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Evaluation of antioxidant and antibacterial activities of *Crotalaria pallida* stem extract

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Abstract

Background: Antioxidants play an important role in protecting cellular damage by reactive oxygen species. The aim of the present study is to assess the phytochemical nature, antioxidant and antibacterial activities of petroleum ether, chloroform, methanol, and aqueous extracts of *Crotalaria pallida* stem.

Methods: The preliminary screening of the various extracts was carried out using standard methods. Total phenolic content (TPC) was determined by the modified Folin-Ciocalteu method and antioxidant activities were assayed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, reducing power assay, total antioxidant capacity (TAC) and reduction of ferric ions. Antibacterial activity of different extracts were assayed against Gram-positive and Gram-negative bacterial strains by observing the zone of inhibition using disc diffusion method, where *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* and *Pseudomonas aeruginosa* were used as bacterial strains.

Results: Petroleum ether, chloroform, methanol and aqueous extracts of stem exhibited remarkable antioxidant activity in terms of all the assays tested. Petroleum ether extract (PEE) showed DPPH radical scavenging activity with IC_{50} value of 126.96 μg/ml. In antibacterial assay, the PEE and chloroform extract (CE) showed mild to moderate antibacterial activity against all the bacterial strains except *B. cereus* while methanol extract (ME) showed no inhibitory effect against any of the tested bacteria.

Conclusion: PEE, CE and ME of *C. pallida* stem were found to be the most effective free radical quencher and a potent source of natural antioxidants as well as antibacterial activity. Thus justifying their traditional use in green therapeutics.

Keywords: Antioxidant, Antibacterial, *Crotalaria pallida*, Phytochemicals

Background

At low or moderate concentrations, ROS and RNS are necessary for body's defense mechanism against disease, cellular signaling systems and induction of a mitogenic response [1, 2]. But at high concentration, highly reactive free radical and oxygen species produced either by exogenous chemicals or by body metabolic process leads to the oxidation of nucleic acid, lipid, protein and eventually high oxidative stress may result in breaking and coupling of DNA. As a result gene mutation, cell damage and ultimate death can happen [3]. Beside this cancer, cardiovascular disease, neurological disorder, pulmonary disease and ocular disease are also related to oxidative stress [4, 5].

Antioxidants prevent the radical chain reactions of oxidation by inhibiting the initiation and propagation step leading to the termination of the reaction and a delay in the oxidation process [6]. A no of synthetic phenolic antioxidants such as, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been widely uses as antioxidants in food industry, cosmetics, and therapeutic industry. Due to high volatility and instability at elevated temperature, carcinogenic nature of some synthetic antioxidants and consumer preferences motivate the manufacturers for using natural antioxidants [7]. Although antioxidant can neutralize the sick effects of free radicals

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by scavenging or chain breaking but most of the naturally occurring antioxidant compounds (plant sources) have been identified as free radical or active oxygen scavengers. Recent investigations recommend that the plant source antioxidants with free radical scavenging properties may have awesome therapeutic importance in free radical mediated diseases [8].

Due to the indiscriminate use of antibiotics, microorganisms have evolved into resistant strains and a numbers of antibiotics have lost their effectiveness against those microorganisms in recent years [9]. In this regard, natural products derived from medicinal plants may offer an alternative source of antimicrobial agent that might have a great impact on infectious diseases and overall human health [10]. According to the estimation of World Health Organization, in developing countries 80% of the population still depends on traditional medicines, mostly plant drugs for their primary healthcare needs [11] and literature shows that the interest on the use of phytochemical constituents of medicinal plants with pharmacological activity is increasing [12]. Hence, the study for screening of antibacterial properties of herbs is a crying demand which may help to find out proper treatment of several diseases caused by microorganisms. This species is used in traditional medicine, the leaves are used as curative agent of urinary tract infections and vermifuge [3], Mikirs of Assam used the leaves extracts to kill intestinal worms [13]. The plant has antimicrobial, antioxidant and anti-inflammatory properties [3].

To the best of our knowledge, very few pharmacological studies have been reported so far on *C. pallida* stem. As a part of the continuation of our research on bioactivity screening of Bangladeshi medicinal plants [14–16], present study was carried out to assess the phytochemical nature, antioxidant and antibacterial profiling of PEE, CE, ME and aqueous extract (AE) of *C. pallida* stem in order to scientifically evaluate the claimed biological activities.

