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In vitro assessment of antioxidant activity and anti-bacterial screening of *Pithecellobium dulce* leaves against resistant bacteria

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Abstract
Oxidative stress resulted from free radicals and reactive oxygen species are associated with many diseases. Several studies are going on worldwide directed towards finding natural antioxidants of plant origin. The present study was designed to investigate the *in-vitro* antioxidant and antibacterial activity of *Pithecellobium dulce* leaves extract. The ethyl acetate fraction showed the highest total phenolic (201.22 mg GAE/g extract) and flavonoid content (252.11 mg CE/g extract) and antioxidant properties as shown by DPPH radical, reducing power, hydroxyl radical and hydrogen peroxide scavenging assay among different fractions of methanol extract. Antibacterial activity was assessed by disc diffusion method. Methanol extract showed highest zone of inhibition (45 mm) against *B. Cereus* which was higher than standard (36mm). Ethyl acetate fraction produced an inhibitory effect against bacteria which are resistant to some antibiotics like azithromycin, cefuroxime, cephalexin and cotrimoxazole. So, this can be used as a good therapeutic approach for infectious disease caused by those resistant bacteria.

Keywords: antioxidant activity, DPPH, resistant bacteria, antibacterial activity

Introduction
World is endowed with a rich heritage of medicinal plants and it has been used for centuries as remedies for disease [1]. Medicinal plants play a golden role not only as traditional medicine but also as trade commodities, meeting the demand of distant markets for the development of new drugs. As a source of medicine, medicinal plants have always been at forefront virtually all cultures of civilizations. During the metabolic process in human body a number of free radicals are formed which can cause harmful effects leading to various diseases, such as, cardiovascular ailments, inflammation, cancer, neural disorders, Alzheimer disease, atherosclerosis, allergies and aging [2]. Free radicals are chemical entities that can exits separately with one or more unpaired electrons produced from various biochemical reactions. They are generated as a result of imbalance between formation and neutralization of pro-oxidants in different metabolic process of biological system [3]. Examples of these are reactive oxygen species (ROS) or reactive nitrogen species (RNS) radicals which include superoxide anion, singlet oxygen, hydrogen, hydrogen peroxide and hydroxyl radicals. These radicals can cause damage to lipid cells, proteins and DNA. These radicals can be scavenged by the protective role of natural and synthetic antioxidant agents. Synthetic drugs such as butylated hydroxytoluene (BHT), rutin, and butylated hydroxyl anisole (BHA) are commonly used. However, they have been reported to cause adverse side effects such as toxicity, cell damage, inflammations and atherosclerosis in animals and humans [4]. The use of natural antioxidant has gained much attention from consumers because they are considered safer than synthetic antioxidants. Recently, there has been a worldwide trend towards the use and ingestion of natural antioxidants presents in different parts of plants due to their phytochemical constituents [5].
Antibacterial screening is essential for drug discovery, epidemiology and prediction of therapeutic outcome. Any chemical substance or biological agents that either destroy or suppress the growth of microorganisms is called antibacterial agent. Antimicrobial activity of any plant extract can be detected by observing the growth of microorganisms when contact with plant extract. The susceptibility of microorganisms to antibacterial agents can be measured *in-vitro* by a number of methods, one of which is the "Disc Diffusion Technique" using different concentration of the agents absorbed on sterile filter paper discs, is widely acceptable for the preliminary evaluation of antimicrobial activity. To date, one of the methods which help preventing microbial resistance is assessment of antimicrobial activity of antibiotics. It is recommended to investigate the antimicrobial activity of the antibiotics during

their consumption, especially in the hospitals where there are more resistant microbes [6]. *P. dulce* are widely distributed in the tropics, chiefly in Asia and America. *P. dulce*, a most versatile medicinal plant, has attracted a worldwide prominence in recent years, owing to its wide range of medicinal properties and diverse utility. This plant is known to possess many medicinal properties such as antimicrobial, astringent, dysentery, dermatitis [7], anti-inflammation [8] emollient, abortifacient and antidiabetic properties [9]. The aim of my present study was to evaluate the *in vitro* antioxidant and antibacterial activity against the clinical isolates of *E-coli*, *Pseudomonas spp*. and *Staphylococcus aureus* which are resistant against some antibiotic.

