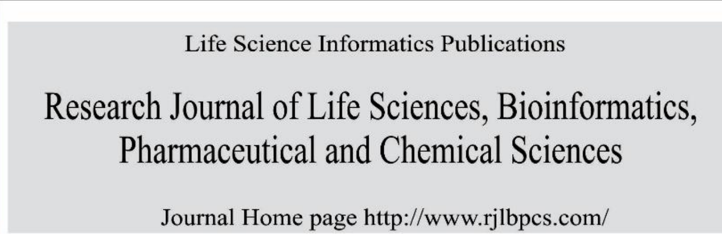

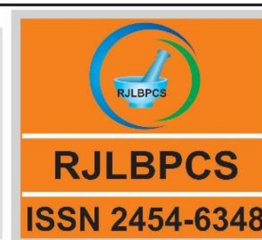

Original Research Article **DOI: 10.26479/2018.0406.03**

EVALUATION OF IN VITRO ANTIOXIDANT POTENTIAL OF *PHYLLANTHUS ACIDUS* FRUIT

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ABSTRACT: *Phyllanthus acidus,* a seasonal tropical plant being used in the traditional medicinal system due to its high bio-efficacy. In the present investigation, it was aimed to evaluate the biopotentials of *P. acidus* by employing *in vitro* experiments. Initially, extraction of *P. acidus* fruits was performed using four different solvents *viz*., hexane, chloroform, ethyl acetate and methanol. Flavonoids, terpenoids and tannins were found to be significant in all the solvent extracts. Antioxidant capacities of fruit extracts were determined. The results ensure the DPPH radical scavenging activity in methanol and ethyl acetate extract which found to be statistically significant when compared with tested solvent systems. It is also worth noting the substantial similarity with the results of ABTS, hydrogen peroxide and nitric oxide activity of ethyl acetate and methanolic factions of *P. acidus* fruit extract where above 90 % of inhibition has resulted at 500µg/ml concentration. Except in scavenging of nitric oxide, where only the methanolic fraction to be significant by recording 75.99 % of scavenging. Thus, the report suggests, the antioxidant potential of *P. acidus* can be an effective therapeutic dietary supplement.

KEYWORDS: *Phyllanthus acidus,* Phyto-medicine, antioxidant activity, radical scavenging activity.

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1.INTRODUCTION

Oxygen is an essential element for aerobic organisms in maintaining cellular homeostasis. Production of energy by respiratory process demands oxygen, which undergoes in forming hydrogen peroxide, hydroxyl and superoxide radicals which exists independently with unpaired electrons in their atomic orbit [1]. They induce oxidative stress [2], and their surplus in the generation led to alter the normal physiological process [3]. Over the years of research efforts in understanding mechanisms of free radicals in changing various biological process have laid to new competent strategies in health and disease management [4]. Advances in the area of preventive medicine are one such strategy, wherein, understanding and use of dietary supplements in prevention/treating of chronic diseases and in reducing risk factors for several diseases [5-6]. *P. acidus*, locally named as gooseberry India, is an edible small yellow berries fruit in the Phyllanthaceae family. Fruits are in loose clusters, which are pale yellow or white, and very sour found most common in South India, Bangladesh and Southeast Asian countries. Some of the medicinal activities of *Phyllanthus* species are antipyretic, anti-inflammatory, analgesic, anti-hepatotoxic and antiviral [7-10]. Fruits of *P. acidus* have reported for a high content of vitamin C, which plays an important role in improving eyesight and memory. It also has a preventive action against diabetes and relief of cough [7]. The antioxidant is one of the essential substances which can protect the body from free radicals generated due to oxidative stress or various physiological functions. To lower the risk of various degenerative diseases, studies on evaluating the antioxidant potential of different plants and dietary supplements is extensively studied [11]. Many studies have been published analyzing the role natural phenolics and flavonoids in treating the various cellular abnormalities posed by oxidative damage [12-14]. With this line of background, the present investigation was designed to assess the free radical scavenging ability of *P. acidus* fruit extract using different *in-vitro* models in search of preliminary experimental evidence to advocate the importance to use *P. acidus* fruits to use as dietary supplements.