Methods

Sample preparation

The stem of *C. pallida* used for this study was collected from the campus of Noakhali Science and Technology University, Noakhali, Bangladesh and identified by an expert. The stems were washed with distilled water and chopped into pieces using knife and then air dried under shade for 2 weeks. The dried stems were grounded into mesh size and kept in an airtight container for further use.

Proximate analysis

Proximate analysis was done to determine moisture, total ash, water soluble and acid insoluble ash content of prepared sample using standard procedures described peeviously [17].

Preparation of extracts

The extracts of *C. pallida* stems were prepared by using sequential extraction method. In this method, sample materials were subjected to successive extraction with different solvents according to their ascending order of polarity (non-polar to polar). The prepared sample (120 grams) was extracted sequentially for 10 h in different solvent, e.g. petroleum ether, chloroform, methanol and distilled water using a Soxhlet apparatus. These extracts were concentrated with a rotary evaporator followed by drying at 35–40 °C employing oven dryer and stored for further use.

Phytochemical screening

The preliminary phytochemical screening of the different extracts of studied plant was performed to detect the presence of active chemical constituents e.g. alkaloid, flavonoid, saponin, tannin, and phenolic compound according to standard procedures reported previously [18, 19].

Total phenolic content

The TPC of the extracts was determined by the modified Folin-Ciocalteu method [18]. Briefly, 0.5 ml of each extract (1 mg/ml) was mixed with 5 ml Folin-Ciocalteu reagent (1:10 v/v in distilled water) and 4 ml of 7.5% sodium carbonate. The mixture was vortexed for 15 s and allowed to stand for 30 min at 40 °C for color development. The absorbance was measured at 765 nm with a UV-Vis spectrophotometer. The standard curve was prepared using 31.25 to 500 µg/ml solutions of gallic acid in 50% methanol.

Antioxidant activity

There is no unique method for measuring antioxidant activity because of complex nature of biological system. The antioxidant properties of the examined various extracts were determined through DPPH scavenging activity, reducing power assay, total antioxidant capacity and reduction of ferric ions.

DPPH scavenging activity

The free radical scavenging activity of the prepared extracts was quantified in vitro by DPPH free radical using the procedure described in Hossain et al., 2016 [20]. The DPPH solution was prepared by dissolving 4 mg DPPH with 100 ml methanol. An aliquot of 1 ml of this solution was mixed with 50 µl of the sample at various concentrations (31.25 - 500 µg/ml). This mixture was then well shaken and allowed in the dark for 20 min at room temperature and absorbance at 517 nm was taken. The percentage inhibition was calculated from $[(A_o - A_1)/A_o] \times 100$, where A_o is the absorbance of the control (DPPH solution) and A_1 is the absorbance of the extract/standard. Ascorbic

acid (AA) and BHT were used as standards. The inhibition curves were prepared and IC_{50} values were calculated.

Reducing power assay

The reducing power of the prepared extracts was determined based on the ability to reduce ferric ions to ferrous ions, which was monitored by measuring the formation of Perl's Prussian blue at 700 nm, according to the method previously described in Islam et al., 2017 [16]. Different concentration of extracts (31.25–500 µg/ml) were mixed with 1 ml of phosphate buffer (0.2 M, pH 6.6) and 1 ml of potassium ferricyanide (1%). The mixture was left for 20 min at 50 °C followed by addition of 2 ml of trichloroacetic acid (10%). The mixture was centrifuged at 3000 rpm for 10 min and a volume of 0.5 ml supernatant from each of the mixture was mixed with 1 ml of distilled water and 0.1 ml of 0.1% (w/v) fresh ferric chloride. After 10 min reaction, the absorbance was measured at 700 nm against a blank in a spectrophotometer. Increased absorbance of the reaction mixture indicated the increased reducing power. BHT was used as the reference standard.

