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**EVALUATION OF ANTIOXIDANT, CYTOTOXIC, ANTIBACTERIAL POTENTIAL
AND PHYTOCHEMICAL SCREENING OF CHLOROFORM EXTRACT OF
PHYLLANTHUS ACIDUS.**

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ABSTRACT: The present study was carried out to evaluate the phytochemical properties, antimicrobial and cytotoxic as well as antioxidant activities of the chloroform extract of *Phyllanthus acidus* fruit. Phytochemical screening showed confirmation of saponin, alkaloid, tannin and flavanoid. Antioxidant activity of PACF (*Phyllanthus acidus* chloroform extract) was assessed by using 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), reducing power, cupric reducing antioxidant capacity and antioxidant activity increased in a concentration dependent manner. In DPPH radical scavenging assay IC₅₀ value found 2745.86 µg mL⁻¹ and compared to ascorbic acid with 13.37 µg mL⁻¹. In brine shrimp lethality bioassay it showed good result with LC₅₀ value 4.46 µg/mL. Antibacterial activity of plant extract was carried out using disc diffusion method with eleven pathogenic bacteria. This extract showed narrow spectrum activity aligned with *Shigella dysenteriae*, *Escherichia coli*, *Staphylococcus aureus* and *Sarcina lutea* at concentration of 500 µg/disc in comparison with standard kanamycin. The range of zone of inhibition of chloroform extract was 0.5 to 2.5 mm.

Key words: *Phyllanthus Acidus*, Antioxidant, Cytotoxicity, Phytochemical screening

INTRODUCTION

Consumption of medicinal herbs is tremendously increasing over a past decade as an alternative approaches to improve the quality of life and maintain a good health. Medicinal plants have been used for centuries as remedies for human diseases (Nostro *et al.*, 2000; Arokiyaraj *et al.*, 2008). Usually, when natural defenses of the organism are overwhelmed by an excessive generation of Reactive Oxygen Species (ROS), a situation of 'oxidative stress' occurs, in which cellular and extra cellular macromolecules (proteins, lipids, and nucleic acids) can suffer oxidative damage, causing tissue injury (Halliwell *et al.*, 1989), several human chronic diseases such as atherosclerosis and cardiovascular diseases, mutagenesis and cancer, several neurodegenerative disorders, and the aging process (Anderson *et al.*, 2000; Tseng *et al.*, 1997). Several drugs currently used in chemotherapy were isolated from plant species or derived from a natural prototype. Over 50 % of all drugs in clinical trials for anticancer activity were isolated from natural sources or are related to them (Cragg *et al.*, 2000). The brine shrimp lethality assay is considered a useful tool for preliminary assessment of toxicity. The acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the available antibiotics has led researchers to investigate the antimicrobial herbal extracts (Bisignano *et al.*, 2000; Hammer *et al.*, 1999). Plants containing flavonoides, terpenoids, steroids, phenolic compounds and alkaloids have been reported to have antimicrobial activity (Hostettmann *et al.*, 1977). Furthermore there are many reports on antibacterial activity of various plants growing in different parts of this region.

DESCRIPTION AND MEDICINAL USES

Phyllanthus acidus L. (Family: Phyllanthaceae), 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, antiinflammatory, antihepatotoxic 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* and *P. emblica* 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). As part of the endeavor for search of medicinal properties in local floristic resources we herein report a study of antioxidant, preliminary cytotoxic and antibacterial activities of the roots of *P. acidus*.

MATERIALS AND METHODS

Collection of Plant Material and Extraction

After cutting and slicing, the collected plant samples were dried in the sun as well as in a mechanical dryer at 60 – 70°C. The dried sample was ground to coarse powder with a mechanical grinder and powdered sample were kept in clean closed glass container. The dried sample then extracted by chloroform with a volume of 800 ml for 3 days for allowing total extraction process. After that the *Phyllanthus acidus* plant extract was filtered with sterilized cotton filter and the filtrate was collected in a beaker. The plant extract then kept in a water bath at 60°C to evaporate the solvent from the solution. The container allowed to airtight for 72 hours and filtrate thus obtained was concentrated by using a rotary evaporator

Chemicals and drugs

DPPH (1, 1-diphenyl, 2-picrylhydrazyl), TCA (trichloroacetic acid) and ferric chloride were obtained from Sigma Chemical Co. USA; Ascorbic acid was from SD Fine Chem. Ltd. India, ammonium molybdate from Merck, Germany.

Phytochemical screening

To identify the chemical constituents of plant extract standard procedures are followed. Freshly prepared crude extracts of *P. acidus* were qualitatively tested for the presence of chemical constituents using the following reagents and chemicals: flavonoids with the use of Mg and HCl; tannins with ferric chloride and potassium dichromate solutions and saponins with ability to produce stable foam and steroids with Libermann Burchard reagent, reducing sugars with Benedict's reagent and observed color change in respective (Ghani., 2003).

