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Antibacterial and antioxidant potential of the acetone extract of the fruit of *Phyllanthus acidus* L.

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Abstract

The *Phyllanthus acidus* is an edible small yellow berries fruit in the Phyllanthaceae family. Fruits are borne in loose clusters, are pale yellow or white, waxy, crisp and juicy. The present study is so designed for phytochemical screening of the plant as well as antibacterial and antioxidant activities of the fruit part of acetone extract of *P. acidus*. The fruit part was collected in and around Chennai. Phytochemical screening of acetone extract of *P.acidus* revealed the presence of flavonoids, tannins, phlobatannin, saponin, terpenoids and glycosides. The antibacterial activity of the crude extract was determined by the well diffusion method against four pathogenic bacteria. In higher concentration the extract showed moderate to good zone of inhibition against *E. coli* and *P. aeruginosa.* The phenolic content was 122.22 mg/g GAE and the amount of flavonoid was 163.15 mg/g of quercetin equivalent. The DPPH radical scavenging activity was found to increase with increasing concentration of the extract and IC_{50} value showed 7.31 µg/mL for plant extract compared to 8.65 µg/mL which was the IC_{50} value for the reference ascorbic acid.

Keywords: well diffusion method, antioxidant, zone of inhibition, phytochemicals

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Introduction

Medicinal plants, since times immemorial, have been used in virtually all cultures as a source of medicine. The widespread use of herbal remedies and health care preparations, as those described in ancient texts such as the Vedas and the Bible and obtained from commonly used traditional herbs and medicinal plants, has been traced to the occurrence of natural products with medicinal properties (Hoareau et al., 1999). The use of traditional medicine and medicinal plants in most countries, as a normative basis for the maintenance of good health, has also been widely observed (UNESCO, 1996). Furthermore, an increasing reliance on the use of medicinal plants in industrialized societies has been traced to the extraction and development of several drugs and chemotherapeutics from these plants as well as from traditionally used rural herbal remedies (UNESCO, 1998). The World Health Organization has estimated that 80% of the world's population use botanical medicine for their primary health care needs (Akerele, 1993). Organisms have self defense mechanisms to protect themselves from the attack of free radicals such as preventive antioxidant system that reduces the rate of free radical formation and to produce chain-breaking antioxidants that scavenge and stabilize free radicals. If free radical production rate exceeds the normal capacity of the antioxidant defense mechanisms, substantial tissue injury results (Rahman and Moon, 2007).

Description and medicinal use

The *P. acidus*, locally named as Arbaroi in Bangladesh and gooseberry or star gooseberry in India, is an edible small yellow berries fruit in the Phyllanthaceae family. Fruits are borne in loose clusters, are pale yellow or white, waxy, crisp and juicy, and very sour, found in Bangladesh, South India, and Southeast Asian countries. The medicinal activities of Phyllanthus species are antipyretic, analgesic, anti-inflammatory, anti-hepatotoxic and antiviral (Unander et al., 1995; Chang et al., 2003; Zhang et al., 2004; Sousa et al., 2007). Fruits of the two well-known species, *P. acidus L.* and *P. emblica L.* contain high contents of vitamin C and have been used for used for improving eyesight and memory and preventive action against Diabetes and relief of coughing (Unander et al.,1990). Another species of the family, *P. amarus* is an important herbal medicine due to its effective antiviral activities especially toward the hepatitis B virus (Unander et al., 1991; Ott et al., 1997; Rai et al., 2005).

Materials and Methods

Plant material

The plant material of fruit part of *P. acidus* was collected from in and around Chennai. It was identified using standard books. The fleshly part of the fruit was shade dried and crushed into fine powder with electric blender. The powdered sample was sealed in polythene bags and was stored in desiccators until further use.

Preparation of acetone extract

Dried and powdered fruit part of *P. acidus* (500 g) were extracted using soxhlet with 100% acetone (1:5 W/V) for about 72 hrs. The extract was removed and it was concentrated to dryness in rotary vacuum evaporator below 50°C and stored until needed for the bioassays at -4°C.