Total antioxidant capacity

The TACs of all extracts were evaluated spectrophotometrically by the phosphomolybdenum method according to the procedure described by Prieto et al., 1999 [21] with minor modification. An aliquot of 100 µl of sample solution was combined in an Eppendorf tube with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated at 50 °C for 90 min. After the samples had cooled to room temperature, the absorbance was measured at 695 nm against a blank. A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the solvent used for the sample, and it was incubated under the same conditions as the rest of the samples. AA was utilized as the standard and the total antioxidant activity was expressed as equivalents of ascorbic acid.

Reduction of ferric ions assay

The reduction of ferric ions by the extracts was determined by o-phenanthroline method reported previously [17]. A reaction mixture containing 1 ml o-phenanthroline (5 mg in 10 ml methanol), 2 ml of 0.2 mM ferric chloride and 2 ml of various concentrations (31.25–500 µg/ml) of the extracts was incubated at ambient temperature for 10 min, then the absorbance was measured at 510 nm. AA and gallic acid (GA) were used as reference standards.

Antibacterial assay

Four bacterial strains, including two Gram-positive as well as two Gram-negative were obtained from the department of Microbiology, Noakhali Science and Technology University, Bangladesh. The strains were *S. aureus*, ATCC 25923; *B. cereus*, ATCC 10707; *P. aeroginosa*, ATCC 27853 and *E. coli*, ATCC 25922.

The antimicrobial activity of different extracts of *C. pallida* against Gram-positive as well as Gram-negative bacterial strains were evaluated using a modified Kirby-Bauer's disc diffusion method repoted previously [22]. The fresh bacterial cultures were suspended into normal saline and compared the turbidity with 0.5 McFarland standards. Bacterial lawns were prepared on Mueller-Hinton Agar plates by dipping sterile swabs with reference bacterial cultures. The sterile paper discs (4 mm) was soaked with the previously prepared solutions of all extracts at a concentration of 20 mg/ml were dried for 3 h. The prepared paper discs of each extract were placed on swab plates. The agar plates were incubated at 37 °C for 24 h and the diameter of zones of inhibition were measured. Commercially available Imipenem (5 µg/disc) antibiotic discs were used as the antimicrobial positive control and solvent wetted discs were used as the negative control for all of these assays.

Results and discussion

Proximate analysis

The proximate analysis of the stem of *C. pallida* was performed to evaluate its moisture, total ash, water soluble, and acid insoluble ash content. Proximate analysis is generally performed to evaluate food components, which are important for product development, quality control or regulatory purposes in the food industry, and for purity and quality of crude drug. The moisture, and ash contents reflect the mass content of the *C. pallida* stem. The low moisture content (8.6%) of the stem of *C. pallida* indicates that it may hinder the growth of microorganisms; therefore, its preservation period will be high. The ash content of 8.73% suggests that the stem is comparatively rich in mineral elements, and may contains inorganic radicals like phosphates, carbonates, and silicates of sodium, potassium, magnesium, calcium etc.

Phytochemical screening

Preliminary phytochemical screening of the different extracts was qualitatively analyzed to observe the presence of secondary metabolites such as alkaloid, flavonoid, saponin, phenolic compounds and tannin in the *C. pallida* stem. Table 1 illustrates the results of preliminary phytochemical screening. ME contains all of the studied secondary metabolites while PEE contains only two, alkaloid and tanin. AE contains all except phenolic compound and CE contains all except saponin.

The phytochemical analysis is a measure of the bioactive compounds which can be used to treat chronic as well as infectious diseases. The leaves contains alkaloids,

Table 1 Qualitative chemical analysis of different extracts of *C. pallida* stem

Phytochemical composition	PEE	CE	ME	AE
Alkaloid	+	+	+	+
Flavonoid	-	+	+	+
Saponin	-	-	+	+
Phenolic compound	-	+	+	-
Tannin	+	+	+	+

PEE-Petroleum ether extract, CE-Chloroform extract, ME-Methanol extract, AE-Aqueous extract

flavonoids, terpenoids, saponins, phenols, steroids and tannins [23]. But it is mentioning that alkaloid of this species of plant is often found to be toxic although its toxicity depends on concentration.

Total phenolic content

The TPC was expressed as gallic acid equivalent (GAE) (mg/g). In the preliminary phytochemical investigations, only CE and ME showed the presence of phenolic compounds. The TPC was calculated from standard gallic acid calibration curve ($y = 0.005x + 0.024; R^2 = 0.998$) and these were 47.6 ± 2 and 44.6 ± 4.6 for CE and ME respectively. Phenolic compounds are very important plant constituents because of their scavenging ability due to their hydroxyl groups. It is known that non-phenolic antioxidants could also contribute to the antioxidant activity of an extract [24].

