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Research Article

# Evaluation of Antioxidant, Cytotoxic and Antimicrobial Activity of *Phyllanthus acidus*

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**ABSTRACT**

The aim of present study was to evaluate antioxidant, cytotoxic and antimicrobial activities of methanolic extracts of pulp and seed of *Phyllanthus acidus*. The antioxidant potential were evaluated in terms of total phenolic content, total flavonoid content and DPPH radical scavenging potential by specific standard procedures. Maximum phenolic (25.672± 0.645 mg gallic acid equivalents/mg of plant extract) and flavonoid (13.893 ± 0.320 mg catechin equivalents/mg of plant extract) contents were found in pulp extract than seed extract. Both the pulp and seed extracts showed the potent antioxidant activity with $IC_{50}$ value of 5.96 µg and 6.79 µg/mL respectively which are very close to the $IC_{50}$ value of standard ascorbic acid having 2.16 µg/mL). The cytotoxic activity was evaluated by using brine shrimp lethality bioassay and compared with vincristine sulfate as standard. The cytotoxicity exhibited by both extract was promising with $LC_{50}$ value of pulp and seed were 6.25 µg/mL and 6.7925 µg/mL respectively comparing with the $LC_{50}$ value 0.4687525 µg/mL of standard vincristine sulphate as a positive control. Furthermore, the extracts were examined for antimicrobial activity against a panel of microorganisms where both the extracts CMEP and CMES showed mild to moderate antimicrobial activity. Moreover, CMEP have exhibited highest zone of inhibition which was 12mm against *Pseudomonous aeruginosa*. The results suggest into the plant extracts could be used as a potential therapeutics in many pathological conditions.

**Keywords:** *Phyllanthus acidus.* CMEP, CMES, Antioxidants, brine shrimp lethality bioassay, antimicrobial activity.

## INTRODUCTION

The plant kingdom has been the best source of remedies for curing a variety of diseases. This is why medicinal plants have been played a key role in the worldwide maintenance of health. Natural products of higher plants are an important source of therapeutic agents; therefore, many research groups are currently screening the different biological activities of plants[1-3]. Approximately 20% of the plants found in the world have been submitted to pharmacological or biological tests[4]. The rapid emergence of multiple drug resistant strains of pathogens to current antimicrobial agents has generated an urgent intensive search for new antibiotics from medicinal plants. Many medicinal plants have been screened extensively for their antimicrobial potential worldwide[5-7]. Cytotoxic screening of plants is the preliminary methods to identify active compounds of plants[8,9]. In addition a greater interest in the antioxidant activity of plant extracts exists because of free radicals (e.g. reactive oxygen species) that can be responsible for several diseases, for example, heart disease, stroke, arteriosclerosis and cancer, as well as the aging process[10]. Plants (fruits, vegetables, medicinal herbs) contain a wide variety of free radical scavenging molecules, such as phenolic compounds, vitamins, terpenoids and some other endogenous metabolites, that are rich in antioxidant activity[11-14]. Plants belonging to genus *Phyllanthus* (Euphorbiaceae) produce useful secondary metabolites such as alkaloids, tannins, flavanoids, lignans, phenolics and terpenes[15]. *Phyllanthus acidus*, locally named as Arbaroi in Bangladesh and gooseberry or star gooseberry in India, is an edible small yellow berries fruit in the Phyllanthus family. Fruits are borne in loose clusers, are pale yellow or white, waxy, crisp and juicy, and very sour, found in Bangladesh, South India, and Southeast Asian countries[16-19]. Several parts of these plants have been used in folk medicine. The roots and seeds are cathartic. The fruit is a liver tonic and a blood purifier and is used in several vitialed conditions of jaundice, bronchitis, constipation and piles in Ayurvedic system of medicine[20]. The leaves are useful to treat fever, piles, small pox, blood vomiting, itching and gum infection[21]. Several therapeutic properties including antiviral[22], antibacterial[23], neuroprotective[24], antifibrosis[25], and anticancer[26] activities have also been reported for *Phyllanthus acidus.* Extensive evaluation of traditional medicine for various medicinal activities is an obligatory step in the isolation and characterization of the active principle and further leading to drug development. In view of these, this study is therefore designed to evaluate the antibacterial, antioxidant and cytotoxic activities of *Phyllanthus acidus.*