Materials and Methods

Collection and identification of plant Sample

The leaves pertained to the study were collected during the months of November and December and was authenticated from National herbarium, Dhaka, Bangladesh. The collected leaves were examined carefully and old, infected and damaged leaves removed. Initially the healthy leaves were washed with tap water and then with distilled water to remove any debris or dust particles. Healthy leaves were spread out and dried at room temperature for about 15 – 20 days and pulverized by mechanical grinder and passed through a 40-mesh sieve to get fine powder and stored in an air tight container.

Preparation of plant extract

1.5kg of *P. dulce* leaves powder was first extracted with n-Hexane. The residue after n-hexane extraction soaked in 3.5 liter of methanol and kept it for 12 days with occasional shaking and stirring. The whole mixture was then filtered through cotton and then through Whatmann No.1 filters paper and was concentrated with a rotary evaporator under reduced pressure at 45°C temperature until needed to collect the crude methanolic extract (CME).Then sequentially fractionated towards polarity using the solvent n-Hexane(NH), chloroform(CH) and ethyl acetate(EA).

Phytochemical Screening

Phytochemical test was carried out on methanolic extracts using standard procedures to identify the constituents such as alkaloids, flavonoids, glycosides, phenols, saponins, steroids, tannins [10].

Estimation of total phenolics content (TPC)

The content of total phenolics of different fractions and crude extract were determined according to Singleton *et al*; 1965[11] in which Folin-Ciocalteu reagent (FCR) was used as oxidizing agent and Gallic acid (GA) was used as standard. 0.5 ml of sample (200µg) and standard gallic acid with varying concentration was taken in different test tubes. Then 2.5 ml of 10% FCR was added to the above test tubes. After 5min 2.0 ml 7.5% sodium carbonate solution was added into test tube and vortex each test tubes 1min for complete mixing. The test tubes were then incubated at room temperature for 25-30min to complete the reaction. The absorbance of the solution was measured at 760nm.A calibration curve was established using varying concentration of Gallic acid. The values were expressed in mg/g of sample

Estimation of total flavonoids content (TFC)

The content of total flavonoids was determined by aluminium chloride colorimetric method. In this method aluminium chloride forms complex with hydroxyl groups of flavonoids present in the sample. This complex has the maximum absorbance at 510nm. Catechin was used as standard and the flavonoid content of the extractives was expressed as mg of catechin equivalent/gm of dried extract [12].

In vitro Free Radical Scavenging Activity

DPPH radical scavenging activity

The ability to scavenge the free radical DPPH was measured as decrease in absorbance at 517nm[13]. To 2ml of varying concentrations of extracts, 3ml of DPPH (0.04%) was added and incubated in dark for 30 min. Percentage of DPPH radical scavenging activity was expressed as follows-

$$\% \text{ of Scavenging} = [(A_0-A_1)/A_0] \times 100$$

Where, A_0-absorbance of blank,

A_1- absorbance of extract

Total antioxidant assay

The total antioxidant capacity of different parts of CME and other fractions of *P. dulce* were determined according to Prieto *et al*. 1999 [14].0.5 ml varying concentration extracts were taken into a test tube.Then3 ml of reaction mixture containing 1ml of 0.6M sulphuric acid, 1ml of 28mM sodium phosphate and 1ml of 1% ammonium molybdate was added into the test tubes. A typical blank solution contained 0.5 ml of methanol and 3 ml of reaction mixture also taken and all test tubes were incubated at 95 °C for 10min.The test tubes were then cooled at room temperature for 10min and absorbance was measured at 695nm using a spectrophotometer.

Reducing power assay

1 ml of varying concentration of extract was mixed with 2.5 ml phosphate buffer and 2.5 ml of potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Aliquots of 2.5 mltrichloroacetic acid (10%) were added to the mixture, which was then centrifuged at 4000 rpm for 10min. The upper layer of solution (2.5 ml) was mixed with equal volume of distilled water, to this to mixture 0.5ml of freshly prepared ferric chloride (0.1%) solution was added and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates increase in reducing power [15].

Hydroxyl radical scavenging assay

Scavenging of the hydroxyl free radical was measured by the method of [16]. To 0.5ml of varying concentration of extract, The Fenton reaction mixture containing 100µl of 28mM 2-deoxy-D-ribose prepared in 20mM KH_2PO_4-KOH buffer, 100µl of 1.04mM EDTA, 100µl of 1mM Ascorbic acid, 100µl of 200µM $FeCl_3$ and 100µl of 1.0mM hydrogen peroxide was added to the each test tube. The reaction mixtures were then incubated at 37 ^{0}C for 60 min. After incubation, 1 ml of 1% TBA & 1ml of 10% TCA were added in each test tube and placed in a boiling water bath at 100 ^{0}C for 20min. The resultant mixture was allowed to cool up to room temperature and absorbance was recorded at 532nm in a UV-VIS spectrophotometer. Inhibition was calculated as above.