Antioxidant activity

Antioxidants are characterized as substances that even at low concentration significantly delay or avoid oxidation of simple oxidizable substrates. It works through preventing the generation of free radicals produced in the body or reducing/chelating the transition metal composition [25].

DPPH radical scavenging activity

The free radical scavenging activities of the extracts of *C. pallida* stem were determined through DPPH method and the results are presented in Fig. 1. The percentage of inhibition by PEE steadily increased linearly with the increase of concentration but the rate was very lower than other extracts and standards. On the other hand, the percentage of inhibition by other extracts were increased steadily which was almost similar to the standards. It is evident from the data that, the value of 50% scavenging activity (IC_{50}) of PEE was significantly lower (126.96 µg/ml) in contrast to other extracts and AE was abruptly high (898.46 µg/ml) are presented in Fig. 2. Lower IC_{50} value indicates higher antioxidant activity.

The role of antioxidants is their interaction with oxidative free radicals. The essence of DPPH method is that

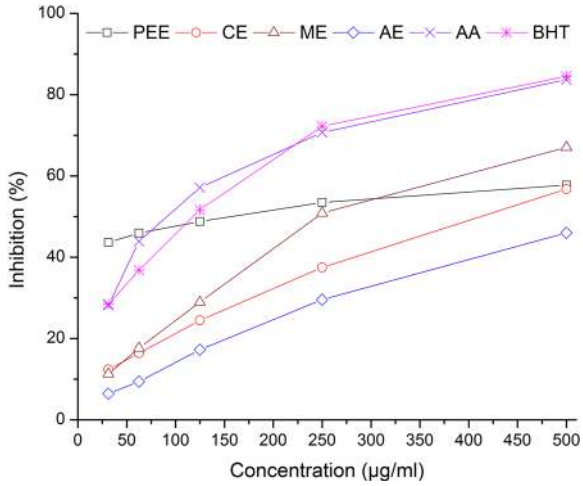

Fig. 1 DPPH radical scavenging activity of different extracts of *C. pallida* stem and standard antioxidants. All tests were done three times. PEE-Petroleum ether extract, CE-Chloroform extract, ME-Methanol extract, AE-Aqueous extract, AA-Ascorbic acid, BHT-Butylated hydroxytolune

the antioxidants react with the stable free radical 2,2-diphenyl-1-picrylhydrazyl (deep violet color) and convert it to 2,2-diphenyl-1-picrylhydrazine with discoloration. The degree of discoloration indicates the scavenging potentials of the sample antioxidant. In the present study, the extracts of *C. pallida* stem were able to decolourise DPPH and the free radical scavenging potentials of the extracts were found to be in order of PEE > ME > CE > AE. It has been found that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (hydroquinone, pyrogallol, etc.), and aromatic amines

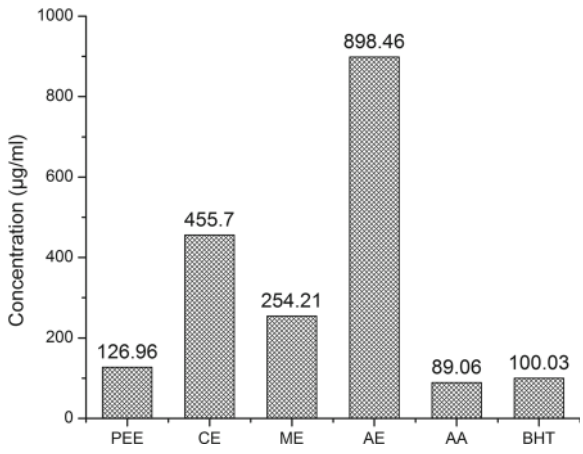

Fig. 2 IC_{50} of different extracts of *C. pallida* stem and standard antioxidants. All tests were done three times. PEE-Petroleum ether extract, CE-Chloroform extract, ME-Methanol extract, AE-Aqueous extract, AA-Ascorbic acid, BHT-Butylated hydroxytolune

(p-phenylene diamine, p-aminophenol etc.), reduce and decolourise DPPH by their hydrogen donating ability [26]. It appears that the extracts from the *C. pallida* stem process hydrogen donating capabilities to act as antioxidant.

