JOURNAL OF NATURAL REMEDIES
DOI: 10.18311/jnr/2025/47754
RESEARCH ARTICLE

Anti-oxidant and Anticoagulant Effects of *Phyllanthus acidus* Leaves Extracts: An *In Vitro* and *In Vivo* Study

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Abstract

Background: *Phyllanthus acidus* is used traditionally in various diseases, analgesics, inflammation, rheumatism, bronchitis, asthma, respiratory disorders, hepatic diseases and diabetes in Bangladesh, South India and Southeast, Asian countries. The plant is a good source of antioxidants. **Aim:** This study aims to investigate the *in vitro* and *in vivo* anticoagulant activities of an aqueous extract (Aq.) and methanolic extract (MeOH) derived from *P. acidus* leaves. **Method:** The Ultrasonic-Assisted Extraction (UAE) method was used to extract various solvents. ether (PE), chloroform (Chlo.), methanol (MeOH), and aqueous (Aq.), phytochemical testing were done. All the extracts were processed for the antioxidant test (DPPH and FRAP), Total phenolic content, activated partial thromboplastin time, prothrombin time and clot lysis activity. The MeOH and Aq. fractions of *P. acidus* were used to assess *in-vivo* anticoagulant activity using the clotting time and tail amputation model. **Results:** All the extract shows the presence of different phytoconstituents. The Total Phenolic Content (TPC) was found high in MeOH (2.125 GAE mg/gm) and Aq. (1.870 GAE mg/gm). Clotlysis activity of PE, Chlo., MeOH, and Aq. were respectively found (300 (mg/ml)-49.05%,43.13%,53.84%, and 66.00%). MeOH and Aq. exhibited significant results in Activated Partial Thromboplastin Time (APTT), Prothrombin Time (PT) and *in-vivo* studies. **Conclusion:** The species *P. acidus (*PA) has been historically utilised for various pathological conditions, possessing substantial traditional significance. The polyphenolic compounds and proteases within the plant influence the coagulation mechanism, as evidenced by the present study, which evaluated both intrinsic and extrinsic coagulation pathways, yielding statistically significant outcomes in both assessments.

Major Findings: Significant antioxidant and anticoagulant qualities are displayed by plant extracts, which suggests that this plant may find use in the treatment of cardiovascular diseases.

Keywords: Anticoagulant, Antioxidant, Activated Partial Thromboplastin Time (APTT), Clotting Time, *Phyllanthus acidus*, Prothrombin Time (PT), Tail Amputation

1. Introduction

The predominant contributor to mortality on a global scale is thrombotic pathologies[1]. Hemostasis refers to the physiological condition wherein blood circulation is preserved and haemorrhage is prevented[2]. The mechanism through which a thrombus is formed is termed Hemostasis; however, it remains perplexing why there is an increasing trend among the populace towards the consumption of anticoagulant agents[3]. In contemporary times, thrombotic disorders, antiphospholipid syndrome[4], atherosclerosis[5], cardio vascular diseases[6] and Disseminated Intravascular Coagulation (DIC) are notably widespread[7]. Substantial quantities of anticoagulants are administered in each of these scenarios; nevertheless, there has been a notable shift towards holistic remedies, as patients encounter challenges associated with synthetic anticoagulant therapies[8]. Research indicates that traditional medicinal practices are the principal therapeutic approach employed by 80% of individuals residing in developing nations for various ailments[9]. Botanicals, specifically

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Article Received on: 11.12.2024 Revised on: 05.02.2025 Accepted on: 21.02.2025

lactiferous species from diverse taxonomic families, have been extensively studied. In circumstances that initiate coagulation, both extrinsic and intrinsic factors are involved, particularly when specific constituents influence blood clotting[10].

An additional concern pertains to the adverse effects associated with anticoagulant pharmacotherapy, which include haemorrhagic manifestations, excessive bleeding (particularly in women during menstrual cycles), and, frequently, life-threatening allergic responses[7]. Nonetheless, a paradigm shift may occur as a greater number of individuals begin to utilise natural remedies. A substantial number of nations have embraced the traditional paradigm of herbal medicine[11,12], with numerous studies focusing on botanical species exhibiting properties such as antioxidants[13], phenolic compounds, polyphenols, and proteolytic enzymes, which are implicated in the anticoagulant effect[14,15]. Although coagulation can arise from a myriad of circumstances and factors, only two elements—intrinsic and extrinsic—are responsible for thrombus formation. The PT assesses prothrombin, fibrinogen, factors V, VII, X, and additional vitamin K-dependent components of the extrinsic coagulation pathway. Conversely, activated partial thromboplastin time APTT evaluates all clotting factors and components of the intrinsic pathway, except factors VII and XIII[16,17].

Cicca distica also known as *P. acidus*, commonly referred to as gooseberry, belongs to the Euphorbiaceae family (Figure 1). This botanical has historically been employed for myriad applications, encompassing analgesic, antipyretic, and anti-rheumatic effects[18]. Documented cases of smallpox and jaundice illustrate its therapeutic efficacy. Certain indigenous groups utilise the plant's stick for dental hygiene, alleviating gum inflammation, and purifying their blood[19]. The leaf extract of this plant is utilised for the treatment of hepatic disorders. Furthermore, methanolic extracts of the plant's leaves exhibit potential neurogenic effects relevant to Alzheimer's disease[20]. The fruit of the plant is rich in potent bioactive compounds. Among the 77 distinct chemical constituents identified within the fruit are phenols, acids, terpenes, alcohols and esters[21].