## MATERIALS AND METHODS

*Collection and Identification of the Plant Sample*

Ripe fruit of *Phyllanthus acidus* were collected in the vicinity of Mirpur, Dhaka Bangladesh during the month

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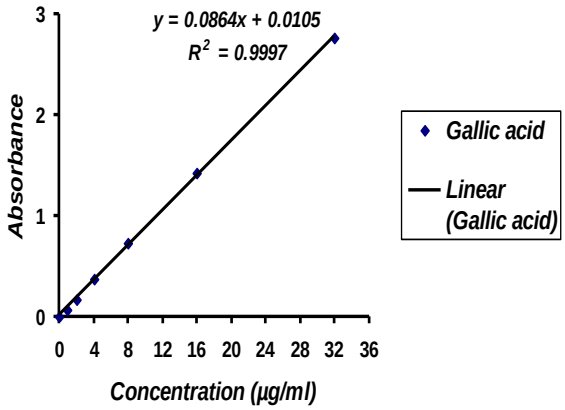


Figure 1: Standard curve of gallic acid for the determination of total phenolic content.

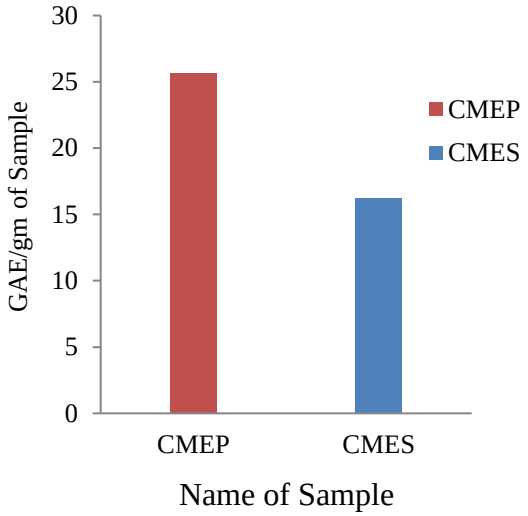


Figure 2: Total phenolic content (mg/gm plant extract in Gallic acid equivalent) of the CMEP and CMES of *Phyllanthus acidus.*

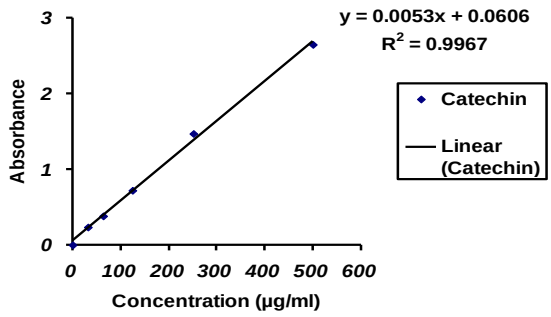


Figure 3: Standard curve of catechin for the determination of total flavonoids.

March 2014. The plant was identified by an expert taxonomist.

*Preparation of Plant Sample*

After collecting, the pulps and seeds were washed thoroughly in tap water and shade dried for several days with occasional sun drying. These were then dried in an oven for 24 hours at considerably low temperature (not more than 45ºC) for better grinding. Dry samples of fruits were ground into a fine powder in a grinding mill. The coarse powder was then stored in an air tight container and kept in cool and dry place for further use.

*Extraction and Solvent Evaporation*

The powdered plant materials were extracted by cold extraction process. Powdered plant materials were taken in an amber colored reagent bottle and soaked in 500mL of methanol. The bottle with its contents were sealed and kept for period of about 7 days with occasional shaking and stirring. The whole mixture was then filtered through cotton and Whatman no.1 filter paper and was concentrated with a rotary evaporator under pressure at 50ºC temperature to afford crude extract known as crude methanolic extract (CM).

*Determination of Total Phenolics*

Total phenolic content of methanolic extracts of pulp and seed of *Phyllanthus acidus* were determined employing the method as described by Singleton *et al.*,1965[27] involving Folin-Ciocalteu reagent as oxidizing agent and gallic acid as standard. Firstly, 0.5 ml of plant extract or standard of different concentration solution was taken in a test tube and 2.5 ml of Folin – ciocalteu (Diluted 10 times with water) reagent solution was added into the test tube. Then 2.5 ml of Sodium carbonate (7.5%) solution was added and incubated for 20 minutes at $25^0$C to complete the reaction. Then the absorbance of the solution was measured at 760 nm using a spectrophotometer against blank. A typical blank solution contained all reagents except plant extract or standard solution.

