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Study of Antioxidant, Antidiabetic and Antibacterial Activities of Mangrove Plant *Phoenix Paludosa*.

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ABSTRACT

Leaf and stem ethanol extracts of Sundarbans mangrove plant *Phoenix paludosa* Roxb were explored for its antioxidant, antidiabetic and antibacterial activities by using different methods. After phytochemical investigation, the leaf extract showed higher DPPH and hydrogen peroxide radical scavenging activities than the stem extract. In ferric reducing and reducing power capacity assays, both leaf and stem extract exhibited dose-dependent reducing capacity but stem extracts showed the marked response. Moreover, total phenol (TP) in mg GAE/g extract, total flavonoid (TF) in mg QE/g extract and total tannin (TT) in mg GAE/g extract contents are higher in stem extracts (TP:185.42±1.62; TF:70.01±0.19; TT:30.31±0.18) than leaf extract (TP: 176.22±1.06; TF:61.79± 0.09; TT:27.97±0.14). The stem extracts predominantly ($p<0.05$) reduced the blood glucose level at the dose of 500mg/kg in glucose-induced hyperglycemic mice. The leaf extracts didn't show any anti hyperglycemic activity. The stem extract showed significant antibacterial activity that means the zone of inhibition (ZOI) against all tested microorganisms with more potent activity against *Salmonella typhi* (ZOI: 15.00mm) than leaf extract. The present study reveals the potential of stem extracts of *P. paludosa* as the antioxidant, antidiabetic and antibacterial agent than leaf extract.

Keywords: *Phoenix paludosa* Roxb, Antioxidant, Anti hyperglycemic, Antibacterial.

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INTRODUCTION

Nature contains structurally diverse array of compounds that help to meet the global demand for new therapeutically active substances. Plants, in general, have traditionally been recognized as the main source of novel bioactive compounds (Salini et al., 2015). The mangroves are an enriched source of natural products from where researchers have isolated various bioactive compounds (Akter et al., 2016). Demographically, mangrove is a source of food, industry matter and ethno medicine. We can utilize mangrove plant to treat diarrhea, cough, ulcer, hepatitis and infection. Moreover, these can be used as insecticide and pesticide. Some previous research indicates that mangrove plant has antibacterial, antifungal, antioxidant, anticancer, and antidiabetic activities. According to the research report, mangrove plant contains steroid, triterpene, saponin, flavonoid, alkaloid and tannin (Hardoko et al., 2012). These natural medicines are in great demand both to promote wellness and for healing purpose. Free radicals create barriers to sustain a well life in every country. Insulin resistance and diabetes are caused by free radicals as reactive oxygen species (Fatima et al., 2012). And here, antioxidants prevent radicals induced disorders. Many hypoglycemic compounds possess antioxidant properties (Coman et al., 2012). Antioxidants from natural sources can be another source of prevention or healing of diabetes and other diseases. According to the functions of antioxidants in the body, they are classified into preventive antioxidants, radical-scavenging antioxidants and repair and de novo antioxidants (Noguchi and Niki, 1999). In recent years, scientists are engaged to replace synthetic antioxidants with natural antioxidants from plant materials due to the carcinogenic properties of the synthetic antioxidants (Sasaki et al., 2002). In this study, the mangrove plant *P. paludosa* has been chosen to scientifically validate the usage of the plant in traditional medicine or ethno medicine. The two parts of the plant as leaf and stem are used for these study. *P. paludosa* grows all over the sundarbans in Bangladesh and commonly known as hantal. The genus Phoenix is reported to have diuretic, analgesic, ameliorative, antioxidant and anti mutagenic activities (Alam F. et al., 2009). Conventionally, *P. paludosa* is well recognized for its antipyretic and anti-inflammatory action in different areas of Bangladesh. In cosmetic industries, *P. paludosa* is also used (Saha S. et al., 2012). Therefore, the aim of this present study was to search antioxidant, antidiabetic and antibacterial activities of these plant parts of sundarbans by using various methods.

MATERIALS AND METHOD

Drugs and Chemicals

Aluminium chloride, 2, 2-Diphenyl-1-picryldydrazyl (DPPH), ascorbic acid, gallic acid, quercetin, butylated hydroxy toluene were purchased from Merck, Germany. FC reagent

(Folin-Ciocalteau) was bought from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). Glibenclamide hydrochloride was obtained from Square Pharmaceuticals Ltd, Bangladesh. Including the solvents used, all other chemicals, were of analytical grade.