2. MATERIALS AND METHODS

2.1 Plant material collection and preparation of the extract

The mature fruits of *P. acidus* were brought from the local market, Mysuru, Karnataka. The nuts were cleaned and washed under running tap water; then the fruits were cut into small pieces followed by shade drying for two weeks. The dried fruits were powdered using a food processer. A soxhlet apparatus was used for the extraction using four different solvents such as hexane, chloroform, ethyl acetate and methanol. For extraction 30 g of the ground fruit powder was loaded to the thimble, and 250 ml of the given solvent was added into the flask and refluxed for 6 h. To ensure the maximum extraction, the process was repeated twice. The extraction process was carried out in the ascending order of the solvent strength, i.e., hexane, chloroform, ethyl acetate and methanol. The obtained extracts were concentrated using a rotary evaporator under reduced

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pressure [15-16].

2.2 Phytochemical analysis

The preliminary phytochemical screening of extracts of *P.acidus* was carried out according to the standard methods. The presence of important phytochemicals like flavonoids, terpenoids, Arthro-quinone, Phenols, saponins, and tannins was evaluated. Standard protocols were employed for the confirmation of the phytochemicals such as flavonoids, tannins and phenolic compounds with ferric chloride test and gelatine test, terpenoids with Libermann Burchard`s analysis, Arthro-quinones with ammonia solution and saponins with the ability to produce stable foam [17].

2.3 Determination of Total Phenolic Content

The total phenolic content of different extracts was measured by FC (Folin–ciocalteau) reagent described by Slinkard and Singleton (1977) [18]. The blue colour was read at 765 nm using spectrophotometer produced by the reaction of phenol with the phosphomolybdate in alkaline condition. The results were expressed as gallic acid equivalence (GAE) in micrograms. 50µl of each sample was incubated with 2.5 ml of FC reagent (1:10 dilution) for three mins. 2ml of Na2CO3 was added to this mixture and incubated for 15 mins at 45°C. Absorbance was measured at 765nm using spectrophotometer using methanol with FC as blank. The assay was conducted concerning the standard Gallic acid concentration. Results were expressed in terms of gallic acid equivalence (GAE) in micrograms [19-20].

2.4 Estimation of Total Antioxidant Assay

2.4.1 Determination of Scavenging Effect on DPPH Radicals

The DPPH free radical scavenging activity of different extracts of *Phyllanthus acidus* was determined according to the method by Brand-Williams *et al.* (1995) [21] with slight modification [22]. The stock solution was prepared by dissolving 24 mg DPPH in 100ml methanol and refrigerated until further use. The working solution was prepared by diluting the DPPH stock solution with methanol to obtain an absorbance of about 0.98 (±0.02) at 517nm [23]. In a test tube, 3ml DPPH working solution was mixed with 100µl plant extract. The mixture was incubated in the dark for 30 mins at room temperature. The absorbance of the samples was measured at 517nm. DPPH with methanol was used as the positive control. Percentage of DPPH radical scavenging was calculated by using the following equation. This was compared with the percentage of radical scavenging activity of ascorbic acid as standard.

$$\text{Scavenging activity (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

Where,

A_0 = absorbance of a standard that was prepared in the same conditions, but without extract,

A_1 = absorbance of plant extract samples.

Ascorbic acid was used as positive control.