2. Materials and Methods

2.1 Chemicals

L-Ascorbic Acid, Gallic Acid, Folin and Ciocalteu's Phenol Reagent, DPPH, Methanol, Petroleum Ether, Chloroform, and various other phytochemical reagents were procured from Research-Lab Fine Chem Industries, located in Mumbai.

2.2 Plant Authentication

Mature leaves of *P. acidus* were harvested during December from the botanical garden of Shree Dhanvantary Pharmacy College, situated in Kim, Surat, Gujarat. The collected specimens were subsequently submitted to the botany department of Veer Narmad South Gujarat University (VNSGU), Surat, for herbarium documentation (voucher no.-VNSGU/BVBRC/2024/08/TC-54).



Figure 1. *Phyllanthus acidus* family *Euphorbiaceae* - **A.** whole plant, **B.** Arial portion of the plant.

2.3 Animals

In the context of this study, female Wistar rats with a body weight ranging from 150 to 200 grams were employed. Before the commencement of the experimental procedures, the animals were acclimatised in standard wire-meshed plastic cages for a duration of four to five days at ambient temperature (28 ± 5 degrees Celsius) and relative humidity (55 ± 5%). Throughout the experimental period, the rats had continuous access to water and were fed a standard pellet diet. The handling and management of the animals adhered strictly to the guidelines established by the Institutional Animal Ethics Committee. The animals were sourced from the Institutional Animal Ethics Committee at Dr APJ Abdul Kalam University, located in Indore, M.P., India (AKU/IAEC/2023/09).

2.4 Extraction

The aerial parts and mature leaves of the plant were excised, thoroughly washed, and subjected to a comprehensive drying process in a designated shed. The desiccated leaves were subsequently comminuted utilising a mechanical mixer to facilitate the extraction process. The crude powdered material underwent extraction via the Ultrasonic Assisted Extraction (UAE) technique (Figure 2) as described by Kumar K. *et al.*[22]. The extraction of the crude material was accomplished by immersing it in various solvents, namely pet ether, chloroform, methanol, and aqueous solutions, for a continuous duration of 36 hours, during which it was intermittently subjected to sonication cycles of 15 minutes each[23]. The resultant filtrate was then dried and the resulting semi-solid extracts were stored in a sealed container at ambient temperature until further use, as referenced by Lesellier[24] and Liu *et al.*[25].

2.5 Phytochemical Screening

All extracts were subjected to preliminary phytochemical screening assays to ascertain the presence of alkaloids, glycosides, flavonoids, tannins, steroids, phenolic compounds, and saponins. These assessments were executed by the methodologies delineated by Junaid and Patil[26] and Madike *et al*[27].

2.6 Total Phenolic Content (TPC)

The Folin-Ciocalteau method was utilised to quantify the TPC. Gallic acid at varying concentrations acted as the standard reference, while 0.5 ml of different dilutions of plant extracts (0.1, 1, 2, 5, and 10 µg/µl) were employed. Subsequently, 2.5ml of 10% Folin-Ciocalteau reagent and 2ml of 7% sodium carbonate solution were incorporated. Following a saturation period of 30 minutes, the absorbance was recorded at 760nm. The concentration of gallic acid was employed to determine the total phenolic content expressed in mg/g of extract[28-30].

2.7 Antioxidant Activity

2.7.1 DPPH

A volume of 1 ml of 1-diphenyl-2-picryl hydroxyl (DPPH - 0.1mM in ethanol) was combined with 3 ml of plant extract at various concentrations, followed by

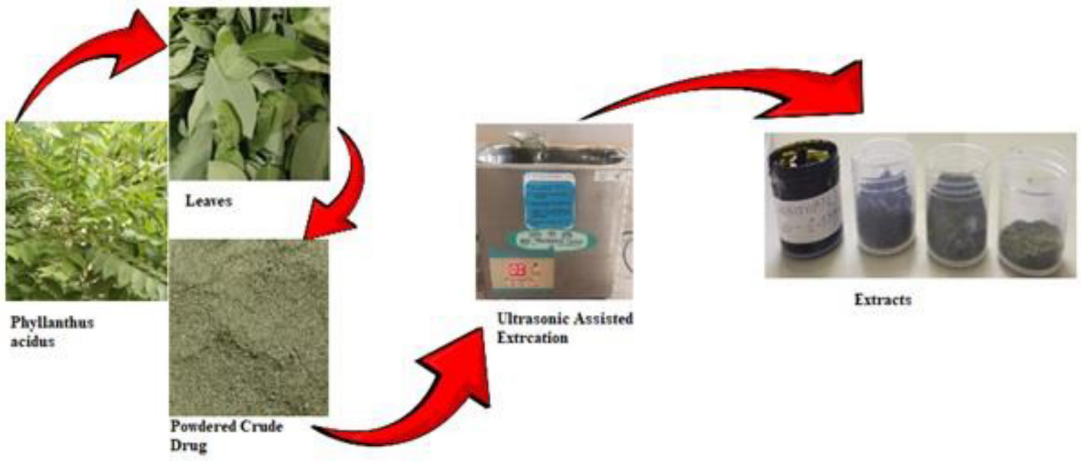


Figure 2. Ultrasonic-assisted extraction process of the *Phyllanthus acidus* leaves.

incubation for 30 minutes in a dark environment at a temperature of 25°C. The resultant reaction mixture was analysed utilising a spectrophotometer at a wavelength of 517nm[10].