*Determination of Total Flavonoids*

Total flavonoid content was determined by following the procedure by Dewanto *et al.*, 2002[28]. Catechin was used as standard and the flavonoid content of the extracts were expressed as mg of catechin equivalent/gm of dried extract. Firstly, one milliliter of aqueous extract containing 0.1 g/ml of dry matter was placed in a 10 ml volumetric flask, then 5ml of distilled water added followed by 0.3ml of 5% $NaNO_2$. After 5 minutes, 0.6 ml of 10% $AlCl_3$ was added and volume made up with distilled water. The solution was mixed and absorbance was measured at 510 nm.

*Antioxidant Assay*

*DPPH (1, 1-diphenyl-2-picrylhydrazyl) Radical Scavenging Assay*

The antioxidant activity of different fractions and isolated compounds were determined in terms of hydrogen donating ability, using the DPPH method with a minor modification[28,29]. Firstly, 2 ml of methanol solution of plant extract or standard at different concentration was taken in a test tube. Then 3 ml of methanol solution of DPPH was added into the test tube. The test tube was incubated at room temperature for 30 minutes in dark place to complete the reaction. Then the absorbance of the solution was measured at 517 nm using a spectrophotometer against blank. A typical blank solution contained all reagents except plant extract or standard solution.

Table 1: Absorbance of Gallic acid at different concentrations after treatment with Folin-Ciocaltue reagent.

| Concentration (µg/ml) | Absorbance | | | Absorbance Mean ± STD |
|---|---|---|---|---|
| | a | b | C | |
| 1 | 0.078 | 0.075 | 0.076 | 0.076±0.001 |
| 2 | 0.176 | 0.171 | 0.181 | 0.176±0.005 |
| 4 | 0.364 | 0.368 | 0.372 | 0.368±0.004 |
| 8 | 0.722 | 0.718 | 0.726 | 0.722±0.004 |
| 16 | 1.413 | 1.417 | 1.423 | 1.417±0.005 |
| 32 | 2.758 | 2.752 | 2.764 | 2.758±0.006 |

*Determination of Total Antioxidant Capacity*

Total antioxidant capacity was measured spectrophotometrically through phosphomolybdenum method by Prieto *et al.,* (1999)[30] with some modifications. An aliquot of 0.5 ml of sample solution was combined with 3ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 1% ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95ºc for 10 minutes. After the sample had cooled to room temperature, the absorbance of aqueous solution of each was measured at 695 nm against a blank. A typical blank solution contained 3 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions. Catechin, Ascorbic acid, ∞ tochopherol can be used as standard.

*Cytotoxicity Studies*

Brine shrimp lethality bioassay (Mclaughlin, 1990; Persoon, 1980)[31,32] is taken to determine cytotoxicity. In this study dimethyl sulfoxide (DMSO) was used as a solvent and negative control while vincristine sulfate served as a positive control. For the experiment, 2 mg of sample was dissolved in 400µl DMSO and solutions of varying concentrations (10, 20, 40, 80 and 160 µg/ml) were obtained by serial dilution technique.

*Antimicrobial screening*

The plant extracts were tested for antimicrobial activities by the Disc Diffusion Method (Bauer AW *et al*, 1996)[33].

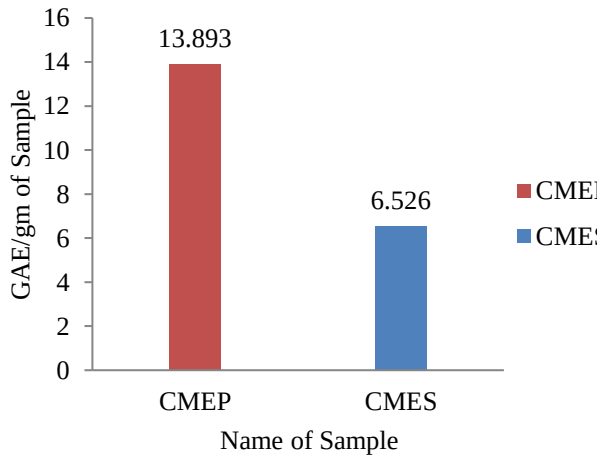


Figure 4: Total Flavonoid content (mg/gm plant extract in catechin equivalent) of the CMEP and CMES of *Phyllanthus acidus.*