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2.4.2 ABTS Assay

Antioxidant activity of *Phyllanthus acidus* extracts as per ABTS decolourisation assay was measured using the method reported by Re *et al*. (1999) [24] with some modification [25-26]. The working solution of ABTS radical was made by reacting ABTS (9.5 ml,7mM) with potassium persulfate (245µl, 100mM), and the volume was made up to 10ml with distilled water. The solution was kept in the dark at room temperature for 18h and then diluted with potassium phosphate buffer (0.1 M pH 7.4) to get an OD of 0.7 at 734 nm. The plant sample was prepared in methanol with dilution 100-500µg/ ml. The sample (10µl) was placed in a test tube and mixed thoroughly with 2.99ml ABTS radical working solution. The absorbance of the resulting clear mixture was recorded at 734nm. The per cent antioxidant activity of the sample was determined using the following formula:

$$\text{Antioxidant activity (\%)} = \frac{(Ac - As)}{Ac} \times 100$$

Where A_c and A_s is the absorbance of the control and the sample, respectively. 10µl of the methanol in place of the sample was used as the control.

2.4.3 Hydrogen peroxide scavenging assay

Hydrogen peroxide is the least reactive molecule among reactive oxygen species and is stable under physiological pH and temperature in the absence of metal ions. It can be generated through a dismutation reaction from superoxide anion by superoxide dismutase; it can produce the radical hydroxyl ion in the presence of metal ions [27]. A solution of 40mM H_20_2 was prepared in phosphate buffer (pH 7.4). The different extracts of *Phyllanthus acidus* fruit extract (1µg/ml) were added to the hydrogen peroxide solution (0.6ml). After 10 minutes of incubation, the absorbance of hydrogen peroxide at 230 nm was determined against a blank solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid was used as the standard.

$$\text{\% scavenging activity} = \frac{\text{(OD of control-OD of test)}}{\text{OD of control}} \times 100$$

2.4.4 Nitric oxide scavenging assay

Nitric oxide radical scavenging activity was measured spectrophotometrically [28] 1.0 ml of sodium nitroprusside in phosphate buffer was mixed with the different concentration of extract mg/ml in phosphate buffer the tubes were then incubated at 25°c for two hours. At the end of the second hour 1.5ml of the reaction mixture was removed and diluted with 1.5ml Greiss reagent (1% sulphanilamide, 2% orthophosphoric acid and 0.1% naphthylethylenediamene dihydrochloride), The absorbance was immediately measured at 546nm. The tube without extract was taken as control

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3. RESULTS AND DISCUSSION

3.1 Phytochemical analysis

Preliminary biochemical screening of various solvent extracts of *P. acidus* fruits suggested the presence of various phytoconstituents which have medicinal properties. As shown in table 1, flavonoids and terpenoids are present in all the studied extracts namely, hexane, chloroform, ethyl acetate and methanol. However, high concentrations were observed in the case of ethyl acetate and methanol. High concentration of phenol was observed in the extracts of ethyl acetate and methanol, but it is absent in the extracts of hexane and chloroform. Arthro-quinone and saponin were absent in all the extracts of *P. acidus*. The presence of tannin was seen in all the extracts except hexane.

Table 1: Phytochemical analysis of *P. acidus* extracts

Phyto-chemical test	Hexane	Chloroform	Ethyl acetate	Methanol
Flavonoids	+	+	++	++
Terpenoids	+	+	+	+
Arthro-quinone	-	-	-	-
Phenols	-	-	++	++
Saponin	-	-	-	-
Tanin	-	+	+	+

3.2 Determination of total phenolic content

Result obtained from the present study suggested that the total phenolic content in selected solvent extracts of *P. acidus* fruit and was expressed as gallic acid equivalent. Fig.1. Reflects the concentration of total phenols which revealed the presence of total phenols in ethyl acetate (0.61 mg GAE/g) and methanolic (0.72 mg GAE/g) extracts and contains the high amount of polyphenols but very less concentration was observed in case of hexane and chloroform extract.