$$DPPH\ scavenging\ (\%) = \frac{\left(Absorbance\ of\ Control - Absorbance\ of\ Test\ Sample\right)}{Absorbance\ of\ Control} \times 100$$

2.7.2 Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay employed potassium ferricyanide as a reactive agent[31]. Specifically, 2.5 millilitres of plant extract, 2.5 millilitres of phosphate buffer (0.2 M, pH = 6.6), and 2.5 millilitres of 1% potassium ferricyanide were amalgamated. The mixture was incubated for twenty minutes at a temperature of 50°C. Upon the addition of 2.5ml of 10% trichloroacetic acid, the reaction mixture was centrifuged for 10 minutes at 3000 rpm. Finally, 0.5ml of ferric chloride (FeCl3, 0.1%) was combined with 2.5 ml of distilled water and 2.5ml of the supernatant. The absorbance was subsequently assessed at a wavelength of 700nm[32]. The results were expressed as Trolox Equivalent Antioxidant Capacity (TEAC) in mM/L Trolox. The TEAC was calculated by constructing a Trolox standard curve for the FRAP assay (standard curve equation: y = 0.0007x + 0.0645, R^2 = 0.9998) ranging from 31.25 µg/mL to 1.0 mg/mL of standard Trolox solution. Each experiment was performed in triplicate.

2.8 Clot-lysis Activity (Clot Dissolving)

Proteolytic enzymes facilitate clot lysis, with lactiferous plants exhibiting elevated concentrations of proteolytic enzymes. To induce clot formation, fresh blood was extracted from healthy rats and introduced into Eppendorf tubes, which were then allowed to remain undisturbed for a duration of 90 minutes[33,34] (Figures 3 A, B and C). After 90 minutes, the serum was removed, and the initial weight of the clot was documented. Subsequently, the extract concentrations were combined in the following ratios: 75, 100, 250, and 300 µg/µl with a clot that was allowed to remain undisturbed for an additional 150 minutes. Use the below-mentioned formula to determine the percentage of the lysed clot[20,30].

$$\%\ of\ Clot-lysis = \frac{Weight\ of\ Clot\ (after\ lysis)}{Weight\ of\ initial\ Clot} \times 100$$

2.9 *In Vitro* Anticoagulant Study

For the *in vitro*, coagulation test PT and APTT were measured at different concentrations of plant extract. Blood was collected from the retro-orbital sinus in a citrated tube (contains 9:1, blood: sodium citrate). Collected blood was subjected to the separation of plasma and 50µl of plasma was separately taken for APTT and PT tests[35]. Different graded concentrations of plant extract were taken (25 µl) with separated plasma volume. For the APTT test, APTT reagent (50µl) were mixed and incubated for 5 minutes at 37°C, then clotting was induced by the addition of $CaCl_2$ (50µl, 0.025 µmol/µl), incubation for 10 min at 37°C, clotting time was recorded. For the PT test, Different graded concentrations of plant extract were taken (25 µl) with separated plasma volume. Then PT reagent (100µl) was mixed and incubated for 10 minutes at 37°C, and then clotting was induced[36].

2.10 *In Vivo* Anticoagulant Activity

2.10.1 Clotting Time

The clotting time was evaluated using the capillary technique. The rats were split up into four groups and each group had pre-treatment for a string of seven days. Group 2 (treatment -1) received 100 mg/kg of the plant extract, Group 3 (treatment -2) received 200 mg/kg of the plant extract, and Group 4 (treatment -3) received 400 mg/kg of the plant extract[37]. Group 1 (control) received normal saline. The clotting time was measured one hour after the therapy on the first, third, and seventh days as long as the treatment was maintained. A lancet was used to pierce the tail tip to get blood. Up to 3/4 of the capillary's capacity was filled with blood, and every 15 seconds (Figures 3 G, H and I), a little section of the capillary split open to reveal the fibrin thread[38].

2.10.2 Tail Amputation

The tail amputation was used for the assessment of the bleeding time. The rats were divided into 4 groups. All the groups received pre-treatment for consecutive seven days. Group 1 (control)- the control group received

normal saline, Group 2 (treatment -1)- received 100mg/kg of the plant extract, Group 3 (treatment -2)- received 200mg/kg of the plant extract, Group 3 (treatment -3)- received 400mg/kg the plant extract. On the seventh day, one hour after the last treatment dose, the bleeding time was recorded (Figures 3 D, E and F). According to the Committee for Control and Supervision of Experiments on Animals (CCSEA) protocol, anaesthesia was administered to each animal, and a scalpel was used to amputate the tails (starting at 3mm in diameter)[39,40]. The damaged tail was submerged for 30 minutes at 37°C in a 15ml falcon tube filled with normal saline, and the amount of bleeding was noted[25]. Animals were sacrificed for histopathology[41].

3. Results

3.1 Extraction

The extraction was conducted utilising the Ultrasonic Assisted Extraction methodology, which resulted in a notable percentage yield for the Chloroform extract (13.4%), Petroleum ether extract (6.6%), Aqueous extract (4.56%) and Methanolic extract (4.39%), respectively.

3.2 Phytochemical Screening

The bioactive constituents belonging to various classifications were systematically assessed through a series of tests for each component, and the resultant data are documented in Table 1.

T3.3 Total Phenolic Content

The total phenolic content of the leaves of *P. acidus* was calculated by the regression equation (y= 0.0087X + 0.0645, R^2 = 0.9812) expressed in an equivalent weight of Gallic acid mg/g of plant extract, the result mentioned in Table 2.