Test organisms were collected from the Microbiology research laboratory, Department of Pharmacy, Southeast University. To perform this test the extracts were dissolved in the same solvent used for their extraction and sterilized by 0.22µm sterile Millipore filter. Then 100 µl inoculums ($10^6$CFU/ml) was spread on the surfaces of the agar plate prepared for the growth of bacteria. The discs (6 mm in diameter) contain 10 µl of the extracts as 100mg, 200mg and 500mg per disc was placed on the surface of the inoculated media. Standard Kanamycin disc (30µg/disc) was used as positive control and negative controls were prepared with the same solvents used to dissolve the sample extracts. The zone of inhibition generated around each disc is carefully measured to determine the antimicrobial activity. The experiment for this activity is performed three times.

## RESULTS AND DISCUSSIONS

*Determination of Total Phenolic Content*

The total phenolic content of the methanolic pulp extract (CMEP) & seed extract (CMES), calculated from the calibration curve ($R^2$= 0.9997), were 25.672 ± 0.645 & 16.220 ± 4.344 GAE/gm of dried sample showed that CMEP yielded 25.672 ± 0.645 GAE/gm (gallic acid equivalent/gm) of dried sample while CMES yielded 16.220 ± 4.344 GAE/gm of dried sample (Table 1, 2 and figure 1, 2). The result demonstrated that the total phenolic content of CMEP is higher than that of CMES.

*Determination of Total Flavonoids*

Quantitative determination of total flavanoids was done on the basis of a standard curve of catechin (R2=0.9967) and the otal flavanoid content of CMEP & CMES were 13.893 & 6.526 mg of Catechin equivalent/gm of dried extract respectively (Table 3, 4 and figure 3, 4). The methanolic extract of the pulp contents of flavanoids in comparison to seed is given in figure 3.4.

*Antioxidant activity Determination*

*DPPH Radical Scavenging Activity*

Plant rich in secondary metabolites including phenolics, flavanoids, have antioxidant activity due to their redox properties and chemical structures. The methanolic pulp & seed extracts, both had strong antioxidant activity against the DPPH free radical scavenging activity. CMEP had $IC_{50}$ value of 5.96 µg/ml, which is closer to the Ascorbic acid (Standard) while CMES showed DPPH radical scavenging activity with $IC_{50}$ value of 6.79 µg/ml. (Table 3 and figure 5, 6).

*Total antioxidant activity*

The assay was based on the reduction of Mo ((VI) to Mo (V) by the test agents and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. The total antioxidant activity was measured and compared among crude methanolic pulp (CMEP) and seed (CMSE) extract of *P. acidus* and catechin (standard) at different concentration. The high absorbance values indicated that the sample possessed significant antioxidant activity. The results revealed that both of the CMEP and CMES had significant antioxidant activity and the effects increased with increasing concentration (table. 6 and figure 7). The absorbance value of CMEP and CMES at 100 µg/ml

Table 2: Determination of total phenoliccontent of CMEP & CMES of *P. acidus.*

| Sample | No. of sample | Concen-tration (µg/ml) | Absorbance | GAE/gm of dried sample | GAE/gm of dried sample Mean ± STD |
|---|---|---|---|---|---|
| Crude methanolic | 1. | 500 | 2.229 | 25.67 | |
| Extract of pulp | 2. | 500 | 2.226 | 25.64 | 25.672± 0.645 |
| | 3. | 500 | 2.231 | 25.70 | |
| Crude methanolic | 1. | 500 | 1.412 | 16.22 | |
| Extract of seed | 2. | 500 | 1.408 | 16.17 | 16.220± 4.344 |
| | 3. | 500 | 1.415 | 16.25 | |

Table 3: Absorbance of catechin (standard) at different concentrations for quantitative determination of total flavonoids.

| Concentration (µg/ml) | Absorbance | | | Absorbance |
|---|---|---|---|---|
| | a | b | C | Mean ±STD |
| 31.25 | 0.241 | 0.225 | 0.260 | 0.242 ± 0.017 |
| 62.5 | 0.380 | 0.398 | 0.362 | 0.380 ±0.018 |
| 125 | 0.726 | 0.722 | 0.731 | 0.726 ± 0.004 |
| 250 | 1.476 | 1.481 | 1.468 | 1.475 ± 0.006 |
| 500 | 2.667 | 2.670 | 2.599 | 2.645 ± 0.040 |