Plant materials and extraction

The leaf and stem of *P. paludosa* were collected from the mangrove forest of Khulna region, Bangladesh and identified by the experts of Bangladesh National Herbarium, Dhaka (Accession no. DACB- 42133). Cold extraction process in ethanol was used to get the extract. A refrigerator is used to store the crude extract at 4°C until experiment to commence.

Test microorganisms

Ten pathogenic bacterial strains (Four gram positive bacteria and six gram negative bacteria) were taken from Microbiology Laboratory of Pharmacy Discipline, Khulna University, Bangladesh.

Experimental animals

Swiss-albino mice of 4-5 weeks age and weighing 20-25g were procured from Jahangirnagar University, Savar, Dhaka-1342, Bangladesh. The mice were housed in polypropylene cages under pathogen free condition at an ambient temperature of 24 ± 1^0C; 12 h light/dark cycle with 55 ± 5 % controlled relative humidity.

Acute toxicity study

Animals were separated into five groups and each group contained ten animals. A stomach tube was used to administer different doses (250, 500, 1000, 2000, and 4000 mg/kg) of ethanol extract to mice. Then the mice were seen for general signs of toxicity.

Phytochemical screening

Different standard qualitative chemical test of two extracts were carried out to recognize different phytochemical constituents such as carbohydrates, alkaloids, tannins, steroids, gums, glycosides, phenolic compounds, flavonoids, and saponins (Ghani, 2005).

Evaluation of Antioxidant activity

1. DPPH radical scavenging activities

After performing Qualitative free radical antioxidant activity, the in vitro antiradical activity was quantitatively determined by the reported method (Sharma and Bhat, 2009) with minor modification. At first, 1ml of extract solution from each concentration (512, 256, 128, 64, 32, 16, 8, 4, 2, 1 µg/mL) was added to the 3ml of immediately prepared 0.004% w/v DPPH solution. The mixture was allowed to stand in dark place for 30minutes at ambient room temperature and then absorbance was taken at 517nm. Here, the standard was ascorbic acid. The antiradical activity of the extract and standard was calculated using the formula: % discoloration = $[(Abs_0-Abs_1)/Abs_0] \times 100$; where Abs_0 and Abs_1 are the absorbance of control

and absorbance of extract or standard respectively. Extract concentration that provides 50% discoloration of DPPH solution (IC_{50} value) was measured from the obtained data.

2. Hydrogen peroxide (H_2O_2) radical scavenging assay

Hydrogen peroxide (H_2O_2) radical scavenging assay was conducted based on the reported method (Nabavi *et al.*, 2009). Different concentrations of standard and extracts solution were prepared and added to a H_2O_2 (6 ml, 40 mM) solution. From this mixture, 1 ml was drawn and put into test tube. Then, phosphate buffer (3 ml) was taken to each mixture. Ten minutes later, absorbance of hydrogen peroxide was observed at 230 nm. The result was measured by the equation of: % Scavenged = $[(Ao - A_1)/Ao] \times 100$; ($A_1$ and Ao indicate the absorbance of standard or extract and absorbance of control respectively).

3. Reducing power assay

The reducing power of extracts was determined by a established method (Oyaizu *et al*., 1986) with modifications. At first, 2.5ml phosphate buffer (0.2mol/L; PH 6.6) and 2.5ml potassium ferricyanide solution (1% w/v) were mixed to 1ml of various concentrations of extract solution. Then mixture was incubated (50°C) for 20 minutes. After cooling at room temperature, 2.5ml trichloro acetic acid (10% w/v) was added to the mixture. The mixture was centrifuged at 3000 rpm. for 10minutes. 2.5ml aliquot of supernatant was mixed with 2.5ml distilled water and 0.50ml 0.1% w/v ferric chloride with continuous shaking. 10minutes later the absorbance of the resultant chromospheres was taken at 700nm. Butylated Hydroxy Toluene (BHT) was used to compare the reducing power of the extracts. When absorbance increases, we mean that reducing capacity of extracts increases.