Phytochemical screening

The freshly prepared crude extract was qualitatively tested for the presence of chemical constituents and they were identified by the characteristic colour changes using standard procedures as described by Sofowara (1993), Trease and Evans (1989) and Harborne (1973).

Estimation of Total phenol

Total phenol of acetone extracts of fruit was determined with the Folin-Ciocalteu reagent (Singleton et al., 1977) method. One hundred mg acetone extract of fruits were dissolved in 10 mL of acetone and diluted to different concentration. The diluted sample was used to measure the total phenol contents. One mL of diluted sample mixed with 1 mL of folin-phenol reagent (1:1 v/v) and 2 mL of sodium carbonate (2% w/v). The reaction mixture was incubated at room temperature for 2 hrs with intermittent shaking. The absorbance of samples was measured at 760 nm using spectrophotometer. Results were expressed as milligrams of Gallic acid equivalent per gram of dry weight (mg GAE/g DW).

Estimation of flavonoid

Aluminium chloride colorimetric method was used for flavonoids determination (Chang et al., 2002). One hundred mg acetone extract of fruits were dissolved in 10 mL of acetone and diluted to various concentrations. The diluted samples were used to measure the total phenol contents. Each extract of leaf and stem (0.5 mL) in acetone was separately mixed with 1.5 mL of acetone, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled

water. It was kept at room temperature for 30 mins. The absorbance of the reaction mixture was measured at 415 nm using spectrophotometer. Results were expressed as milligrams of quercetin equivalent per gram of dry weight (mg GAE/g DW).

Microbial strains and inoculum preparation

The microorganisms used in this study were human pathogens namely *Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus* and *Staphylococcus aureus.* Bacterial strains stock cultures were maintained at 4°C on Muller Hinton Agar medium. Active cultures were prepared by inoculating fresh nutrient broth medium with a loopful of cells from the stock cultures at 37°C for overnight. To get desirable cell counts for bioassays, overnight grown bacterial cells were sub-cultured in fresh Muller Hinton Broth at 37°C.

In-vitro antibacterial screening

In vitro antibacterial activity of the acetone fruit extracts of *P. acidus* was screened against a total of the four bacterial strains.

Well diffusion method: The well diffusion test (Bennet et al., 1966; Janssen et al., 1987; Magaldi et al., 2004) was performed using MHA medium. The medium was prepared and autoclaved at 15 lbs pressure (121ºC) for 15 mins immediately cooled in a 50-55 ºC water bath after removed from the autoclave. The cooled medium was poured into sterile petriplates to a uniform depth of 4 mm; this is equivalent to approximately 25 mL in a 90 mm plate. Once the medium was solidified, then the culture was inoculated on the medium. Within 15 mins of adjusting the density of the inoculum, a sterile cotton swab was dipped into the standardized bacterial suspension. The sterile swab was used to streak on the surface of the MHA containing plates. The plates were allowed undisturbed for 3 to 5 min to absorb the excess moisture. Sterilized 9 mm cork borer was used to make agar wells 25, 50, 75 µg of extract stock solutions were placed into each wells and 100% DMSO as a control. Positive control was made by tetracycline 30 µg which were suspended in 100% DMSO solvent. Zone of inhibition (ZI) were measured by 1 mm accuracy scale prescribed method and calculated the zone of inhibition percentage also by the following formula

Percentage of inhibition = I/diameter of the petriplate in mm×100

In vitro antioxidant activity

DPPH free radical scavenging activity

The acetone extract of the fruits of *P. acidus* were subjected to *in vitro* antioxidant assay (Yen and Hsieh, 1997). For acetone extracts reaction mixture consisted of 1 mL of 0.1 mM di-phenyl-p-picrylhydrazyl radical (DPPH) in methanol and 1 mL of different concentrations (8, 16, 32, 64, 125 and 250) were prepared. Acetone extracts of fruit were prepared and diluted to concentrations (8, 16, 32, 64, 125 and 250 µg/mL). One mL of DPPH and 1 mL of methanol were used as control. The reaction mix was left in the dark at room temperature for 30 mins. The OD was measured using spectrophotometer at 517 nm. Quercetin was used as standard. The inhibition percentage was calculated according to the formulae.