Table 4: Determination of total flavonoid content of the Crude methanolic extract of pulp and crude methanolic extract of seed.

| Sample | No. of sample | Conc. (µg/ml) | Absorbance | GAE/gm of dried sample | GAE/gm of dried Sample Mean ± STD |
|---|---|---|---|---|---|
| Crude methanol | 1. | 500 | 0.793 | 13.818 | |
| extract of pulp | 2. | 500 | 0.797 | 13.894 | 13.893 ± 0.320 |
| | 3. | 500 | 0.801 | 13.969 | |
| Crude methanol | 1. | 500 | 0.403 | 6.46 | |
| extract of seed | 2. | 500 | 0.407 | 6.53 | 6.526 ± 0.155 |
| | 3. | 500 | 0.410 | 6.59 | |

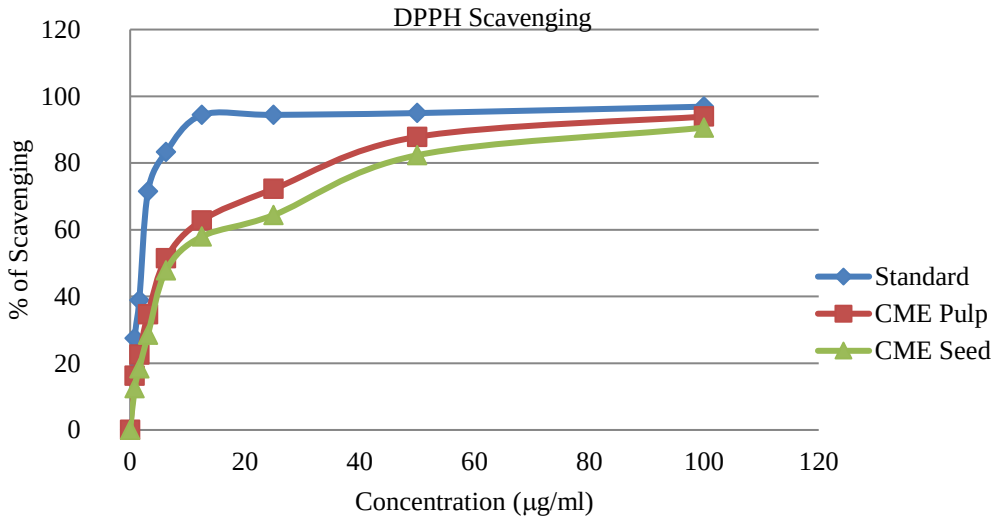


Figure 5: DPPH radical scavenging activity of different fruit parts of crude methanolic extract of *Phyllanthus acidus* at different concentrations in comparison to Standard (Ascorbic acid)

were 2.202 ± .172and 1.661 ± .109 respectively, which demonstrated that the total antioxidant activity of CMEP is higher than that of CMES.

*Cytotoxicity study*

The $LC_{50}$ values of CMEP of *P. acidus* were 3.8. At the conc. of 12.5 µg/ml, 70 % nauplii died but above the 50 µg/ml concentration, all are toxic which indicates very good cytotoxic effects. The $LC_{50}$ values of CMES of *P. acidus* were 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.* (figure 7, 8)

*Antimicrobial assay*

Table 5: DPPH radical scavenging activity of the Ascorbic acid (Standard) and different fruit parts of crude methanolic extract of *Phyllanthus acidus* at different concentrations.