4. FRAP (Ferric reducing antioxidant power) assay

The Ferric Reducing Antioxidant power of ethanol extract of plants was determined by the reported method (Jaslin *et al.*, 2011). After preparing sample solution, 0.15 ml solution of standard and extracts were taken to make reaction with FRAP solution (2.85 ml) especially in the dark condition. After 30 minutes, absorbance was observed at 593 nm.

5. Determination of Total phenol, Flavonoids and Tannin content

The total phenol content of the extracts was calculated by the Folin-Ciocalteu technique (Wolfe *et al*., 2003). After mixing 0.5 ml extract solution with 5 ml 1:10 v/v Folin-Ciocalteu reagent, 4ml 7.5% w/v sodium carbonate was taken to the mixture. The mixture was permitted to incubate in at 40^0C for 30 minutes. Then the absorbance was taken at 765 nm. By using different concentrations (0-1mg/ml) of gallic acid, the standard calibration curve was drawn and finally total phenol content was calculated as mg of gallic acid equivalent (GAE) per gram of dry extract.

Total flavonoids content was determined according to a reported technique (Shah *et al*., 2012). In 1ml extract solution, 4ml distilled water and 0.3ml 5% w/v sodium nitrate was sequentially mixed. After five minutes, 0.3ml aluminum chloride (10 % w/v) was taken to the mixture and then 2ml 1M sodium hydroxide was taken and the volume was adjusted to 10ml. Then absorbance of the mixture was taken at 510 nm. For this assay, quercetin was used for standard calibration curve and total content of flavonoids was indicated as mg quercetin equivalent (QE) per gram of dry plant extract.

Total tannin content was determined by a recognized method (Amorim *et al*., 2008). 0.1 ml of the extract solution was added to 7.5 ml distilled water. Then 0.5ml Folin-Ciocalteu reagent and 1ml 35% w/v sodium carbonate were sequentially added to the mixture and finally the volume was adjusted to l0ml. After 30minutes, absorbance was taken at 725nm. Gallic acid was used as standard to serve the calibration curve and total tannin was expressed as mg gallic acid equivalent per gram of dry mass.

Oral glucose tolerance test

Test for oral glucose tolerance was performed by following an established process (Joy and Kuttan *et al*., 1999). At the very beginning, overnight fasted mice were kept into 4 groups and each contains five mice. Mice of Group I were used as control and group II was treated with glibenclamide (5mg/kg, orally) as reference drug. Mice form groups III and IV were given extract at 2 doses (500 mg/kg and 250 mg/kg respectively orally). After waiting for 30 minutes, glucose (10 g/kg) was orally loaded to all mice. From tail vein, blood samples for oral glucose tolerance were collected prior to (0 min) and at 1^{st}, 2^{nd} and 3^{rd} hour after extract administration and by using a glucometer, the glucose levels in blood were measured.

Antibacterial Activity

Disc diffusion technique is used to measure antibacterial activity of extracts (Ahmed *et al*., 2003). The antibacterial activity of the extracts was observed at the dose of 250µg/disc, and 500µg/disc and kanamycin (30µg/disc) was used as standard antibacterial agent. Sterile nutrient agar is used to prepare bacterial medium and then a suspension of the tested bacteria was added to it at 45°C and poured into sterile Petri dishes to freeze. Blank disc filled with different doses of sample extract, control and also the standard kanamycin disc were placed in such a manner that possible chance of overlapping of zone of inhibition can be prevented. After that at least 120 minutes was permitted to diffuse the drug into the agar. Then the petri dishes were incubated at 37^0C for twenty four hours. Finally, the observed zone of inhibition was measured in millimeters (mm).

Statistical analyses

EXCEL package is used for statistical analyses. Regression analysis was made to find out the relationship between the antioxidant activities and antioxidant components. Statistical significance of tolerance test of oral glucose was estimated by student's t-test. The significance level was evaluated at the probability of $P<0.05$ and $P<0.01$. All values are expressed by mean ± SD (three parallel measurements).

RESULTS AND DISCUSSION

Phytochemical Screening

Phytochemical screening (Table 1) indicates that the leaf extract and stem extract of *P. paludosa* showed the positive results for carbohydrates, alkaloids, glycosides, phenolic compounds, flavanoids, tannins, steroids, proteins and acidic compounds but both extract demonstrated negative result for saponin.