Inhibition percentage = A_C-A_S/A_C×100. Where, A_C-Absorbance of Control; A_S-Absorbance of Sample. The 50% inhibitory concentration (IC_{50}) values were calculated by plotting an x, y scatter trendline with regression equation.

Reducing power assay: The reducing power of crude acetone extracts of fruit were determined by the method

of (Oyaizu, 1986). Various concentrations of the plant extracts in 1 mL of solvent were mixed with phosphate buffer (2.5 mL) and potassium ferric cyanide (2.5 mL) and incubated at 50°C for 20 mins. Trichloroacetic acid (10%; w/v) 2.5 mL were added to the mixture, which was then centrifuged at 3000 rpm for 10 mins whenever necessary. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and a freshly prepared ferric chloride (0.1%; w/v) solution 0.5 mL. The Absorbance was measured at 700 nm. A blank was prepared without adding extract. Ascorbic acid with various concentrations was used as the standard reference. Increase in absorbance of the reaction mixture indicates the increase in reducing power.

Phosphomolybdate reduction assay

The antioxidant capacity of crude acetone extracts of fruits were assessed by (Prieto et al., 1990). Extract with different concentrations (64,125, 250, 500 and 1000 µg/mL) was combined with 1mL of reagent solution containing ammonium molybdate (4 mM), sodium phosphate (28 mM), sulphuric acid (600 mM).The reaction mixture was incubated in water bath at 90°C for 90 mins. The absorbance of the coloured complex was measured at 695 nm. For reference the appropriate solutions of ascorbic acid has been used.

Results

Phytochemical screening

The qualitative studies of crude acetone extract indicate the presence of flavonoids, tannins, phlobatannin, saponin, terpenoids and glycosides while absence was observed in steroids (Table 1).

Total phenol and flavonoid content

The total phenol and flavonoid content from acetone extract of fruits of *P. acidus* were expressed in gallic acid and Quercetin equivalents respectively and are presented in Table 2. The content of phenolics in this extract showed 122.22 mg/g GAE and the amount of flavonoid was 163.15 mg/g quercetin equivalent.

Table 1. Qualitative analysis of *P. acidus* (fruit) of acetone extracts (+ = Present; - = Absent)

Phytochemicals	*P. acidus*
	Fruit
Flavonoid	+
Tannin	+
Phlobatannin	+
Saponin	+
Terpenoids	+
Glycosides	+
Steroids	-

Table 2. Total phenol and flavonoids of acetone extracts of *P. acidus* (fruit)

Total phenol mg/g DW		Flavonoids mg/g DW
Fruit	122.22±2.66	163.15±4.19

Values are mean ± standard deviation of triplicates

Antibacterial activity

Antibacterial activities of the crude extracts were tested against four pathogenic bacteria and they were compared with standard antibiotic tetracycline by measuring the zone of inhibition diameter and expressed in mm showed in Table: 3. The average zone of inhibition ranges from 13-20 mm. Highest inhibitory activity was observed against the growth of *E. coli*, *P. aeruginosa* with the zone of inhibition 19 and 20 mm respectively. The concentration above 50 µg/mL showed slight effect against the all four bacteria (*E. coli, P. aeruginosa, B. cereus* and *S. aureus*)

In vitro antioxidant activity

DPPH radical scavenging assay

The DPPH radical scavenging activity from the acetone extract of fruit has strong scavenging activity. Maximum inhibition activity for fruit extract was

Table 3. Antibacterial activity of acetone extracts of *P. acidus* (fruit) against human pathogens