3.4 Antioxidant Activity

The aqueous extract also exhibits satisfactory results 233.73 **µg/ml** and 250 µg/ml in both DPPH and FRAP methods respectively. The results of DPPH and FRAP assay are mentioned in Figures 4 and 5.

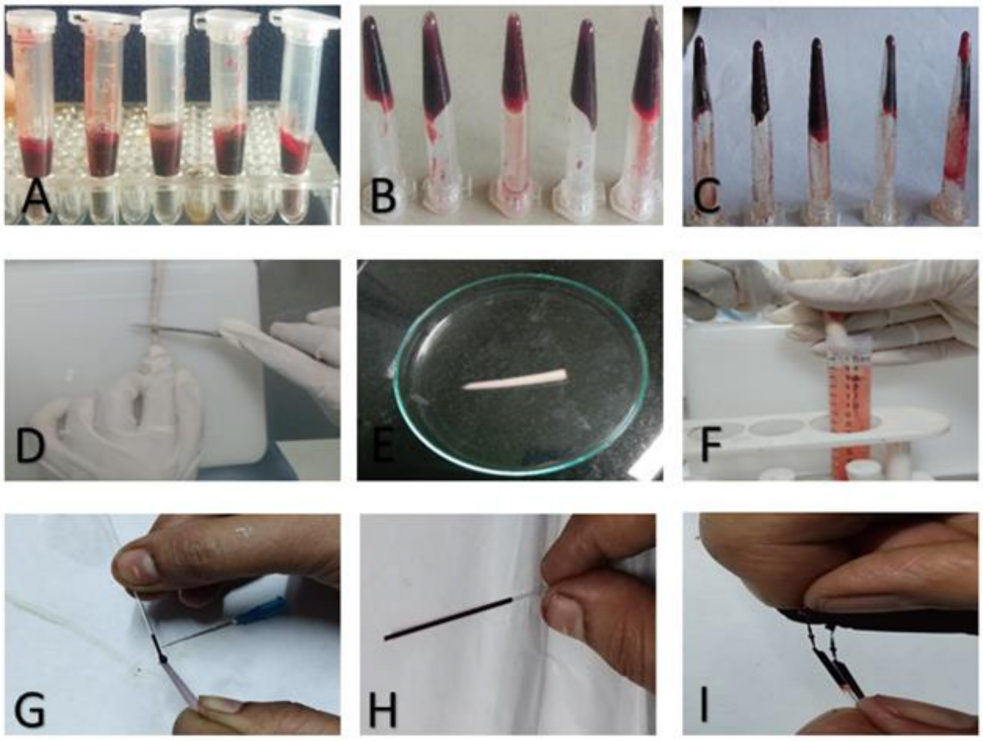


Figure 3. Images A, B, and C represent the clot-lysis activity, D, E and F represent the tail amputation and images G, H and I represent the blood clotting time. Where, A- Formation of a clot, B is the initial clot after serum separation, and C- is a lysed clot after incubation of extract for 150 min. D- amputation of the tail from 3 mm diameter, E- amputated tail, and F- bleeding from the injured tail. The clotting time recorded by capillary puncture method, Images G, H- blood is withdrawn through a capillary and I- formation of fibrin thread.

Table 1. Results of preliminary phytochemical screening

S. No.	Phytoconstituents	Test Employed	Prescence and Absence of Phytoconstituents			
			PE	Chlo.	MeOH	Aq
1	Alkaloids	Dragendroff's test	-	+	+	+
		Hager's test	+	-	-	+
		Mayer's test	+	-	+	-
		Wagner's test	-	+	+	+
		Picric acid test	+	-	+	+
2	Carbohydrates	Barfoed's test	+	+	-	-
		Molish's test	+	-	+	+
		Seliwanoff's Test	-	+	+	+
3	Reducing Sugar	Benedict's test	-	+	+	+
		Fehling's test	-	+	+	+
4	Glycosides	Borntrager's test	+	-	+	+
		Aqueous NaOH test	-	+	+	+
		Conc. H_2SO_4 test	+	+	+	+
		Keller-Killani test	-	-	+	-
5	Proteins and Amino Acid	Biuret test	-	-	+	+
		Millon's test	-	-	-	+
		Ninhydrin test	-	-	+	+
		Xanthoproteic test	-	-	+	-
6	Flavonoids	Conc. H_2SO_4 test	+	+	-	-
		Ammonia test	-	+	+	-
		Ferric Chloride test	+	+	+	-
		Lead Acetate test	+	-	+	-
7	Phenols	Iodine test	+	+	-	+
		Ferric Chloride test	+	+	+	+
		Lead Acetate test	+	+	+	+
		Potassium Dichromate test	+	+	+	+
8	Tannin	10% NaOH test	-	-	-	+
		Bromine water test	-	-	+	+

Where (+) shows presence and (-) shows absence of the constituents.

3.5 Clotlysis Activity

Extracts were evaluated at different doses 50, 75, 100, 200, and 300 mg/ml, and the results were mentioned in Table 3. The clotlysis potential of the plant extract was shown to be significant potential.

3.6 *In Vitro* Anticoagulant Study

3.6.1 APTT and PT

Activated Partial Thromboplastin Time (APTT) and Prothrombin Time (PT) are used to assess the intrinsic and extrinsic clotting factors. The results of APTT and PT are mentioned in Table 4.