Table 1: Results of phytochemical screening

Phytochemical Constituents											
Samples	**Carbohydrate**	**Alkaloids**	**Glycosides**	**Phenolic compounds**	**Flavonoids**	**Tannins**	**Steroids**	**Protein & Amino acids**	**Saponin**	**Gum**	**Acidic compounds**
P. paludosa leaf	+	+	+	+	+	+	+	+	-	-	+
P. paludosa stem	+	+	+	+	+	+	+	+	-	+	+

(+) Indicates the presence & (-) Indicates the absence of chemical constituents

Antioxidant activity evaluation

Leaf extract of *P. paludosa* was found to exert significant DPPH Radical Scavenging Activity than stem extract (Table 2). IC_{50} (µg/ml) for *P. paludosa* leaf and stem extract were 25.2 ± 0.52 & 35.9 ± 0.95 respectively against Ascorbic Acid (Standard), which IC_{50} (µg/ml) is 12.9 ± 0.11.

Table 2: Antioxidant activity analysis of the extracts by DPPH radical-scavenging, reducing power, FRAP and H_2O_2 radical scavenging assay

Samples	DPPH Scavenging activity	Reducing power assay [a]	FRAP assay [a]	H_2O_2 radical scavenging assay [a]
	(IC_{50} in µg/ml)	(absorbance at 1 mg/ml)	(absorbance at 1 mg/ml)	(IC_{50} in µg/ml)
P. paludosa leaf	25.2 ± 0.52	0.275±0.0007	0.310±0.002	85.5±0.75
P. paludosa stem	35.9 ± 0.95	0.298±0.001	0.425±0.001	142.6±0.63
Ascorbic acid*	12.9 ± 0.11	-	0.988±0.001	13.6±0.19
BHT*	-	0.882±0.002	-	-

*Antioxidant standards; [a] Values expressed as mean ± SD of triplicate measurements

In Table 2, extract of *P. paludosa* leaf exhibited a significant IC_{50} (µg/ml) value: 85.5±0.75 hydrogen peroxide radical scavenging, which was closed to reference compound Ascorbic acid which IC_{50} (µg/ml) value was 13.6±0.19 but for *P. paludosa* stem extract, IC_{50} (µg/ml) value was 142.6±0.63.

In reducing power assay and FRAP assay, the absorbance of standard compound and *P. paludosa* leaf and stem extracts raised markedly with increased concentration which were seen in Table 2. *P. paludosa* stem extracts exhibited better response than leaf extract.

Total phenolic content for leaf extracts of *P. paludosa* was 176.22±1.06 mg/gm, GAE but stem extracts showed higher value (185.42±1.62 mg/gm, GAE) than leaf extract (Table 3).

Extracts of *P. paludosa* stem demonstrated a remarkable higher value 70.01± 0.19 mg/g, QE of total flavonoid content whereas leaf extract showed values 61.79± 0.09 mg/g, QE which were given in Table 3.

Table 3: Levels of Total phenolic content (TPC), Total flavonoids content (TFC) and Total tannin content (TTC) of the extracts.

Plants	Total Phenolic Content [a] (mg GAE/g extract)	Total Flavonoids Content [a] (mg QE/g extract)	Total Tannin Content [a] (mg GAE/g extract)
P. paludosa leaf	176.22±1.06	61.79± 0.09	27.97±0.14
P. paludosa stem	185.42±1.62	70.01± 0.19	30.31±0.18

[a] Values expressed as mean ± SD of triplicate measurements

Unlike total phenol content and total flavonoid content, total tannin content of the extracts didn't vary such a great extent. The values of total tannin content of leaf and stem extracts of *P. paludosa* were 27.97±0.14 mg/g, 30.31±0.18 mg/g, GAE respectively (Table 3).

Oral glucose tolerance test

After orally administered of *P. paludosa* stem (500mg/kg) extract, blood glucose level was 6.12 ± 0.43 m Mol/L at Zero hour and was 4.94 ± 0.23 m Mol/L at 3rd hour (Table 4). Blood glucose level was appreciably decreased in time dependent manner by the extract which was comparable to the standard drug (Figure 1).