Hydrogen Peroxide Scavenging Assay

Hydrogen peroxide activity of the extract was estimated by replacement titration method [17].To 1ml of varying concentration of extract, 1 ml of (0.1 M) H_2O_2 and 10 ml of (2M) H_2SO_4 and 100µl of (3%) ammonium molybdate, 7 ml of (1.8 M) potassium iodide was added. This reaction mixture

was titrated against sodium thiosulphate until the disappearance of yellow color.

% inhibition = $[(V_0-V_1)/V_0] \times 100$
Where, V_0-Volume of thiosulphate used to titrate blank,
V_1- Volume of thiosulphate used to titrate against the extract.

Antibacterial Screening

The methanol crude extract, ethyl acetate and chloroform fractions of the plant were screened at three concentrations (50, 100, and 200µg/disc) against Gram-positive and Gram-negative bacteria and some resistant bacteria (Table 1) using the disc diffusion method [18]. Solutions of known concentration (10mg/mL) of the test samples were prepared by dissolving measured amounts of samples in calculated solvent volumes. Dried and sterilized filter paper discs (6-mm diameter) were then impregnated with known amounts of the test substances using a micropipette. Discs containing the test material were placed on nutrient agar medium (Merck) uniformly seeded with the pathogenic test microorganisms. The prepared inoculum size was approximately106 cfu/ml. Standard antibiotic discs (kanamycin, 30µg/disc) and blank discs (impregnated with solvents) were used as positive and negative controls, respectively. These plates were then, kept at 4 °C for a 4-h diffusion of the test material. There was a gradual change in concentration surrounding the discs. The plates were then, incubated at 37 °C for 24 h to allow organism growth. The test materials having antibacterial activity inhibited microorganism growth, and a clear, distinct zone of inhibition surrounding the discs was visualized. The antibacterial activity of the test agents was determined by measuring the diameter of the zone of inhibition expressed in mm.

Results and discussion

The phytochemical constituents present in the leaves of CME extract of *P.dulce* are shown in the Table.1. The sample showed the presence of various phytochemicals namely alkaloids, flavonoids, steroids, glycoside, phenols and terpenoids.

Table 1: phytochemical screening of CME of *P. dulce*

Phytochemical Test	CME
Alkaloids	+
Terpenoids	++
Flavonoids	+
Glycosides	++
Phenols	+
Tannins	+
Saponins	-

Total phenolics and flavonoids content

The total phenolics and flavonoids content of the various extracts of *P. dulce* leaves are given in Table-2 and 3. The highest phenolic and flavonoid content was found in EAF (201.22±3.95) mg GAEs/gm extract and (252.11±1.21) mg of CE/gm of extract, respectively amongst all the fractions. Phenolic compounds have redox properties, which allow them to act as antioxidants [19]. As their free radical scavenging ability is facilitated by their hydroxyl groups, the total phenolic concentration could be used as a basis for rapid screening of antioxidant activity. Flavonoids, including flavones, flavanols and condensed tannins, are plant secondary metabolites, the antioxidant activity of which depends on the presence of free OH groups, especially 3-OH. Plant flavonoids have antioxidant activity *in vitro* and also act as antioxidants *in vivo* [20, 21]. The active phenolic and flavonoid components responsible for the antioxidant activity of EAF should be analyzed to identify.

Table 2: Total phenolic content of CME and different fractions of *P. dulce* leaves

Sample	Conc. (µg/ml)	Absorbance	GAE Conc. C (mg/ml)	TPC A=(C*v)/m (mg/gm)	Mean ± SD
CME	200	1.615	0.032	79.13	81.07 ± 2.64
	200	1.632	0.032	80.00	
	200	1.712	0.034	84.08	
NHF	200	0.512	0.009	22.86	19.74 ± 2.76
	200	0.409	0.007	17.60	
	200	0.432	0.008	18.78	
CHF	200	2.347	0.047	116.48	115.46 ± 2.18
	200	2.356	0.047	116.94	
	200	2.278	0.045	112.96	
EAF	200	3.947	0.079	198.11	201.22 ± 3.95
	200	3.982	0.080	199.90	
	200	4.095	0.082	205.66	
AQF	200	0.478	0.008	21.12	20.15 ± 1.72
	200	0.42	0.007	18.16	
	200	0.479	0.008	21.17	

