis or tumour generation[31,49]. Results showed that extract have potential to inhibit lipid peroxidation significantly, implying their beneficial effect against cell membrane lipid oxidation.

Polyphenolic compounds like phenolic and flavonoid component are important for their diverse pharmacological action including antioxidant, antimutagenic and in other diseases caused by oxidative stress. Hydroxyl groups present in the phenolic compounds are important because of their scavenging ability[39,50]. Result indicates that polyphenolic component present may responsible for its activity.

5. Conclusion

The present study demonstrated that methanol extract obtained from the leaves of *Phyllanthus acidus* presented the potential antioxidant, anti-inflammatory and anti-nociceptive activities, which are comparable with the reference drugs. This might be correlated with the presence of phenolic constituents and flavonoids in the extract. Due to the remarkable analgesic, antiinflammatory and antioxidant activity of the plant, further studies are in progress in our laboratory for the isolation and identification of the bioactive components.

Conflict of interest statement

We declare that we have no conflict of interest.

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