Table 2. Results of the total phenolic content of the leaves of *Phyllanthus acidus* leaves

S. No.	Plant Extract	Total Phenolic Content ± SEM (SD) (GAE mg/g of dry material)
1	PE	25.20±0.602 (1.108)
2	Chlo.	44.92±0.729 (1.286)
3	MeOH	72.96±0.574 (2.125)
4	Aq.	55.68±0.801 (1.870)

Note: Analysis was performed in triplicate n=3, and results were expressed in mean ± SEM (SD).

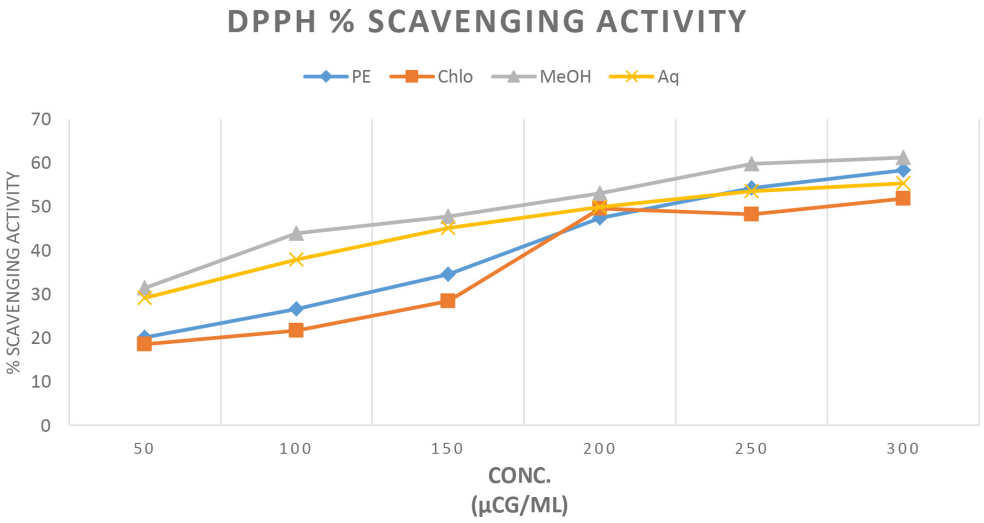


Figure 4. The graphical representation of results of DPPH scavenging activity.

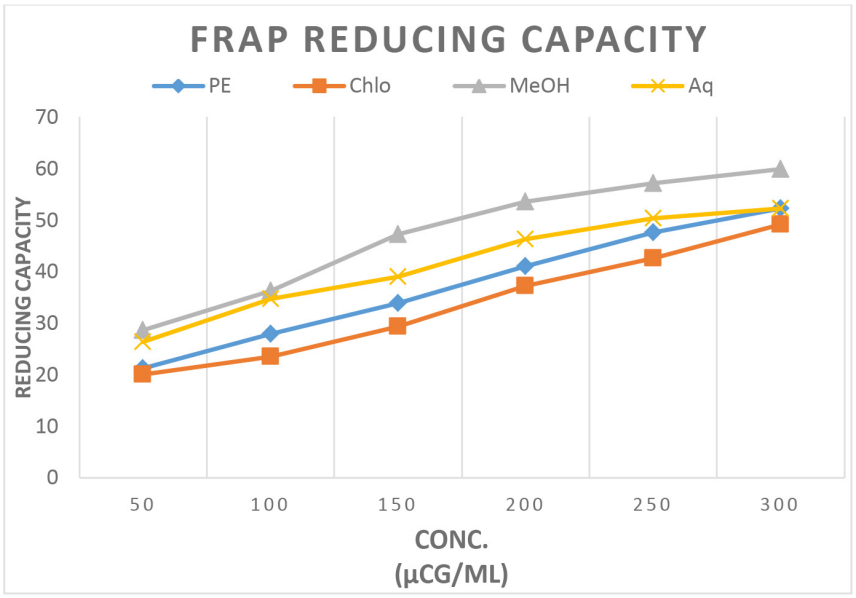


Figure 5. The graphical representation of results of FRAP assay.

Table 3. Result of the clotlysis potential of plant extract

Sr. No.	Conc. of Plant Extract (mg/ml)	*Phyllanthus acidus* Extracts (% of clotlysed by extract)			
		PE	Chlo.	MeOH	Aq
1	Water (Control)	0	0	0	0
2	50	14.867±0.174	12.007±0.027	18.980±0.044	5.997±0.142
3	75	21.300±0.025	18.067±0.058	30.073±0.094	10.330±0.266
4	100	34.707±0.055	39.760±0.107	46.777±0.251	39.640±0.204
5	200	41.900±0.095	37.023±0.071	49.963±0.143	50.893±0.145
6	300	49.033±0.033	43.067±0.041	53.530±0.271	65.737±0.253

Note: *** = p <0.001 (Highly significant), ** = p<0.01 (Moderate significant), * = p<0.05 (low significant). n=6, and values are presented as Mean ± SEM. After comparing the control group to the drug-treated groups using a one-way comparison ANOVA, Dunnett's comparison test was applied. All the drug-treated groups found *** = p <0.001 (Highly significant).

3.7 *In Vivo* Anticoagulant Activity

3.7.1 Clotting Time

The assessment of coagulation time was conducted utilising the capillary technique. The outcome of all the extracts, *Phyllanthus acidus* Methanolic (PAM) and *Phyllanthus acidus* Aqueous (PAW) are mentioned in Table 5.

3.8 Tail Amputation

The tails of the rats were amputated and tail bleeding time and volume were recorded. The results of the bleeding volume and time are mentioned in Table 6.