Determination of total phenolic content

Folin-Ciocalteu method was used to determine the total phenolic content; Folin-Ciocalteu oxidized the extract whereas sodium carbonate neutralized it (Singelton *et al.*, 1999) Blue color formed and the absorbance was measured at 760 nm after 60 min by using gallic acid (GA) as standard. Total Phenolic content was expressed as mg GA equivalent/gm of extract.

Determination of total flavonoid content

Kumaran and Karunakaran method was followed to determine the flavonoid content (Kumaran *et al.*, 2007) where quercetin is used as standard. 1 mg of plant extract in methanol was mixed with 1 ml of aluminium trichloride in Ethanol (20 mg/ml) and a drop of acetic acid added. Then diluted up to 25 ml with ethanol and measured the absorbance at 415 nm after 40 min. The absorption of blank samples and standard quercetin solution (0.5 mg/ml) in methanol was measured under the same conditions.

DPPH radical scavenging activity

The free radical scavenging capacity of the plant extract was determined using DPPH (Hasan *et al.*, 2006; Alam *et al.*, 2008)). The solution of methanol DPPH (0.004% w/v) was mixed with serial dilution (0 to 500 µg) of *P. acidus* extract. The absorbance was taken at 517 nm by using the spectrophotometer after 10 min. Ascorbic acid used as standard then plotted the inhibition curve and calculated the IC₅₀ value.

Reducing power

The reducing power of *P. acidus* extract was determined according to the method of Oyaizu (Oyaizu *et al.*, 1986) Different concentration of *P. acidus* extract in 1 ml of distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K₃Fe(CN)₆] (2.5 ml, 1%) then the mixture was incubated at 50°C for 20 min. Trichloroacetic acid (10%) slightly added (2.5 ml) to the mixture and centrifuged at 3,000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl₃ (0.5 ml, 0.1%) then taken the absorbance at 700 nm. The reference standard was Ascorbic acid and the Blank solution contained Phosphate buffer.

Cupric Reducing Antioxidant Capacity (CUPRAC)

Cupric ion reducing capacity was determined according to the method of Resat (Resat *et al.*, 2004) in this procedure, CuCl₂.2H₂O solution; ammonium acetate buffer (pH 7.0), neocaproin solution and distilled water were added to *P. acidus* extract to adjust the final volume to 4.1 ml. The mixture was incubated for 1 hour at room temperature and the absorbance was measured at 450 nm using a spectrophotometer against blank solution. Ascorbic acid was used as a standard.

Brine Shrimp Lethality Bioassay

Brine shrimp lethality bioassay was used for testing cytotoxic potential of the extract (Meyer *et al.*, 1982; Persoone *et al.*, 1988) The eggs of Brine shrimp (*Artemia salina* Leach) were collected and hatched in a tank at a temperature around 37°C with continuous oxygen supply. Two days were allowed to hatch and mature the nauplii. Stock solutions of the sample were prepared by dissolving required amount of extract in specific volume of pure dimethyl sulfoxide (DMSO). 4 ml of seawater was given to each of the vials. Then specific volume of sample was transferred from the stock solution to the vials to get final sample concentrations of 3.125, 6.25, 12.5, 25, 50, 100, 200 and 400 µg/ml. In the control vials same volumes of DMSO (as in the sample vials) were taken. With the help of a Pasteur pipette 10 living nauplii were put to each of the vials. After 24h the vials were observed and the number of nauplii survived in each vial was counted. After that, the percentage of lethality of Brine Shrimp nauplii was calculated for each concentration of the extract.

Antibacterial assay

The antimicrobial assay was performed by using the disc diffusion method (Bauer *et al.*, 1996; Barry *et al.*, 1980) eleven pathogenic bacteria were used as test organisms for antibacterial activity of dried sample extract. The bacterial strains were collected from BCSIR Chittagong, Bangladesh. 0.5 mg/disc of the sample extract were used to observe the antimicrobial activity of the plant extract and compared with the standard kanamycin (0.1 µg/disc). The test organisms were inoculated on 10 ml previously sterilized nutrient agar media, mixed thoroughly and transferred immediately to the sterile Petri dish in an aseptic condition using a sterile loop. Prepared sample and standard solutions were applied to the corresponding Petri dish. The plates were incubated for overnight at 37° C. After proper incubation, clear zone of inhibition around the point of application of sample solution were measured which is expressed in millimeter (mm).