| Name of sample | Conc. (µg/ml) | % of scavenging | | | % of scavenging Mean ± STD | $IC_{50}$ (µg/ml) |
|---|---|---|---|---|---|---|
| | | a | b | c | | |
| Ascorbic acid (standard) | 0.8 | 26.36 | 27.29 | 28.81 | 27.49±1.23 | |
| | 1.6 | 39.4 | 37.61 | 39.7 | 38.90±1.05 | |
| | 3.1 | 73.23 | 69.86 | 71.33 | 71.47±1.69 | |
| | 6.25 | 85.11 | 81.54 | 83.18 | 83.28±1.19 | 2.16 |
| | 12.5 | 95.23 | 94.5 | 95.19 | 94.97±0.37 | |
| | 25 | 94.15 | 95.72 | 96.38 | 94.43±1.12 | |
| | 50 | 95.18 | 94.52 | 93.41 | 94.37±0.89 | |
| | 100 | 96.54 | 98.49 | 95.64 | 96.89±1.425 | |
| CME Pulp | 0.8 | 15.23 | 16.14 | 16.94 | 16.20±0.86 | |
| | 1.6 | 23.89 | 22.53 | 21.37 | 22.60±1.26 | |
| | 3.1 | 34.57 | 33.29 | 36.25 | 34.70±1.48 | |
| | 6.25 | 50.53 | 51.56 | 52.18 | 51.42±0.83 | 5.96 |
| | 12.5 | 62.61 | 61.51 | 64.2 | 62.77±1.35 | |
| | 25 | 72.95 | 70.42 | 73.25 | 72.21±1.42 | |
| | 50 | 87.09 | 86.95 | 89.48 | 87.84±1.27 | |
| | 100 | 93.20 | 93.41 | 95.11 | 93.90±0.96 | |
| CME Seed | 0.8 | 11.67 | 13.54 | 12.38 | 12.53±0.94 | |
| | 1.6 | 18.67 | 19.24 | 17.49 | 18.47±0.88 | |
| | 3.1 | 28.65 | 27.55 | 29.31 | 28.50±0.88 | 6.79 |
| | 6.25 | 47.57 | 47.61 | 48.16 | 47.78±0.30 | |
| | 12.5 | 58.49 | 59.17 | 56.32 | 57.97±1.43 | |
| | 25 | 67.22 | 69.29 | 65.73 | 64.41±1.78 | |
| | 50 | 82.37 | 82.44 | 82.17 | 82.33±0.14 | |
| | 100 | 90.23 | 91.74 | 89.84 | 90.60±0.95 | |

Table 6: Total antioxidant activity of crude methanolic pulp (CMEP) and seed (CMSE) extract of *P. acidus* and catechin (standard) at different concentration.

| Name of sample | Concentration (µg/ml) | Absorbance | | | Absorbance Mean ± STD |
|---|---|---|---|---|---|
| | | a | B | C | |
| Catechin (standard) | 5 | 0.412 | 0.412 | 0.420 | 0.415± .023 |
| | 10 | 0.669 | 0.678 | .680 | 0.675± .027 |
| | 20 | 1.139 | 1.147 | 1.152 | 1.146± .038 |
| | 40 | 1.552 | 1.561 | 1.563 | 1.559± .227 |
| | 80 | 2.074 | 2.080 | 2.083 | 2.079 ± .567 |
| | 100 | 2.249 | 2.250 | 2.255 | 2.251 ± .069 |
| Crude methanolic extract of pulp (CMEP) | 5 | 0.382 | 0.392 | 0.394 | 0.389 ± .009 |
| | 10 | 0.647 | 0.652 | 0.654 | 0.649 ± .052 |
| | 20 | 0.937 | 0.947 | 0.949 | 0.944 ± .006 |
| | 40 | 1.316 | 1.319 | 1.328 | 1.321 ± .019 |
| | 80 | 1.849 | 1.853 | 1.855 | 1.852 ± .136 |
| | 100 | 2.196 | 2.204 | 2.207 | 2.202 ± .172 |
| Crude methanolic extract of seed (CMES) | 5 | 0.363 | 0.359 | 0.370 | 0.364 ± .013 |
| | 10 | 0.541 | 0.550 | 0.547 | 0.546 ± .069 |
| | 20 | 0.759 | 0.765 | 0.758 | 0.761 ± .067 |
| | 40 | 1.137 | 1.147 | 1.152 | 1.145 ± .056 |
| | 80 | 1.482 | 1.491 | 1.493 | 1.489 ± .061 |
| | 100 | 1.656 | 1.660 | 1.667 | 1.661 ± .109 |

In the antimicrobial screening, the extracts showed moderate antibacterial activity where zone of inhibition were between 8-12 mm (Table 7). Most inhibitory activity was noticed with CMEP against the growth of *Bacillus cereus* with the zones of inhibition 13 mm. In contrast, the CMES extract showed zone of inhibition between 8.5 - 11.5 mm (Table 7). The concentrations above 500 µg/disc, also showed moderate effect against a number of bacteria including *Shigella dysenteriae, Salmonella typhi, Vibro cholera, Pseudomonous aeruginosa, Staphylococcus aureus, Bacillus cereus, Escherichia coli.*

Table **7**: Susceptibility to different microbial organisms and their range (zone of inhibition) for CMEP & CMES of *Phyllanthus acidus*.