Table 4: Effect of extracts on oral glucose tolerance test (OGTT)

Groups	Treatment	Blood glucose level [a] (mmol/L)			
		0 hr	1st hr	2nd hr	3rd hr
I	Control (10ml/kg)	5.6 ±0.13	21.04 ±0.44	8.28 ±0.24	7.6 ± 0.32
II	Glibenclamide (5mg/kg)	5.24±0.29	16.32±0.40*	4.9±0.30**	2.54±0.25**
III	*P. paludosa* stem (500mg/kg)	6.12±0.43	17.28±0.83*	6.34±0.25*	4.94 ± 0.23*
IV	*P. paludosa* stem (250mg/kg)	6.04±0.15	18.8 ±0.69*	7.72±0.59*	5.94 ± 0.34*

[a] Values represent as mean ± SD, n = 5, *=P<0.05 and **=P<0.01 compared to control

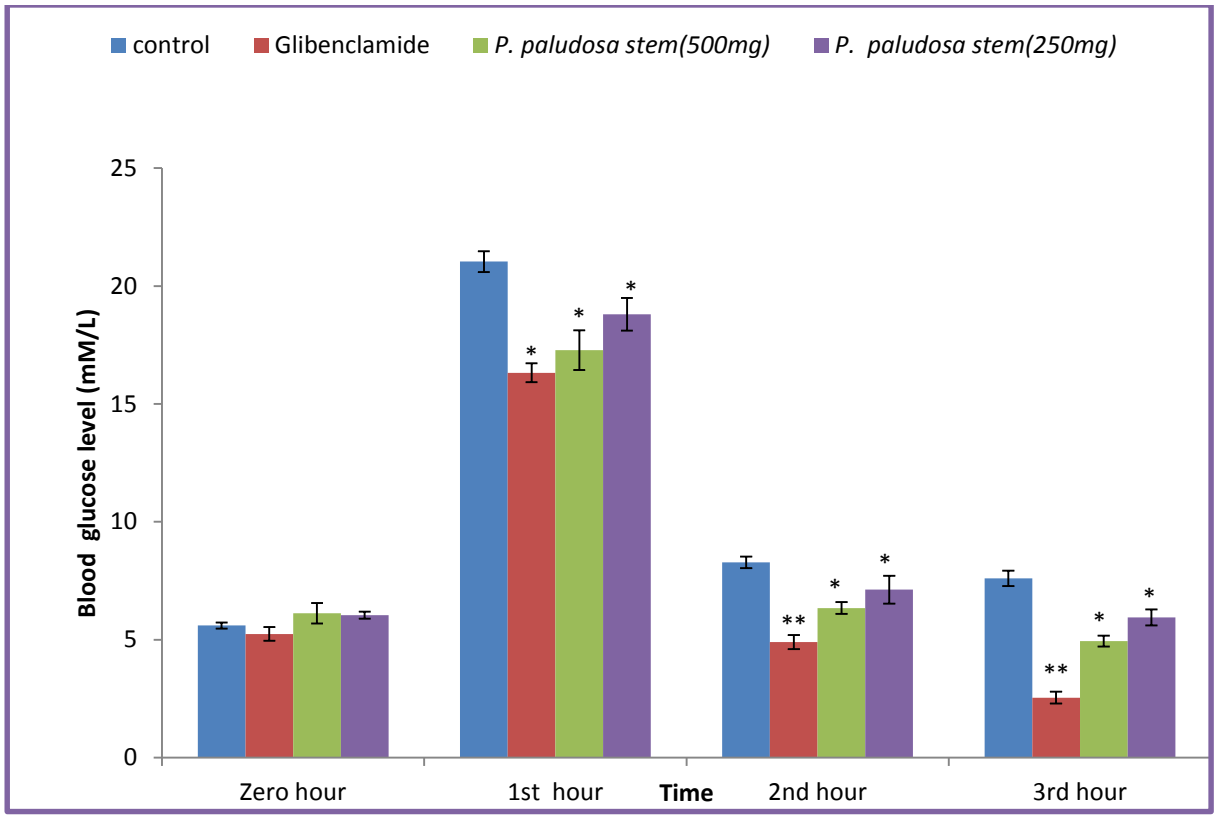

Figure 1: Result of OGTT using *P. paludosa* stem extract

Antibacterial activity

The leaf and stem extract of *P. paludosa* were found to provide results of zone diameter (mm) of inhibition against the experimental bacterial strains. Among them the stem extract (500 µg) showed ZOI 15.00 mm against *Salmonella typhi* where for leaf extract (500 µg) 12.00 mm (*Table* 5) which was comparable to standard (Figure 2, Figure 3).