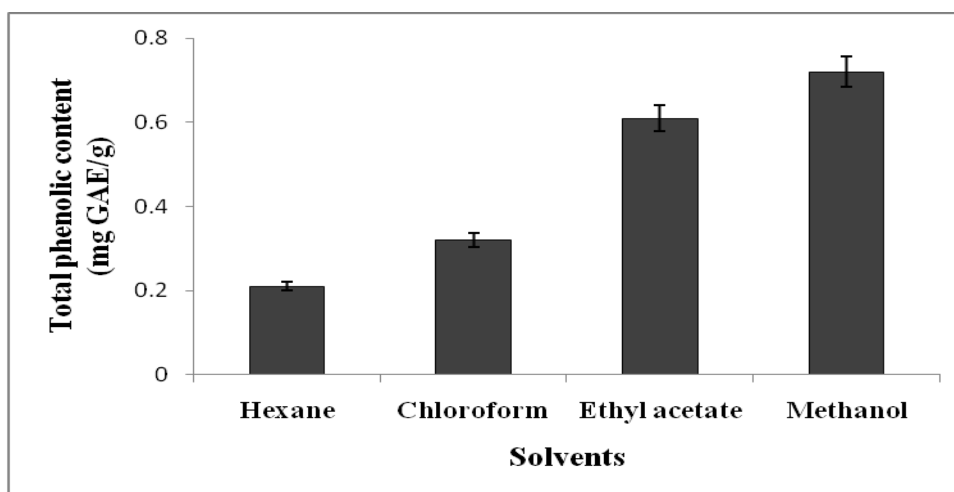

Figure 1: Total phenolic content in different solvent extracts of *P. acidus*

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3.2 Total Antioxidant activities

3.3.1 Determination of DPPH radical scavenging activity

DPPH assay was performed with the different concentration of extracts which were extracted with selected solvents revealed the dose-dependent scavenging ability of *P. acidus* as shown in table 2. Result also concluded that the scavenging activity of methanolic extracts was excellent and found to be almost similar to ascorbic acid. The inhibition activity was 92.20 ± 0.72% at the concentration of 500µg/ml. The scavenging activity of ethyl acetate fraction also showed significant potential but the extracts which were extracted with hexane and chloroform shown lower inhibition activities as compared with ascorbic acid.

Table 2: DPPH radical scavenging activity of *P. acidus*

Sl. No.	Concentration (µg/ml)	DPPH assay (% of Inhibition)				
		Ascorbic acid	*P. acidus*			
			Hexane	Chloroform	Ethyl acetate	Methanol
1.	100	57.93±1.01	11.80±0.78	23.93±0.49	23.94±0.67	44.87±0.94
2.	200	63.18±1.46	26.18±1.49	28.26±1.01	45.59±0.54	59.72±0.72
3.	300	86.22±1.45	30.39±1.52	33.63±2.10	51.96±2.65	72.71±1.21
4.	400	95.23±1.39	33.36±1.15	43.58±1.83	73.77±1.74	78.96±1.64
5.	500	95.92±0.86	36.68±2.22	56.10±2.38	82.18±2.32	92.20±0.72

DPPH radical scavenging activities of *P. acidus* fruit extracts. Values were expressed in % of scavenging, mean ± SE in triplicates, where $p< 0.05$.

3.3.2 Determination of ABTS scavenging activity

The result of antioxidant activity by ABTS method was shown in table 3. Extracts extracted from ethyl acetate and methanol indicated that the highest percentage of inhibition which was 95.62±1.31% and 96.98±0.40% respectively at the concentration of 500 µg/ml and result also support the result obtained in case of DPPH scavenging assay. In case of hexane and chloroform extracts inhibition activity increases with an increase in concentration, but the scavenging potential was less when compared with ethyl acetate and methanolic extracts of *P. acidus* fruit.