Samples	Pathogens	Concentration of acetone extracts (µg)					
		25		50		75	
		ZI	% I	ZI	% I	ZI	% I
Phyllanthus acidus (fruit)	*E. coli*	16.33±2.12	18.11±1.26	16.00±1.07	17.7±1.23	17.66±1.11	19.50±1.3
	P. aeruginosa	13.33±1.44	14.77±1.03	15.33±2.01	17.33±1.21	18.33± 2.22	20.3±1.42
	B. cereus	14.00±1.55	15.55±1.08	16.33±2.09	18.11±1.26	17.33±1.23	19.20±1.34
	S. aureus	14.00±1.55	15.55±1.08	16.00±1.77	17.77±1.24	16.33±0.99	18.11±1.26

Values are mean ± standard deviation of triplicates

Table 4. DPPH assay of acetone extracts of *P. acidus* (fruit)

Samples	Concentration of acetone extracts (µg/mL)	Percentage of Inhibition (%)	IC_{50}
P. acidus (fruit)	8	54.71± 0.64	7.31
	16	68.43± 0.25	
	32	75.66± 0.81	
	64	80.61± 0.41	
	125	84.47± 0.56	
	250	88.22± 0.67	
Quercetin	3	35.32±2.47	8.65
	6	43.26±3.02	
	9	52.56±3.67	
	12	65.45±4.58	
	15	68.66±4.80	
	18	79.86±5.59	
	21	93.98±6.29	

Values are mean ± standard deviation of triplicates

Table 4a. Reducing power assay of acetone extracts of *P. acidus* (fruit)

Samples	Concentration of acetone extracts (µg/mL)	Absorbance at 700 nm
P. acidus (fruit)	64	0.139± 0.016
	125	0.188± 0.021
	250	0.241± 0.024
	500	0.343± 0.031
	1000	0.397± 0.028
Ascorbic acid	64	0.328±0.022
	125	0.480± 0.033
	250	0.530±0.037
	500	0.620±0.043
	1000	0.780±0.054

Values are mean ± standard deviation of triplicates

Table 4b. Phosphomolybdate assay of acetone extracts of *P. acidus* (fruit)

Samples	Concentration of acetone extracts (µg/mL)	Absorbance at 765 nm
P. acidus (fruit)	64	0.159± 0.016
	125	0.226± 0.021
	250	0.289± 0.024
	500	0.323± 0.031
	1000	0.384± 0.028
Ascorbic acid	64	0.308±0.011
	125	0.424±0.024
	250	0.516±0.037
	500	0.624±0.048
	1000	0.716±0.053

Values are mean ± standard deviation of triplicates

observed at 250 µg/mL respectively. Antioxidant activity of fruit extract was compared with standard quercetin. IC_{50} value of fruit extract less than quercetin, showed more activity (Table 4).

Reducing Power assay

Reducing power was assayed from the acetone extract of fruit of *P. acidus* showed increasing activity of Fe^{3+}. Strong reducing power was observed in lower concentration of fruit extract. The concentration ranged from 64-1000 µg/mL. The acetone extract of fruit of *P. acidus* was very potent and the power of the extract increased with the quantity of sample. The concentration of extract in fruit showed absorbance of 0.397 at 1000 µg/mL when compared with standard ascorbic acid the fruit extract showed less activity (Table 4a).

Phosphomolybdate assay

Phosphomolybdate assay was assayed from the acetone extract of fruit of *P. acidus* showed increasing activity of the sample. The concentration ranged from 64-1000 µg/mL. The acetone extract showed strong dose dependent activity. The concentration of extract in fruit showed absorbance of 0.384 at 1000 µg/mL when compared with standard ascorbic acid the fruit extract showed less activity (Table 4b).