Table 3: Total flavonoid content of CME and different fractions of *P. dulce* leaves

Sample	Conc. (µg/ml)	Absorbance	Catechin mg/ml	Catechineq mg/gm	Mean ± SD
CME	500	0.703	0.105	104.83	105.72±0.84
	500	0.709	0.106	105.83	
	500	0.713	0.107	106.50	
NHF	500	0.663	0.098	98.17	97.17±1.09
	500	0.65	0.096	96.00	
	500	0.658	0.097	97.33	
CHF	500	0.673	0.100	99.83	101.89±1.87
	500	0.695	0.104	103.50	
	500	0.688	0.102	102.33	
EAF	500	1.583	0.252	251.50	252.11±1.21
	500	1.582	0.251	251.33	
	500	1.595	0.254	253.50	
AQF	500	0.465	0.065	65.17	65.67±1.17
	500	0.463	0.065	64.83	
	500	0.476	0.067	67.00	

DPPH free radical scavenging activity

The DPPH radical is widely used in assessing free radical scavenging activity because of the ease of the reaction. The percentage radical scavenging activity and thus the degree of discoloration of free radicals by different extracts were determined against DPPH. The results of free radical scavenging effects of all fractions and standard BHT in terms of IC_{50} values are given in Figure.1 and Figure.2. The highest percentage of discoloration of DPPH was observed in EAF and lowest in NHF. The IC_{50} values (concentration of extracts required to scavenge 50% DPPH free radicals) showed inverse relationship between IC_{50} value and percentage scavenging potential of a sample. The strongest DPPH radical scavenging activity was exhibited by EAF with IC_{50} = 9.44µg/ml, while the lowest activity was found in NHF with IC_{50} = 66.85µg/ml.A lower value of IC_{50} indicates a higher antioxidant activity. DPPH radical scavenging activity of each extracts is directly proportional of the concentration of total phenolics including tannins of respective extracts.

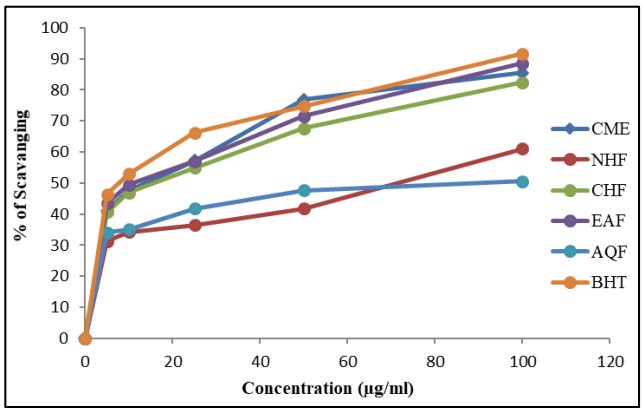


Fig 1: DPPH radical scavenging activity of CME with different fractions of *P. dulce* leaves and standard BHT

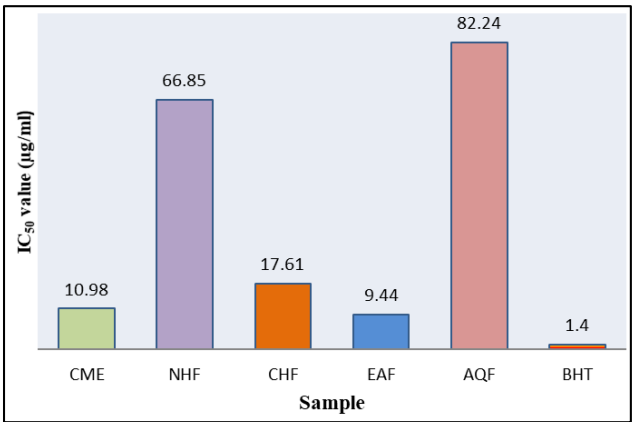


Fig 2: IC_{50} values of CME and different fractions of *P. dulce* leaves and standard BHT

Total antioxidant Assay

The antioxidant effect of each extracts and BHT was investigated and the results are shown in Figure.3. The phosphomolybdenum method is based on the reduction of Mo (V1) to Mo (v) by the antioxidant compound and the formation of green phosphate/ Mo (v) complex. The result expressed in terms of absorbance, the high absorbance values indicated that the samples possessed significant antioxidant activity. EAF showed the highest antioxidant activity than other fractions followed by AQF, CME, CHF and NHF in the decreasing order. All the samples showed a concentration dependent increase in antioxidant activity. Though EAF showed the total highest antioxidant capacity but CHF also had almost the same capacity. The difference in the amount of antioxidant of these samples may be attributed to the differences in the amount and kind of antioxidant compounds in them.