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Table 3: ABTS radical scavenging activity of *P. acidus*

Sl. No.	Concentration (µg/ml)	ABTS scavenging assay (% of Inhibition)				
		Ascorbic acid	*P. acidus*			
			Hexane	Chloroform	Ethyl acetate	Methanol
1.	100	77.66±0.76	12.08±0.82	11.92±0.78	40.68±1.27	73.57±2.29
2.	200	85.07±1.01	20.44±2.41	17.11±1.85	54.24±2.59	86.94±1.67
3.	300	86.44±0.99	24.66±0.27	49.91±1.97	71.67±1.63	92.60±1.16
4.	400	92.36±1.57	33.69±1.80	55.04±0.39	91.02±1.20	94.96±0.62
5.	500	96.56±0.55	35.61±0.51	63.67±0.64	95.62±1.31	96.98±0.40

ABTS radical scavenging activities of *P. acidus* fruit extracts. Values were expressed in % of scavenging, mean ± SE in triplicates, where $p < 0.05$

3.3.3 Hydrogen peroxide radical scavenging activity

The antioxidant potential of *Phyllanthus acidus* fruit extracts was investigated by *in vitro* hydrogen peroxide scavenging experiment as shown in Table 3. In this study, selected extracts were subjected to evaluate the ability of different solvent fractions to scavenge the hydrogen peroxide radicals. Results highlighted that the strength of methanolic and ethyl acetate fractions of *Phyllanthus acidus* fruit was significantly at the concentration of 500 µg/ml, i.e. 94.24±0.60% and 97.55±1.09%, when compared with standard ascorbic acid in the process of scavenging hydrogen peroxide radicals.

Table 4: Hydrogen peroxide radical scavenging activity of *P. acidus*

Sl. No.	Concentration (µg/ml)	Hydrogen peroxide radical scavenging assay (% of Inhibition)				
		Ascorbic acid	*P. acidus*			
			Hexane	Chloroform	Ethyl acetate	Methanol
1.	100	68.82±0.99	13.84±0.54	36.73±1.05	68.76±1.38	79.42±1.20
2.	200	76.88±2.36	14.65±1.56	46.52±0.04	74.92±1.47	83.06±1.29
3.	300	88.00±2.02	18.57±1.74	52.73±0.73	81.74±2.08	84.75±1.66
4.	400	92.4±1.66	22.04±0.70	53.64±0.28	91.93±0.89	93.97±2.53
5.	500	97.48±1.75	23.91±0.37	57.02±0.16	94.24±0.60	97.55±1.09

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Hydrogen peroxide radical scavenging activities of *P. acidus* fruit extracts. Values were expressed in % of scavenging, mean ± SE in triplicates, where $p < 0.05$.

3.3.4 Nitric oxide scavenging activity

Result obtained from nitric oxide radical scavenging activity revealed the ability of extracts to compete with oxygen to react with nitric oxide. The results of the investigation also highlighted the dose-dependent scavenging activity of nitric oxide. Among all the experimented fractions, hexane extracts found almost equally potential to scavenge nitric oxide in comparison with ascorbic acid. The radical scavenging activity of other extract was found to be less effective as compared with hexane extracts, but methanolic extracts have potential to inhibit nitric oxide radical.

Table 5: Nitric oxide radical scavenging activity of *P. acidus*

Sl. No.	Concentration (µg/ml)	Nitric oxide scavaging assay (% of Inhibition)				
		Ascorbic acid	*P. acidus*			
			Hexane	Chloroform	Ethyl acetate	Methanol
1.	100	55.70±0.75	56.27±1.59	22.46±0.96	22.32±1.39	60.14±0.91
2.	200	68.66±1.14	67.54±0.79	25.17±0.23	40.56±2.04	63.46±0.96
3.	300	78.12±0.19	75.23±1.44	26.94±1.44	45.90±1.14	65.23±1.72
4.	400	87.55±1.5	88.87±1.10	28.45±2.01	52.36±1.77	69.38±2.28
5.	500	95.19±1.10	96.44±0.93	30.75±1.46	56.88±0.04	75.99±1.40

Nitric radical scavenging activities of *P. acidus* fruit extracts. Values were expressed in % of scavenging, mean ± SE in triplicates, where $p < 0.05$