3.9 Histopathology

Histopathological examination of all the major organs appeared normal and no abnormalities were reported in gross anatomy (Figure 6).

4. Discussion

The most important phase of this study is extraction; the removal of chemical components from the plant's cells is what keeps the investigation progressing. Numerous variables exist that might modify the impact of the extract, including the extraction process and solvent selection[42]. The UAE approach was selected above all others because it is faster, allows us to extract large amounts of crude drug at once, allows us to control temperature, and yields higher results than other extraction techniques[43]. High-velocity chemical ingredient movement from the plant's cellular portion has been rendered feasible by sonication[44]. The way UAE works is by penetrating the leaf's cell wall and causing a disruption that triggers cavitation. Since the solvent flows freely into this environment, the bioactive chemicals migrate quickly[45].

Table 4. The result of the APTT and PT test of *P. acidus* leaf extract

S. No.	Drug Concentration (mg/ml)	APTT (Seconds)		PT (Seconds)	
		MeOH	Aq	MeOH	Aq
1	Control	26.66 ±0.66	26.66 ±0.66	12.66±0.33	12.66±0.33
2	50	75±6.55	54±3.78	29.33±1.20	26±3.05
3	75	146±1.73	96.33±5.78	54.33±3.28	37±2.64
4	100	155.33±2.08	87±5.03	60.33±0.88	53±2.08
5	200	172.33±4.33	122±1.52	95±2.08	68.33±1.76
6	300	180>	178.33±1.66	98.66±2.33	77±2.51

Note: 180>-not clotted up to 180 seconds. *** = p <0.001 (Highly significant), ** = p<0.01 (Moderate significant), * = p<0.05 (low significant). n=3, and values are presented as Mean ± SEM. After comparing the control group to the drug-treated groups using a one-way comparison ANOVA, Dunnett's comparison test was applied. All the drug-treated groups found *** = p <0.001 (Highly significant).

Table 5. The result of the clotting time activity of *Phyllanthus acidus* leaf extracts

S. No.	Group and Drug Conc.	Clotting Time		
		Day-1	Day-3	Day-7
1	Control	12.66±1.14	13±0.68	12.83±0.79
2	PAM (100mg/kg)	34±1.29	89.33±2.70	97.83±2.44
3	PAM (200mg/kg)	48.66±2.02	100±3.81	112.16±2.86
4	PAM (400mg/kg)	56±2.68	103.16±1.13	113.83±1.74
5	PAW (100mg/kg)	30.83±1.01	68.33±3.20	98.50±1.05
6	PAW (200mg/kg)	42.33±1.52	89.33±3.58	109.83±2.54
7	PAW (400mg/kg)	42.83±2.41	91.50±2.04	110.16±2.89

Note: *** = p <0.001 (Highly significant), ** = p<0.01 (Moderate significant), * = p<0.05 (low significant). n=6, and values are presented as Mean ± SEM. After comparing the control group to the drug-treated groups using a two-way comparison ANOVA, Bonferroni's multiple comparison test was applied. All the drug-treated groups found *** = p <0.001 (Highly significant).

Table 6. Results of bleeding volume and bleeding time of *Phyllanthus acidus* leaf extracts

S. No.	Group	Bleeding Volume (sec.)		Bleeding Time (min.)	
		MeOH	Aq.	MeOH	Aq.
1	Control	0.43±0.03	0.43±0.03	7.16±2.13	7.16±2.13
2	PA (100mg/kg)	1.22±0.08	1.01±0.05	13.66±1.63	11.33±0.81
3	PA (200mg/kg)	1.78±0.08	1.62±0.07	24±2.19	17.16±1.72
4	PA (400mg/kg)	2.12±0.09	1.91±0.04	24.66±2.87	18.66±1.03

Note: *** = p <0.001 (Highly significant), ** = p<0.01 (Moderate significant), * = p<0.05 (low significant). n=6, and values are presented as Mean ± SEM. After comparing the control group to the drug-treated groups using a two-way comparison ANOVA, Bonferroni's multiple comparison test was applied. All the drug-treated groups found *** = p <0.001 (Highly significant).