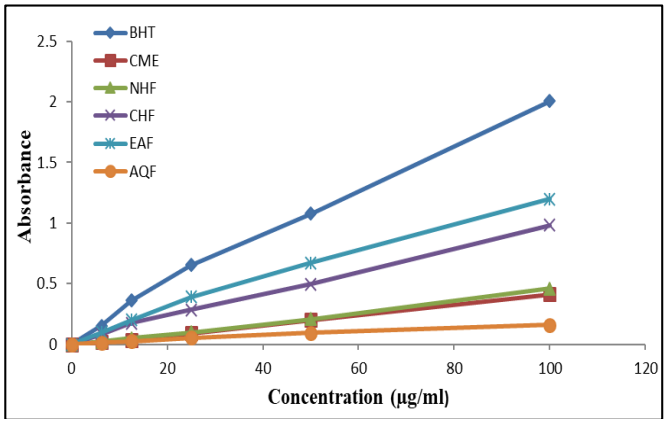


Fig 3: Absorbance of CME and different fractions of *P. dulce* leaves

Reducing Power Assay

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activities of antioxidants have been attributed to various mechanisms such as prevention of chain initiation, decomposition of peroxides, reducing capacity and radical scavenging [22]. Figure. 4 shows that the reducing power of the plant extracts was lower compared with the standard catechin. EAF and CME of leaves showed the higher reducing power and the values were comparable to that of catechin. Methanol and EAF extract of leaves exhibited lower reducing power activity as compare to catechin. All extract shown dose dependent effect. Phenolic contents of all the extracts appears to function as good electron and hydrogen atom donors and therefore should be able to terminate radical chain reaction by converting free radicals and reactive oxygen species to more stable products. At 100µg/ml, the absorbance of the CME, NHF, CHF, EAF, & AQF extract and catechin were (2.832 ±0.063), (1.131± 0.139), (2.353 ±0.123), (3.141 ± 0.016), (1.193 ±0.106) and (4.088 ± 0.091).

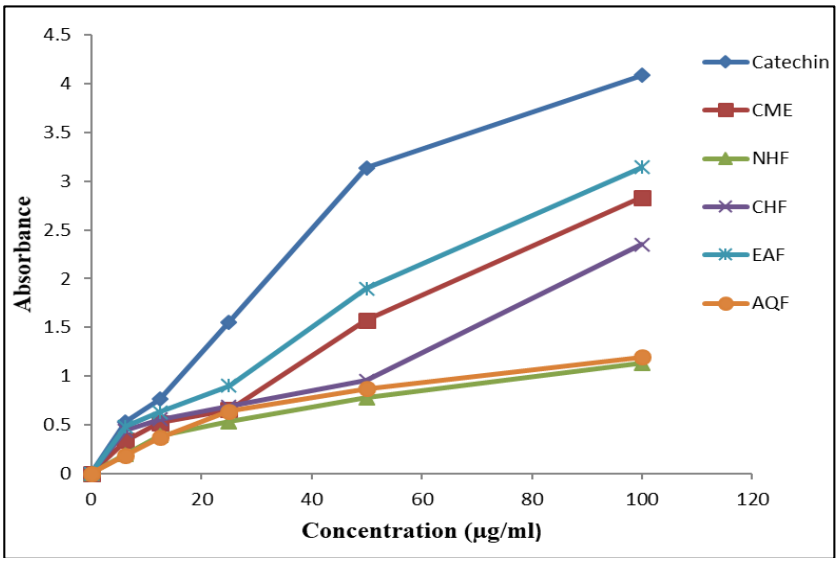


Fig 4: Absorbance of CME and different parts of *P. dulce* leaves and catechin in reducing power assay

Hydroxyl Radical Scavenging Assay

Hydroxyl radicals are the major reactive oxygen species causing lipid oxidation and enormous biological damages [23]. In the hydroxyl radical scavenging assay, the ability of CME and different fractions of *P. dulce* leaves to remove the hydroxyl radical in solution was quantified by measuring the effect on 2-deoxyribose degradation in the presence of EDTA. The Hydroxyl radical scavenging activity of CME and different fractions and standard are shown in Figure.5. The $OH^{\cdot}$ radical scavenging activity was evident at all the concentrations of the samples and correlated well with increasing concentrations. The IC_{50} value of EAF was 18µg/ml and that of standard, catechin, was 6µg/mL.