Table 5: Zone of inhibition in mm against different bacterial strains by extracts

Microorganisms	*P. paludosa* leaf		*P. paludosa* stem	
	250µg	500µg	250µg	500µg
Vibrio cholerae	7.00	9.50	6.50	10.50
Shigella dysenteriae	0	5.00	0	7.00
Escherichia coli	9.00	11.50	11.00	13.50
Proteus spp.	10.00	11.50	8.00	12.50
Pseudomonas aeruginosa	0	5.00	0	6.50
Salmonella typhi	10.00	12.00	11.00	15.00
Staphylococcus epidermidis	8.00	9.00	7.00	10.00
Microbispora coralline	8.00	12.50	8.00	13.50
Streptococcus pyogens	6.50	10.00	6.00	11.00
Staphylococcus aureus	6.00	12.50	5.00	11.50

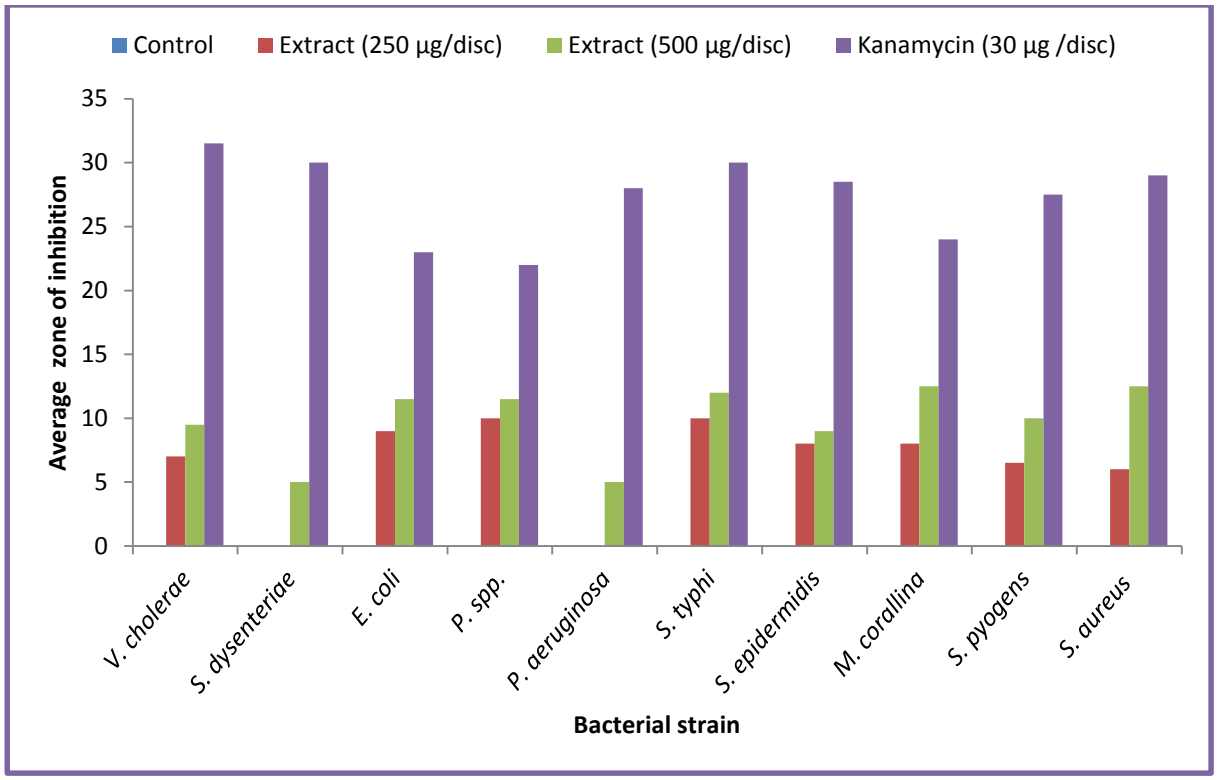

Figure 2: Antibacterial activity of *P. paludosa* leaf at different doses against gram positive and gram negative bacteria in comparison of kanamycin.