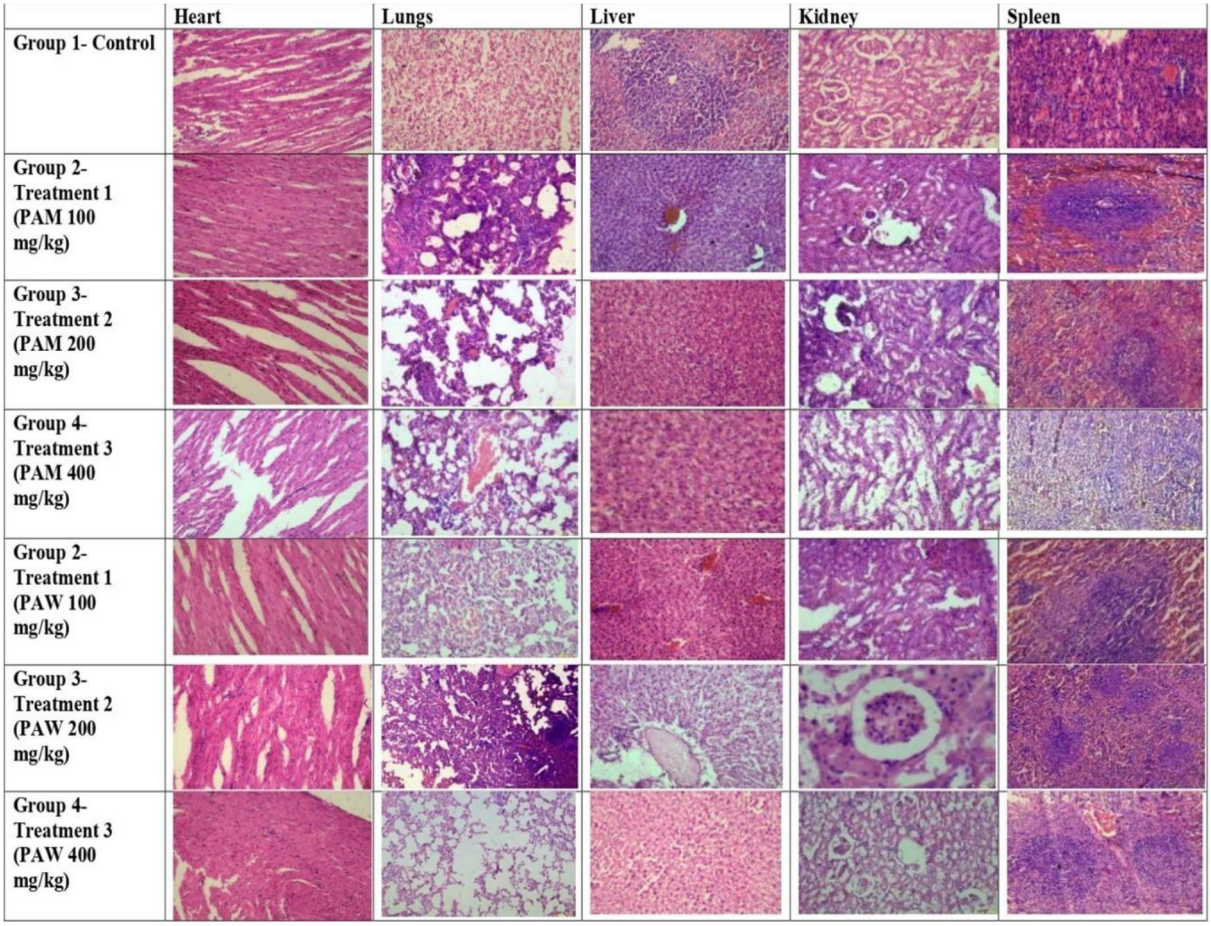


Figure 6. Histopathology of major organs of all the treatment groups.

The phytochemical analysis is necessary to verify whether phytocompounds are present in the plant. Plants have secondary metabolites that are classified into several chemical classes, some of which are recognised for their diverse pharmacological properties[46]. There are several pharmacological actions that phenolic chemicals are accountable for, including antioxidant[47], antibacterial, antifungal, anticancer[48,49] antiaging, cardioprotective, antiatherosclerosis, etc[50]. The extraction yield was high with chloroform but most of the phytoconstituents are present with methanol and aqueous, in the methanolic portion Alkaloids, Glycoside, Amino acids, Flavonoids, Phenols, Tannins, and in the aqueous extract Glycoside, Amino acids, Steroids, Phenols, Tannins, Carbohydrate are present.

The plants have antioxidant potential which is very crucial for their pharmacological potency. We all require antioxidants to detoxify our system regularly otherwise it may impact our systems' normal functioning. The presence of different types of polyphenolic compounds such as alkaloids, phenols, glycosides and tannins enhances the antioxidant potential of the plant each of them is crucial. Our findings show the methanolic extract of the plant exhibits good IC_{50} values that are 188.85 and 186.49 (µg/ml) in both DPPH and FRAP methods respectively, which means most of the components responsible for the antioxidant potential that are soluble in methanol. Reactive Oxygen Species (ROS) are common in all chronic degenerative diseases and the use of preservatives and synthetic antioxidants is the leading cause behind all chronic diseases (diabetes, cardiovascular diseases, neurodegeneration, etc)[51]. Two types of free radical species present behind most of the degenerative diseases are ROS and Reactive Nitrogen Species (RNS) which occur due to some unpaired electrons, and these unpaired electrons lead to the degenerative type of disturbance in the cell. Antioxidants are natural and synthetic both types of positive charge compounds that are paired with free radicals and balance the disturbance[52]. Plants are rich in naturally occurring antioxidants, some phytoconstituents such as phenolic acids, flavonoids, coumarins, tannins, lignans, alkaloids, amines, and terpenoids, which cover phenolic and nitrogenous compounds present in the plants[51]. Plants are rich in phenols and other phytoconstituents that exhibit potent antioxidant activity- IC_{50} value of Pet. Ether, Chloroform, Methanol, and Aqueous present respectively 230.49, 289.85, 188.85, and 233.73 µg/ml, and total phenolic content are 25.20, 44.92, 72.96, and 55.68 mg/g equivalent to gallic acid concentration.

Phyllanthus acidus belongs to lactiferous plants and protease is found in high amounts in lactiferous plants. Protease plays a crucial role in balancing hemostasis due to its catalytic conversions (that will break the peptide bond of the clot-forming protein in the blood and rupture the clot)[53]. Proteases are involved in the many physiological activities researchers call "Molecular Scissors" which participate in various activities like hemostasis, blood coagulation cascade and fibrinolysis[54]. Cysteine proteases, aspartic proteases and FtsH pseudo-protases are responsible for the protein degradation[55]. Proteolytic enzymes have the competence to collapse protein molecules. From the coagulation cascade, the clot is confirmed by forming a fibrin thread is a protein. The dissolution of the clot into liquid also conforms to the potential for anticoagulant action. The aqueous and the methanol extract exhibit strong clotlytic actions, pet. ether and chloroform extracts exhibit satisfactory action against the busting of clots 65.737±0.253, 53.530±0.271, 49.033±0.033 and 43.067±0.041 respectively at 300mg/ml dose. The solubility of the extract affects the reduction of the clot, pet. ether and chloroform extract are sparingly soluble in water so the constituents present in both extracts react less with the blood clot.