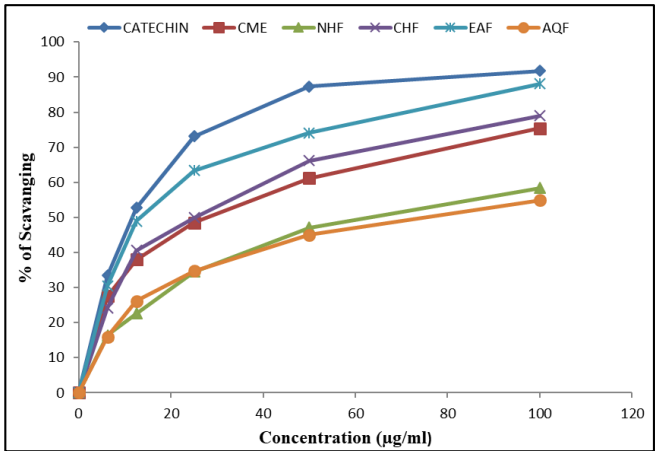


Fig 5: Hydroxyl radical scavenging activity of CME and different fractions of *P. dulce* leaves and standard catechin

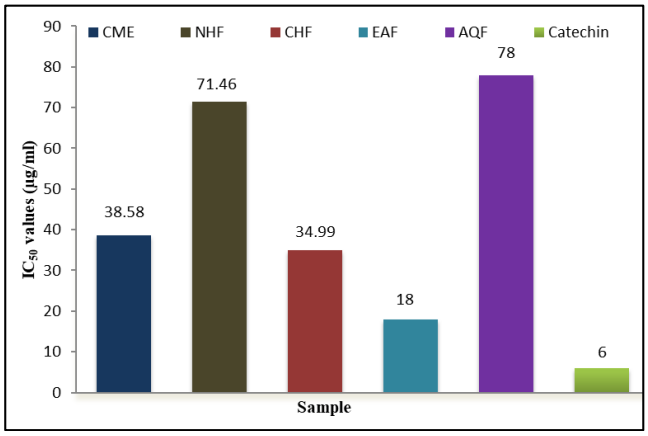


Fig 6: IC_{50} values of CME and different fractions of *P. dulce* leaves

Hydrogen Peroxide Scavenging Assay

The extracts were capable of scavenging hydrogen peroxide in a concentration dependent manner but showed weaker activity than control (BHT). Figure. 7 shows that the plant extract is good scavenger of H_2O_2. At 200µg/ml the percent of scavenging for CME, NHF, CHF, EAF and AQF were 21.96±2.59%, 31.09±1.90%, 46.04±3.80% and 18.64±1.90% respectively, while the values for BHT standard was 56±3.80%. Hydrogen peroxide is a weak oxidizing agent that inactivates a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. It can cross cell membranes rapidly; once inside the cell, it can probably react with $Fe^{2+}$and possibly Cu^{2+} ions to form hydroxyl radicals and this may be the origin of many of its toxic effects [24]. From the results, it appeared that the H_2O_2 scavenging activity of the plant extract less compared to that of the standard BHT.

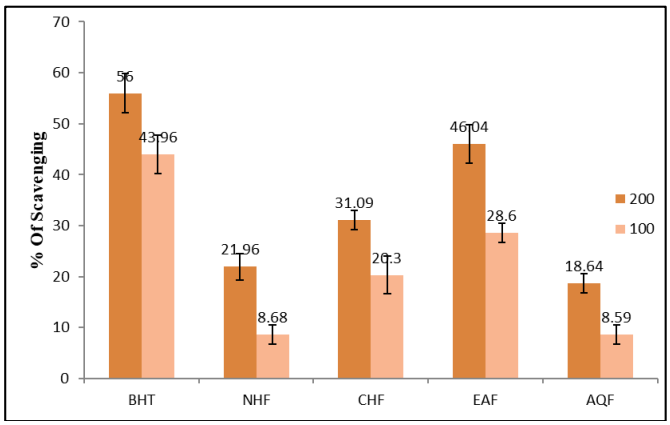


Fig 7: H_2O_2 scavenging activity of different fractions of CME of *p.dulce* leaves and BHT