RESULTS AND DISCUSSION

Phytochemical screening

Phytochemical analyses of the crude extract revealed the absence of carbohydrate glycoside, steroid and presence of alkaloid, saponin, tannin and flavonoid which has been shown in Table I.

Table I: Result of phytochemical screening of chloroform extract of *Phyllanthus acidus*.

Extract	Carbohydrate	Glycoside	Saponin	Steroid	Alkaloid	Tanin	Flavonoid
PACF	-	-	+	-	+	+	+

PACF denote for Chloroform extract of *P. acidus*. (+): Present, (-): Absent.

Total phenol and flavonoid content

The total phenol and total flavonoid contents of *Phyllanthus acidus* of chloroform extract were expressed in gallic acid and quercetin equivalents respectively that are shown in Table II. The content of phenolics of the extract under this investigation showed moderate result 24.82 mg/g GAE and the amount of flavonoid was 30.05 mg/g quercetin equivalent.

Table II: Total amount of plant phenolics and flavonoid content of *Phyllanthus acidus*

Extract name	Total phenol (in mg/g, Gallic acid equivalents)	Total flavonoid (in mg/g, quercetin Equivalents)
PACF	24.82	30.05

Different studies suggest that different types of polyphenolic compounds such as flavonoids, phenolic acids which are found in plants have multiple biological effects, including antioxidant activity (Vinson *et al.*, 1995).

DPPH radical scavenging activity

The DPPH radical scavenging activity of *Phyllanthus acidus* shown in Figure I. Where increasing the concentration of the extract, activity was found to increase slightly and the inhibitory capacity of the plant extract was comparatively lower than the ascorbic acid.

The cupric reducing power of the plant extract (Figure II) shows slight increase while increasing the concentration. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom. (Duh *et al* 1999).

Reducing Power

By using the potassium ferricyanide reduction method the reductive capabilities of the plant extracts was identified in comparison with ascorbic acid which demonstrated at figure III. The reducing power of the extracts was moderately strong while increasing dose it shows little increment.

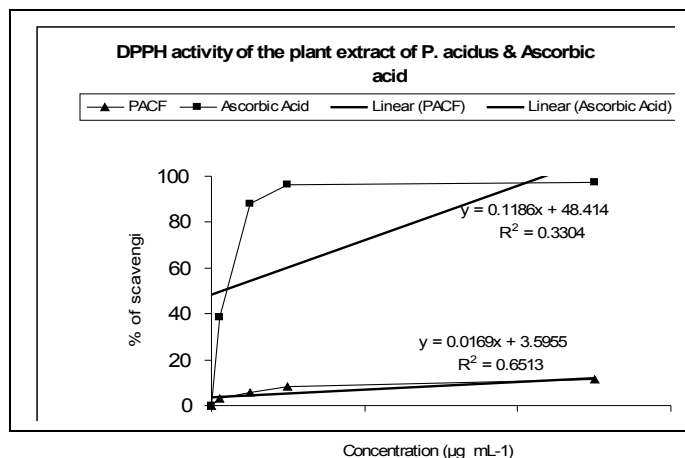


Figure I: DPPH radical scavenging activity of Chloroform extract of *Phyllanthus acidus*.

The Chloroform extract of *Phyllanthus acidus* showed moderate DPPH scavenging activity in which IC_{50} value for the plant extracts was $2745.86 \mu\text{g mL}^{-1}$. In this test, Ascorbic acid was used as the reference antioxidant and the IC_{50} value for ascorbic acid was $13.37 \mu\text{g mL}^{-1}$.

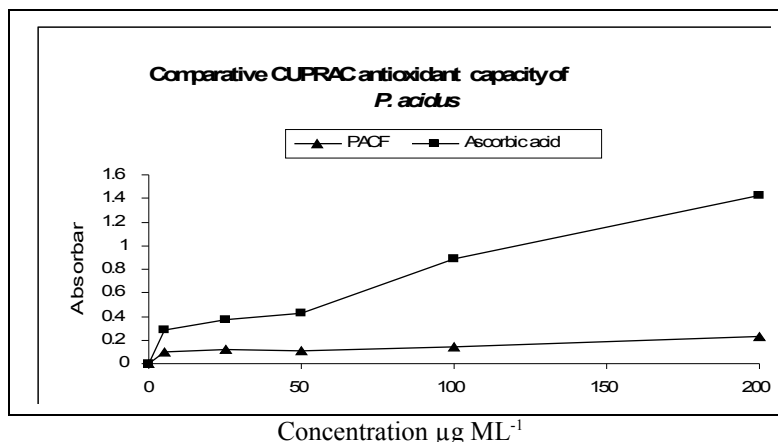


Figure II: Cupric reducing antioxidant capacity of *Phyllanthus acidus* vs Standard.