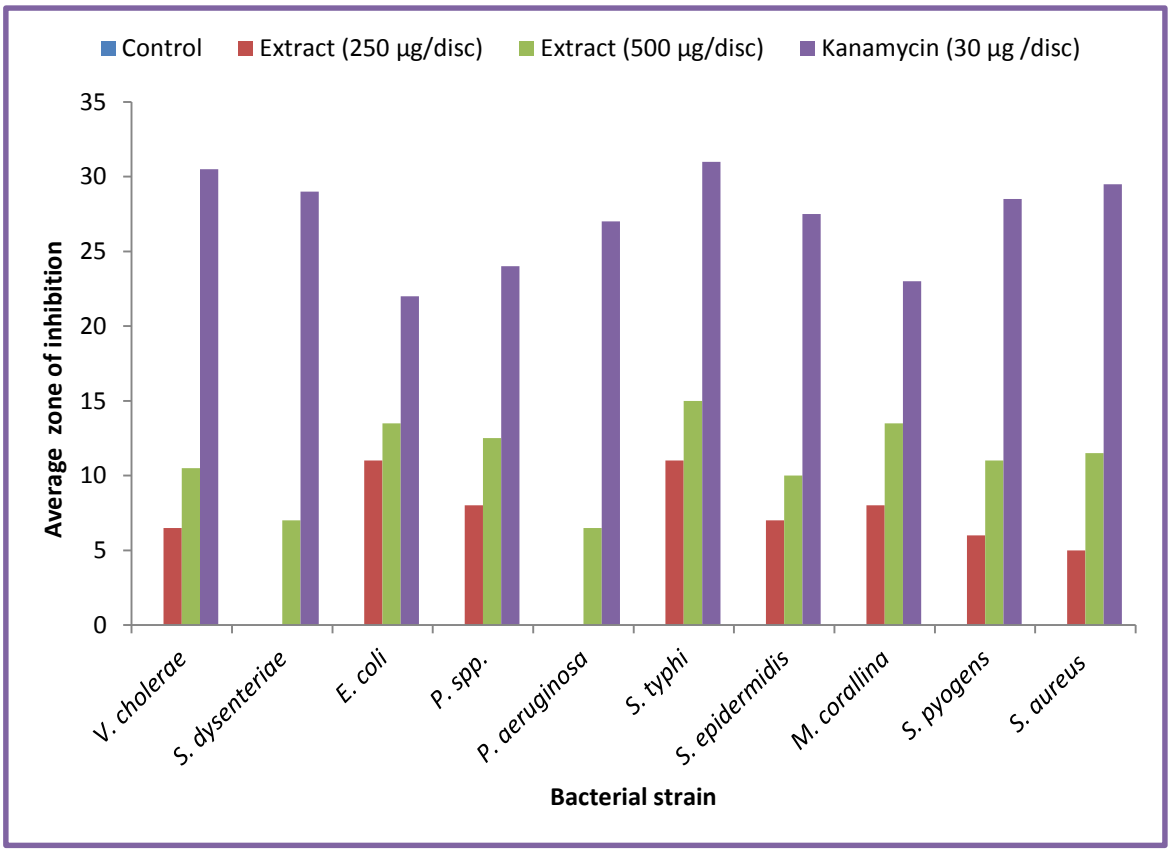

Figure 3: Antibacterial activity of *P. paludosa* stem at different doses against gram positive and gram negative bacteria in comparison of kanamycin.

Antioxidant activity

Antioxidants are agents which slow down lipid peroxidation and other free radical processes. Thus, they always try to protect the human body from several diseases attributed to the reaction of radicals such as aging, cancer, inflammations etc. Use of synthetic antioxidants has been reported to involve toxic side effects thus necessitating the search for natural antioxidants and free radical scavengers (Radulovic et al., 2007). DPPH antioxidant assay is well established in natural product antioxidant studies. The antioxidant effect is proportional to the DPPH free radical conversion to DPPH by anti-oxidant compound (MacDonald-Wicks et al., 2007).