Antimicrobial Activity

Anti-bacterial screening against some pathogenic organism

CME and different fractions of the leaves of *P. dulce* were prepared and screened for their antibacterial activity against six different bacterial strains including both gram negative and gram positive strains (Table.4). Among them CME, EAF and CHF extracts exhibited significant antibacterial activity than other fractions (Table.5 and Figure.8). The results were compared with kanamycin, a known antibiotic or antimicrobial agent. The results confirmed the antimicrobial potential of the plant and indicated that this extract can be used in the inhibition of pathogenic bacteria.

Table 4: List of tested bacteria

Gram positive bacteria	Gram negative bacteria
a) *Bacillus cereus* b) *Staphylococcus aureus*	*a) Escherichia coli* *b) Shegellaboydii* *c) Proteus* *d) Pseudomonas spp*

The EAF was also tested for anti-bacterial activity against three resistant bacteria. The detected resistant organisms were *Pseudomonas spp.*, *E. coli* and *Staphylococcus aureus. Pseudomonas spp.* strains showed sensitivity to Azithromycin and Cephalexin and resistance to Cefuroxime, Cotrimoxazole, Cefepine, Vancomycin, penicillin + Tazobac, *Escherichia coli (E-19)* which are resistant to Azithromycin, Cefuroxime, Cephalexin and Cotrimoxazole. *Staphylococcus aureus* which is sensitive to ciprofloxacin, cefuroxime and resistant to Amikacin, Cephalexin and Azithromycin (Table.6). These bacteria were collected from Microbiology department of Rajshahi Medical College.

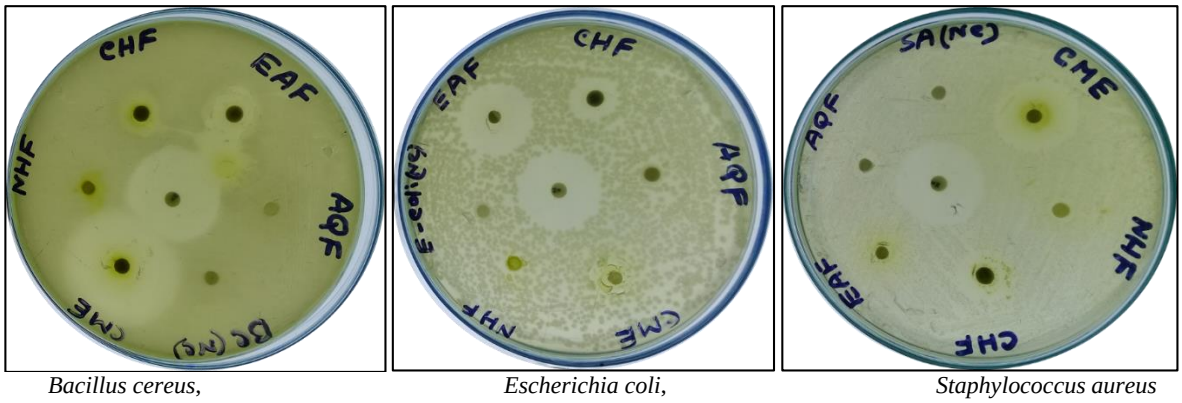
Table 6: List of the drug sensitive and resistant bacteria

Gram positive bacteria	Gram negative bacteria
c) *Staphylococcus aureus*	*a) Escherichia coli (E-19)* *b) Pseudomonas spp(ps-24)*