| Name of Bacteria strain | Zone of inhibition (mm) CMEP | | | Zone of inhibition (mm) CMES | | | Kanamycin |
|---|---|---|---|---|---|---|---|
| | 100µg/disc | 200 µg/ disc | 500µg/disc | 100µg/disc | 200 µg/ disc | 500µg/ disc | 30µg/ disc |
| *Shigella dysenteriae* | NS | NS | 10.5 | NS | NS | 9 | 27 |
| *Salmonella typhi* | NS | NS | 8 | NS | NS | 8.5 | 22 |
| *Vibro cholera* | NS | NS | 9.5 | NS | NS | 11.5 | 28 |
| *Pseudomonous aeruginosa* | NS | NS | 12 | NS | NS | 10.5 | 30 |
| *Staphylococcus aureus* | NS | NS | 11.5 | NS | NS | 9.5 | 27 |
| *Bacillus cereus* | NS | NS | 13 | NS | NS | 11 | 18 |
| *Escherichia coli* | NS | NS | 12 | NS | NS | 10 | 27 |

Here, NS= Not susceptible.

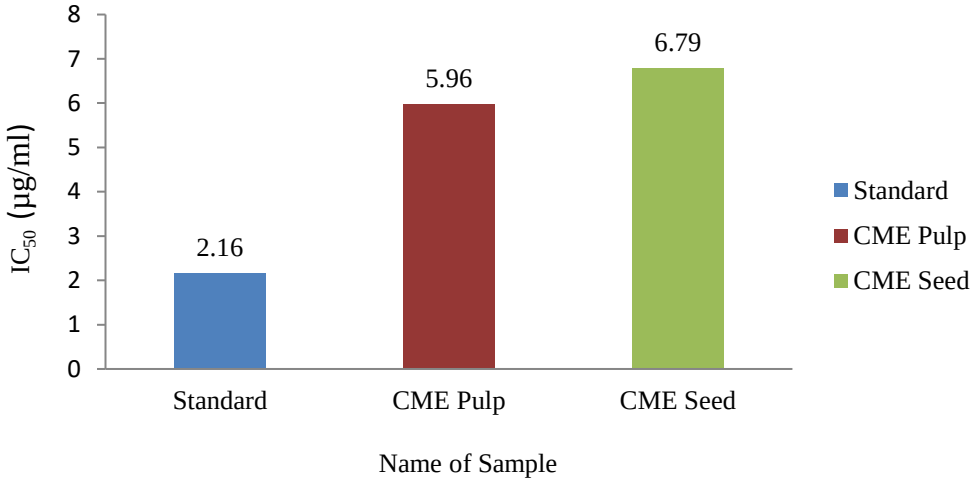


Figure 6: $IC_{50}$ value of different fruit parts of crude methanolic extract of *Phyllanthus acidus* in comparison to Standard (Ascorbic acid) from DPPH scavenging activity.

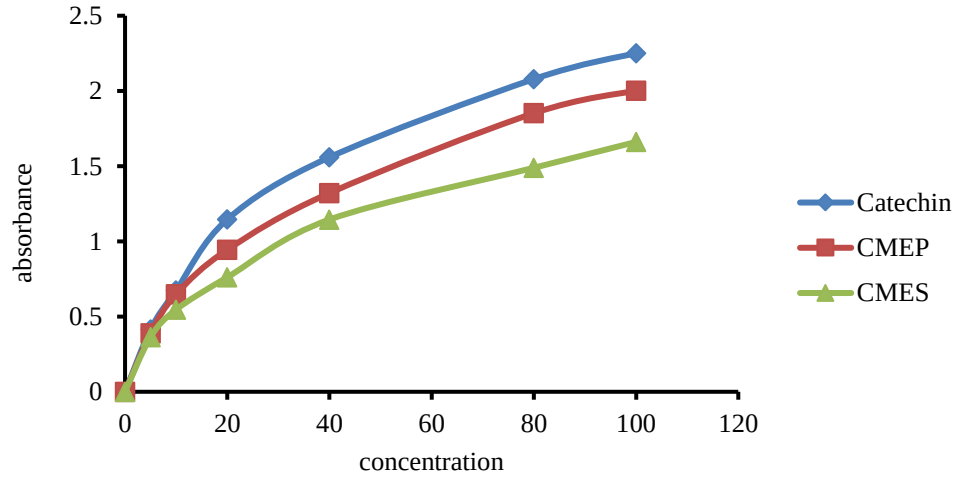


Figure 7: Total antioxidant activity of crude methanolic pulp (CMEP) and seed (CMSE) extract of *P. acidus* and catechin (standard) at different concentration.