DISCUSSION

In the present investigation, preliminary results on the screening of phytochemicals revealed the presence of therapeutically essential constituents like terpenoids flavonoids and phenolics in extracts obtained from different solvent fractions. Both the non-polar (hexane and chloroform) and polar (ethyl acetate and methanol) solvents were used in the extraction of phytoconstituents from the fruits of *P. acidus*. Total phenolics in each extract were estimated by using the Gallic acid method which highlights the significant concentration of phenolic compounds in methanolic and ethyl acetate fractions (Figure. 1). Thus, these results support the efficiency of polar solvents in the extraction of the phenolic compound as advocated by many researchers previously [11, 29-31]. Results also suggest the presence of flavonoids in methanol and ethyl acetate fractions in higher concentration when compared with hexane and chloroform fractions. With these experimental pieces of evidence, further extract fractions were subjected to evaluate the role of *P. acidus* fruit extract in scavenging the free radicals using different *in vitro* experimental models. Free radicals are responsible for many diseases though it generates through normal physiological function but when the concentration exceeds the threshold frequency that leads to many conditions.

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Antioxidants are potent candidates for the management and balance the average level of free radicals in the cells. DPPH is the compassionate and widely used method for evaluating the antioxidant potential of any extracts or purified compound. Initially, DPPH was used to assess the ability of *P. acidus* fruit extracts. Selected fruit extracts found to exhibit DPPH scavenging activity in which the inhibitory activity of polar solvent is better as compared with the non-polar solvent.
ABTS is another compound to evaluate antioxidant potential, and the relative antioxidant potential of selected extracts of *P. acidus* fruit to scavenge ABTS was carried out and compared with standard ascorbic acid. The report suggested that polyphenols are present in most of the fruits which quench free radicals and prevent the body from oxidative damage and it is one of the frequently used methods in food and agriculture industries [32]. Result obtained from this result suggested that antioxidant activity of polar compounds is better than non-polar compounds which directly indicate that it is due to the presence of high concentration of polyphenols which were extracted by using polar solvents. Hydrogen peroxide is an oxidising agent and oxidises thiol groups. It readily crosses the cell membrane and reacts with Fe^{2+} and form hydroxyl radicals and cause toxic effects to the cell [33]. The scavenging activity of *P acidus* fruits against hydrogen peroxide was represented in table 3. The extracts exerted the free radical scavenging activity in the concentration depended on the manner in which as the concentration of extracts were increased the inhibitory activity also increases. Again positive results highlighted towards polar solvent extracts compared with extracts extracted through non-polar solvents in which maximum activity 94.24±0.60% and 97.55±1.09 was seen in ethyl acetate and methanol extracts respectively at the concentration of 500 µg/ml. Results also indicate that the extracts extracted with ethyl acetate and methanol are as useful as the standard reference used in the experiment. Nitric oxide is also an important chemical mediator generated by endothelial cells, neurons, macrophages, etc. and help in the regulation of several physiological processes, but excess concentration is lethal [34]. NO scavenging activity was also used to evaluate the antioxidant potency of selected extracts of *P. acidus* fruits. Table 5 showed the NO scavenging activity of selected samples in the order of hexane> methanol> ethyl acetate> chloroform and hexane extracts inhibitory percentage at the concentration of 500 µg/ml compared with other extracts but less than standard ascorbic acid. Our findings suggested that all four selected extracts of *P. acidus* fruit have the property to counteract against the generation of free radicals due to the presence of phenols and flavonoids

4. CONCLUSION

Current investigation reports on preliminary screening of phyto-chemicals and total phenolic content followed by the antioxidant efficiency of the *P. acidus* fruit extracts. The results concluded the efficacy methanolic extract in scavenging the free radicals using different in vitro experimental models. Which confirms the presence of phyto-constituents and total phenolic compounds may be responsible for the antioxidant potential of the methanolic extract.

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CONFLICT OF INTEREST

None

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