Discussion

The crude extract was qualitatively analysed for the presence of flavonoids, tannins, phlobatannin, terpenois, saponin, glycosides and steroids. According to Habib et al. *(*2011) from the petroleum ether extract of fruit of *P. acidus* showed the presence of carbohydrate, glycoside and steroid while the absence was observed in saponin, tannin and resins. But a mixed result was seen in alkaloids. Habib et al. (2011) showed that the methanol extract of *P. acidus* revealed the presence of carbohydrate, terpenoid, steroid, alkaloid, saponin, glycoside and flavonoid. In the present study from the acetone extract of fruit of *P. acidus* showed the presence of flavonoid, tannin, phlobatannin, saponin, terpenoids, glycosides and while absence was observed in steroids. Total phenol and flavonoid content were expressed in GAE, quercetin equivalent respectively. Earlier study of (Raja Chakraborty et al., 2012) the total phenolic and flavonoid content of methanol extract was found to be 73.08 GAE mg/g and 61.28 mg/g quercetin equivalent. According to (Habib et al., 2011) from the petroleum ether extract of fruit of *P. acidus* showed moderate result

of 159.60 mg/g GAE and the amount of flavonoid was 24.183 mg/g quercetin equivalent. Another study (Habib et al., 2011) from the methanol extract of *P. acidus* the flavonoid content was 24.18 mg/g quercetin respectively. In the present study, the acetone extract of fruit of *P. acidus* revealed that total phenolic content showed 122.22 mg/g GAE and the flavonoid content showed 163.15 mg/g quercetin equivalent respectively.

In the present study, antibacterial activities of the crude extracts were tested against four pathogenic bacteria and they were compared with standard antibiotic tetracycline. The average zone of inhibition ranges from (13-20 mm). Highest inhibitory activity was observed against the growth of *E. coli*, *P. aeruginosa* with the zone of inhibition 19 and 20 mm respectively. The concentration above 50 µg/mL showed slight effect against the all four bacteria namely *E. coli, P. aeruginosa, B. cereus* and *S. aureus*. According to (Habib et al., 2011) from the petroleum ether extract of fruit of *P. acidus* showed average zone of inhibition with 0.5-2.5 mm. Narrow inhibition activity was noticed against the growth of *S. typhi* with zone of inhibition 2.5 mm. One another study of (Habib et al., 2011) from the methanol extract of *P. acidus* showed the average zone of inhibition from 8-12 mm respectively. The DPPH test is based on the ability of stable free radical to decolourize in the presence of antioxidant is a direct and reliable method for determining radical scavenging action. According to (Habib et al., 2011) the methanol extract of *P. acidus* showed IC_{50} value of 2063.42 µg/mL with reference to ascorbic acid where the IC_{50} value was 52.45 µg/mL. Earlier study of (Raja Chakraborty et al., 2012) from the ethyl acetate extract had highest DPPH scavenging effect with IC_{50} value 28.6 µg/mL and IC_{50} value of methanol and petroleum ether extract showed 86.1 and 117.4 µg/mL respectively.

Another study (Habib et al., 2011) from the petroleum ether extract of fruit of *P. acidus* showed IC_{50} value of 1403.76 µg/mL with reference to standard ascorbic acid IC_{50} value was 13.37µg/mL. In the present study the maximum inhibition activity for fruit extract was observed at 250 µg/mL with IC_{50} value 7.31 µg/mL with reference to standard Quercetin IC_{50} value was 8.65 µg/mL respectively. Reducing power assay determine the reductive capabilities of the extract compared with ascorbic acid which has determined using potassium ferric cyanide reduction method. According to Habib et al. (2011) from the methanol extract of *P. acidus* the reducing power was moderatively strong while increasing dose it shows little increment. Earlier study of (Raja Chakraborty et al., 2012) the absorbance value was found to be increasing with methanol, ethyl acetate and petroleum ether extracts with 0.804, 0.735 and 0.588 respectively. In the present study, the concentration of extract of fruit showed absorbance of 0.397 at 1000 µg/mL when compared with reference ascorbic acid the fruit extract showed less activity. In the present study, the concentration range from 500-1000 µg/mL, the acetone extract showed strong dose dependent reducing activity. The result obtained was confirmed by the high potency of the acetone extract towards the transition metal ions. The reducing power of fruit extract of *P. acidus* showed 0.397 at 1000 µg/ml concentration. According to (Habib et al., 2011) from the petroleum ether extract of fruit of *P. acidus* showed 61.27 mg/g ascorbic acid equivalent.

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