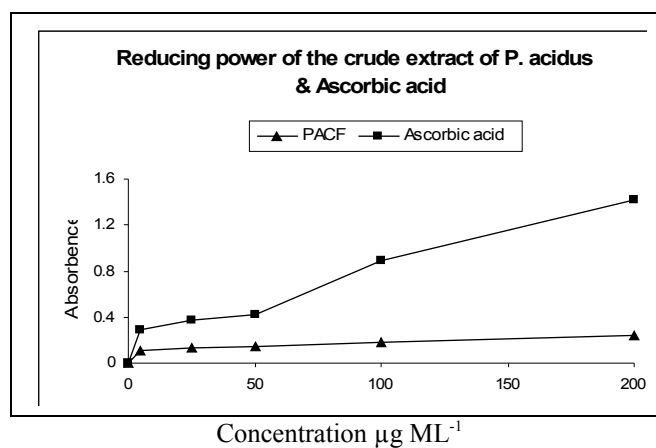


Figure III: Reducing Power of the chloroform extract of *Phyllanthus acidus* vs Standard

Brine Shrimp Lethality Bioassay

The Chloroform extract of *P. acidus* was tested for Brine shrimp lethality bioassay by using brine shrimp nauplii and DMSO as a solvent. Control was used to see whether DMSO had any effect on brine shrimp lethality or not. The control group of brine shrimp nauplii with and without DMSO exhibited no mortality. For the extract, the number of nauplii died and percent mortality was counted. The result is shown in the following table (III).

Table III: Cytotoxic potential of different extracts of *Phyllanthus acidus*

Test solution	Conc. (µg/ml)	Log Conc.	% Mortality	LC ₅₀ (µg/ml)
PACF	3.125	0.49	40	4.46
	6.25	0.79	60	
	12.5	1.096	70	
	25	1.397	90	
	50	1.698	100	
	100	2.000	100	
	200	2.301	100	
	400	2.602	100	

The LC₅₀ values of Chloroform extract of *P. acidus* was 4.46. At the conc. of 12.5 µg/ml, 25 µg/ml, brine shrimp nauplii died 70% and 90% respectively but above the conc. of 50 µg/ml, all brine shrimp nauplii were died which indicates very good cytotoxic effect of *P. acidus*.

Antibacterial assay

Antibacterial activities of the extract were tested against thirteen pathogenic bacteria and were compared with the standard antibiotic kanamycin by measuring the zone of inhibition diameter and expressed in millimeter (mm) showed in table IV.

Table IV: Antibacterial activity of Chloroform extracts of *Phyllanthus acidus*.

Sample code	Name of the Bacteria	Sample Extract Diameter of zone of inhibition (mm) of PACF			
		100 µg/disc	200 µg/ disc	500 µg/disc	Standard (K-30)
B 01	<i>Shigella dysenteriae</i>	NS	NS	2.5	27
B 02	<i>Salmonella typhi</i>	NS	NS	NS	22
B 03	<i>Pseudomonas aeruginosa</i>	NS	NS	NS	30
B 04	<i>Staphylococcus aureus</i>	NS	NS	1.5	27
B 05	<i>Bacillus cereus</i>	NS	NS	NS	18
B 06	<i>Bacillus subtilis</i>	NS	NS	NS	27
B 07	<i>Escherichia coli</i>	NS	NS	1.5	32
B 08	<i>Klebsiella spp</i>	NS	NS	NS	28
B 09	<i>Sarcina lutea</i>	NS	NS	0.5	30
B 10	<i>Shigella sonnei</i>	NS	NS	NS	30
B 11	<i>Bacillus megaterium</i>	NS	NS	NS	28

*Standard: Kanamycin , NS= Not susceptible

In the antimicrobial screening, the extract showed average zone of inhibition 0.5 - 2.5 mm (Table IV). Narrow inhibitory activity was noticed against the growth of *Shigella dysenteriae* with the zones of inhibition 2.5 mm. The concentrations above 500 µg/disc, showed little effect against a number of bacteria including *Shigella dysenteriae*, *Staphylococcus aureus*, *Escherichia coli*, *Sarcina lutea*.

CONCLUSION

From this experiment, it can be concluded that the extract showed average zone of inhibition 0.5-2.5 mm. As apparent from our results it can be revealed that the plant extract has narrow spectrum of antimicrobial activity but it exhibited good cytotoxic effect. The antioxidant activity was found to increase slightly with increasing the concentration of the extract. The antioxidant capacity of plant extract is comparatively lower than the ascorbic acid. This is only a preliminary study and to make final comment the extract should thoroughly investigated phytochemically and pharmacologically to exploit their medicinal and pharmaceutical potentialities.

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