The PT assay evaluates the extrinsic pathway of coagulation. It identifies alterations in the coagulation factors II, V, VII, and X. This assay is predominantly utilised for the monitoring of Vitamin K Antagonists (VKA) therapy, which impacts three of these five coagulation factors and ranks as the most frequently employed anticoagulant medication. The *in vitro* assay of activated Partial Thromboplastin Time (aPTT) influences the constituents of both the intrinsic and common coagulation pathways, which encompass factors II, V, VIII, IX, FX, XI, and XII[56,57].

The capillary tube technique has been employed for an extended period to assess the coagulation duration of blood; within this methodology, platelets, fibrinogen, and vitamin K serve as the principal components responsible for blood coagulation. Methanolic and aqueous extracts derived from the leaves of *P. acidus* demonstrate significant efficacy against the coagulation factors implicated in clot formation, exhibiting remarkable potency in both the inhibition and lysis of fibrin clots. The methanolic extract derived from the leaves demonstrated superior efficacy in comparison to the aqueous extract; nonetheless, no statistically significant disparities were noted between the two experimental groups. In both extract categories, the administration of a 400 mg/kg dosage resulted in the most pronounced outcomes when juxtaposed with the control group (113.83±1.74 and 110.16±2.89 on the 7th day of treatment).

The experimental procedure was conducted over seven days, during which clotting time was systematically assessed on the first, third, and seventh days of the investigation; it was noted that the clotting

duration exhibited a progressive increase correlating with the duration of treatment across all experimental groups, which may suggest that the extract enhances the synthesis of factors that inhibit the coagulation cascade.

The tail amputation model induces a state of haemorrhage within the organism and is evaluated through measurements of both bleeding time and the volume of blood lost. We observed that, under controlled anaesthetic conditions, the rats in the control group experienced a relatively brief bleeding duration (7 minutes) and exhibited a minimal blood loss volume (0.43ml); conversely, the groups that received pretreatment with methanolic and aqueous extracts of *P. acidus* demonstrated significant alterations in both bleeding volume and duration, on the 7th day of pretreatment, amputation was done and the methanolic and aqueous groups exhibited potent anticoagulation action. Methanolic extract on 400, 200, and 100mg/kg were shown anticoagulant potential and bleeding volume was recorded- 2.12±0.09, 1.78±0.08, and 1.22±0.08 respectively. Aqueous extract on 400, 200, and 100mg/kg were shown anticoagulant potential and bleeding volume was recorded - 1.91±0.04, 1.62±0.07, and 1.01±0.05 respectively. The average bleeding time in methanolic 400 mg/kg was observed 24.66±2.87 min and in aqueous 400 mg/kg was recorded 18.66±1.03 min.

In instances of pronounced haemorrhage, no significant haemorrhagic complications were detected in the principal organs; however, some organs displayed mild necrosis and localised lesions, indicating that the extract does not compromise the integrity of vascular permeability. Group-1 (control): all the organs were found normal and no degradation, necrosis, or lesion was observed. PA MeOH extract Group-2 (treatment -1 PAM 100 mg/kg)- no abnormalities were found, Group-3(treatment -2 PAM 200 mg/kg)- in the kidney minimal congestion of glomerular capillary tufts was observed and in other organs, no degradation, necrosis, or lesion was observed, Group-4(treatment -3 PAM 400 mg/kg)- in kidney mild multifocal tubular necrosis was observed and no abnormalities were detected in other organs. PA Aq. extract Group-2 (treatment -1 PAW 100 mg/kg)- no abnormalities were found, Group-3(treatment -2 PAW 200 mg/kg)- in the kidney focal increased bowman's space was observed and in other organs, no abnormalities were observed, Group-4(treatment -3 PAW 400 mg/kg)- in kidney mild multifocal glomerular necrosis was observed and no abnormalities were detected in other organs.

5. Conclusion

The species *P. acidus* has been historically utilised for various pathological conditions, possessing substantial traditional significance. The polyphenolic compounds and proteases found within the plant influence the coagulation mechanism, as evidenced by the present study, which evaluated both intrinsic and extrinsic coagulation pathways, yielding statistically significant outcomes in both assessments. Given its remarkable antioxidant and anticoagulant properties, this plant holds potential for therapeutic applications in the management of cardiovascular disorders.

6. Acknowledgements

For research facilities, the authors are extremely grateful to Dr. M. N. Noolvi, principal of Shree Dhanvantary Pharmacy College in Kim, Surat, Gujarat. They also thank Dr Farzin M. Parabia of Veer Narmada South Gujarat University in Surat, Gujarat, for verifying the plant's authenticity.

7. Authors Contribution

Conceptualisation, A.K.V. and R.A.G.; Methodology, A.K.V., R.A.G and R.K.J; writing—original draft preparation, A.K.V, writing—review and editing, A.K.V., R.A.G and R.K.J, supervision, R.A.G and R.K.J All authors have read and agreed to the published version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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