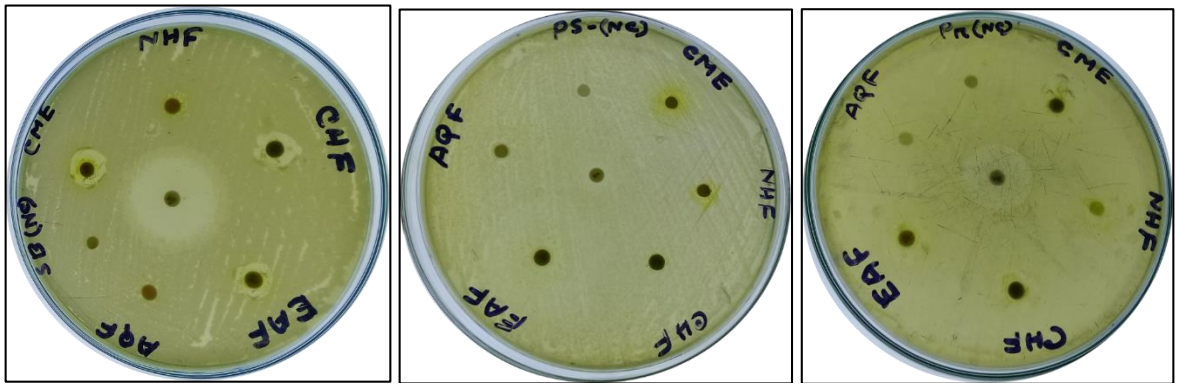
Here at first the activity was measured alone then the sample was given in combination with antibiotic. The zone of inhibition was measured for EAF is shown in (Table.7and Figure.9). From the experiment it has been found that the, EAF might have some compounds, which are responsible for the antibacterial activity.

Table 5: Zone of inhibition of CME and different factions of *P. dulce* leaves

Bacteria	Diameter of Zone of inhibition in mm					
	P. dulce leaves extract					
	NHF	CHF	EAF	AQF	CME	Standard
Escherichia coli	7	18	30	8	20	32
Shegellaboydii	0	13	15	0	14	36
Bacillus cereus	9	8	16	0	45	35
Proteus	8	16	20	0	20	30
Pseudomonas spp	8	0	10	8	12	0
Staphylococcus aureus	8	22	18	12	30	35



Bacillus cereus, *Escherichia coli,* *Staphylococcus aureus*

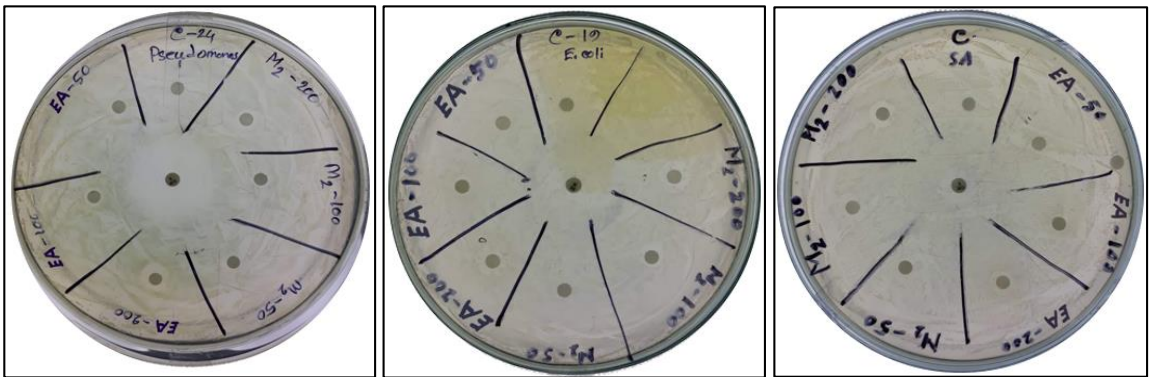


Shegellaboydii, *Pseudomonas spp,* *Proteus*

Fig 8: Zone of inhibition of CME and different factions of *P. dulce* leaves

Table 7: Zone of inhibition of EAF against drug sensitive and resistant bacteria

Bacteria	Zone of Inhibition (mm)			
	Standard	EAF 50µg/disc	EAF 100 µg/ disc	EAF200 µg/ disc
PS-24	30 (K-30)	7	9	11
E-19	0(K-30)	7	10	12
SA	14(CP-30)	0	8	10



Pseudomonas spp *Escherichia coli (E-19)* *Staphylococcus aureus*

Fig 9: Zone of inhibition of EAF against drug sensitive and resistant bacteria

Conclusion

As the main goal of researchers today is to find out natural antioxidants, this plant may play a crucial role in this field. The declined oxidant status and improved antioxidant status evidenced the antioxidant potential of the extract which is attributed to the presence of biologically active ingredients of the extract. The results of present study reveals that ethyl acetate, methanolic and chloroform extract of the leaves of *Pithecellobium dulce* were found to have antioxidant by virtue of their phenolic contents and DPPH free radical scavenging, while the ethyl acetate extract is found to have better antibacterial activities than the other extracts. EAF also showed potent activity against resistant bacteria. When this sample was given in combination with antibiotic a better activity was obtained. Thus, our findings provide evidence that P. *dulce* is a potential source of natural antioxidants.

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