In our investigation, the scavenging activity for DPPH radical of *P. paludosa* leaf was the higher of the two extracts tested, as resulted IC_{50} value was the lowest between two extracts. Same result was found in H_2O_2 radical scavenging assay. The differences in scavenging action may be due to the difference in flavonoid content level as flavonoids are the well recognized to fight free radicals (Gomes de Melo et al., 2010). Though sufficient antioxidant components are present, reduced accessibility of the components to the reaction site may be the possible causes of the lower free radical scavenging activity.

The reducing capability of a compound serves as a considerable indicator of its possible antioxidant activity (Li et al., 2011). Reducing power evaluates the reduction capability of reductants which was determined by the reduction of ferricyanide complex/Fe (III) to the ferrous/Fe (II) form. Although a reductant is not essentially an antioxidant, an antioxidant is generally a reductant. It understood that reductants and antioxidants both are responsible for ferric reducing capacity of the extracts. In comparison of the two extracts higher reducing power of *P. paludosa* stem extract may be due to the existence of higher amount of reductants. Phenolic compounds show antioxidant activity by the ways of inactivating lipid free radicals or preventing breakdown of H_2O_2 into free radicals. Due to presence of hydroxyl groups, conjugated ring structures and carboxylic groups, phenolic compounds are capable to act as free radical scavenger, hydrogen donors, singlet and triplet oxygen quenchers, metal chelator, lipid per oxidator and also exert multiple biological responses (Phang et al., 2013;Gontijo et al., 2012). Between the extracts *P. paludosa* stem extract more efficiently reduce the phosphor molybdic acid of Folin-Ciocalteu reagent in basic condition and formed intense blue color complex of reduced molybdenum. The content of flavonoids was expressed as QE/g of extracts while *P. paludosa* stem extract (70.01± 0.19 mg QE/g) contained the maximum level of TF content between two extracts. Although phenolic compounds have been related to antioxidant activity, in peroxyl radicals quenching tannins are 15 to 30 times more effective than normal phenolics. That's why in the present study it has emphasized on tannins in determining the antioxidant activity of the extracts. Tannin content was highest in *P. paludosa* stem extract.

Oral glucose tolerance test

Mangroves plants have diversified metabolites with some novel chemical structure. Alkaloids, carbohydrates, tannins, acids, amino acids, carotenoids, terpenes, lipids, steroids, phenolics, and their glycosides and related compounds have well-known pharmacological activity (Bandaranayake 2002). It is reported that alkaloids are responsible for anti hyper glycaemic action by pancreatic discharge of insulin from β-cell of islets. (Gulfraz et al. 2011). The phenolic compounds may reveal their hypoglycaemic activities by enhancing the levels of serum insulin, increasing the tissue sensitivity to insulin action, invigorating the enzymatic action of glucose consumption and inhibiting the activity of α-amylase (Arif et al. 2014). Tannins also responsible for preventing diabetic complications by declining the oxidative stress (Soman et al. 2013). Present study indicates that oral test for the glucose tolerance has exposed the glucose tolerance capability of the tested extracts. *P. paludosa* stem extract showed maximum effect at first hour and with increasing the time the extract reduced the glucose more effectively. But the leaf extract did not prove any effect in oral glucose tolerance test.

Antibacterial activity

The phytochemicals of polyphenols, alkaloids, tannins, saponin, and flavonoids show antimicrobial activity (Halilu et al., 2012). The crude extract of *P. paludosa* stem was moderately active in dose dependent manner against all the experimental bacterial strains. The extract showed the maximum zone of inhibition of 11.00mm and 15.00mm against *Salmonella typhi* at the dose of 250 and 500µg/disc respectively. *P. paludosa* leaf extract also showed zone of inhibition against some bacterial strains at highest dose (Table 5). For the differences of possible bioactive compounds, we find differences in sensitivity of the extract of *P. paludosa*.

CONCLUSION

This experimental study has helped to find out that the stem extracts of *P. paludosa* possess antioxidant, antibacterial, and antidiabetic properties and the leaf extracts also possess antioxidant and antibacterial activities. Further research is required to fractionate the extract, to discover the bioactive compounds. Such bioactive compounds should be possible compounds to be developed into medicine and nutraceuticals.

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