## CONCLUSION

In the present study crude methanolic extracts of pulp and seeds of *Phyllanthus acidus* were investigated for antioxidant, cytotoxic and antimicrobial activity. Total phenolic content and total flavanoids content were found greater in pulp than seeds. In addition crude methanolic extract of pulp was potentially active in free radical

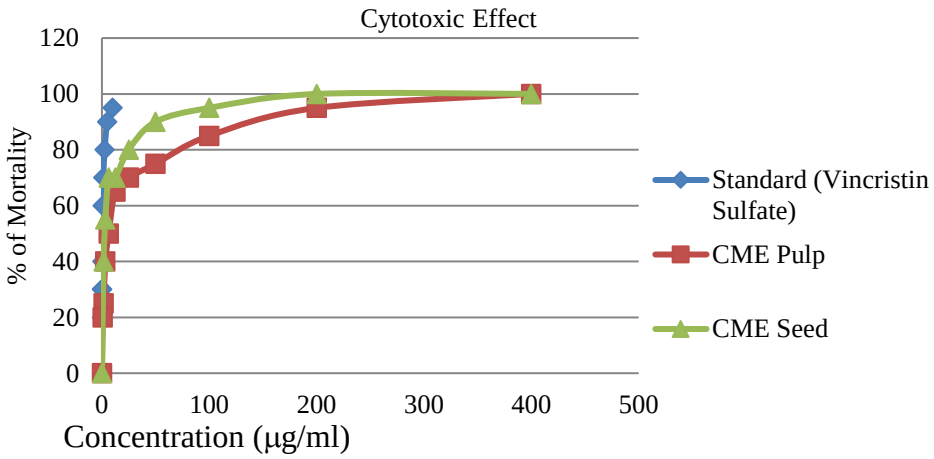


Figure 8: Effect of CMEP and CMES of *Phyllanthus acidus* on brine shrimp nauplii after 34 hours of incubation in comparison to Vincristin sulphate (Standard).

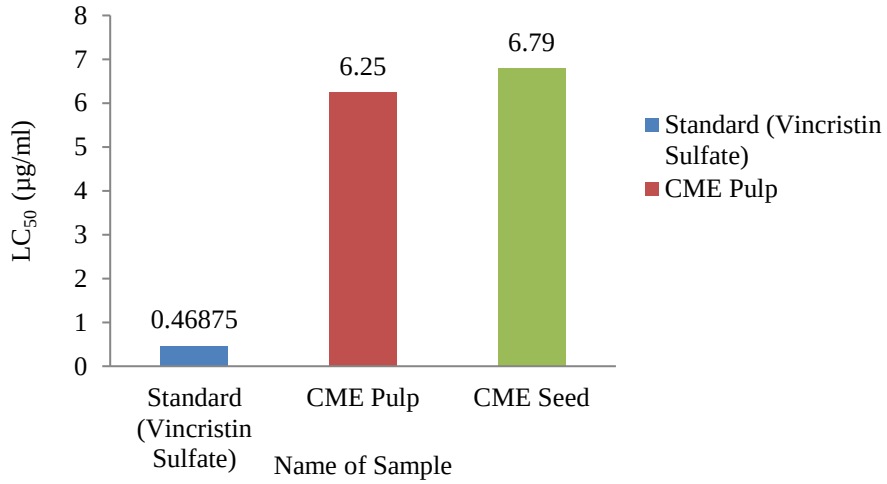


Figure 9: $LC_{50}$ values of different extracts of *Phyllanthus acidus* on brine shrimp viability.

scavenging activity with $IC_{50}$ value of 5.96 µg/ml, which is closer to the Ascorbic acid (Standard) while seeds showed $IC_{50}$ value of 6.79 µg/ml. These extracts may be rich in radical scavengers. Furthermore both the extracts of pulp and seeds showed cytotoxicity having $LC_{50}$ value 6.25µg/ml and 6.79µg/ml respectively when compared with vincristine sulphate. Besides they also showed moderate antimicrobial activity against a range of pathogenic bacteria. Further investigations on the chemical compositions of those extracts are needed to characterize them. To sum up we can say that this plant may serve as potential source of biologically important drug candidates.

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