Reducing power assay

The reducing power abilities of different extracts of *C. pallida* stem, and reference, BHT are shown graphically in Fig. 3. The absorbance of all extracts and standard is a function of their concentrations, and generally, increases linearly with the increase in concentration. Absorbance by PEE was highest compared to all other extracts, but lower than that of standard BHT in all concentrations. In addition, absorbances of PEE, CE and ME were below and shown similar behavior to standard BHT. AE showed lowest absorbances at all concentrations in contrast to others.

The reducing power assay is based on single electron transfer reaction, which is a measure of the antioxidant capacity to reduce ferric ions to ferrous ions in a reaction mixture. The presence of antioxidant causes the conversion of the Fe^{3+}/ ferricyanide complex (Perl's Prussian blue) to the ferrous form, where the intensity is dependent on the concentration of antioxidants [27]. From the results, it is plausible that, the high antioxidant activity observed in the PEE due to its alkaloid(s) contents. In addition, may be the absence of phenolic contents causes the low reduction capability of AE.

Total antioxidant capacity

The TAC of various solvent extracts of *C. pallida* were expressed quantitatively in ascorbic acid equivalents per gram of extracts (AAE) (mg/g) and calculated from ascorbic acid standard equation ($y = 0.001x - 0.015$; $R^2 = 0.999$) and shown in Fig. 4. CE shown significantly highest AAE/g extracts value (576 ± 34 mg/g) over other extracts, and that of lowest (126 ± 12 mg/g) shown by AE.

The TAC mainly concentrates on the thermodynamic conversion and measures the number of electrons or free radicals donated or quenched by a given antioxidant molecule. It is based on the reduction of Mo (VI) to Mo (V) and subsequent formation of green phosphate/Mo (V) complex, which evaluates both water-soluble and fat-soluble antioxidant capacity. The present study reveals that the non-polar extracts showed higher antioxidant activity than polar extracts in general.

Reduction of ferric ions

The measure of the reduction of Fe^{3+} to Fe^{2+} ions is another method to observe the antioxidant activity, which is illustrated in Fig. 5. The absorbance by PEE was steadily increased linearly with the increase of concentrations upto 62.5 µg/ml and sharply increased afterwards, which was exceptionally high in contrast to other extracts, as well as, standards at all but lower concentrations. The absorbance by CE was sharply increased of concentration up to 125 µg/ml and steadily increased afterwards, which was opposite to the PEE. On the other hand, the absorbance by ME and AE were increased steadily which was almost similar to the standards. o-Phenanthroline acts as chelating agent and forms exceptionally stable red colored complex with the reduced Fe^{2+} ions, which was measured by taking absorbance at 510 nm in the visible spectrum. The degree of coloration is a function of reduction potential to reduce Fe^{3+} to Fe^{2+}, which is also a function of

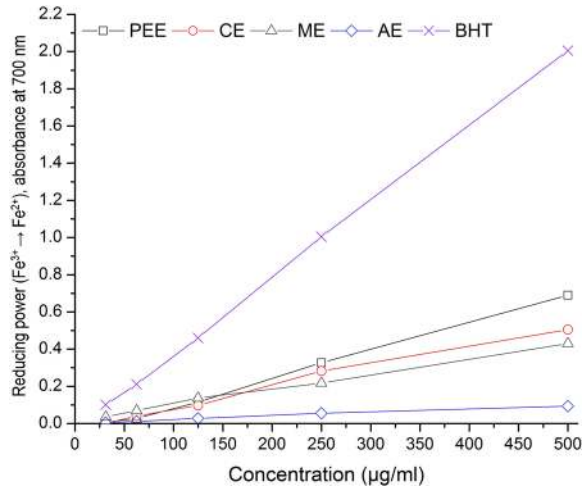

Fig. 3 Reducing power of different extracts of *C. pallida* stem and standard antioxidant. All tests were done three times. PEE-Petroleum ether extract, CE- Chloroform extract, ME-Methanol extract, AE-Aqueous extract, BHT-Butylated hydroxytolune

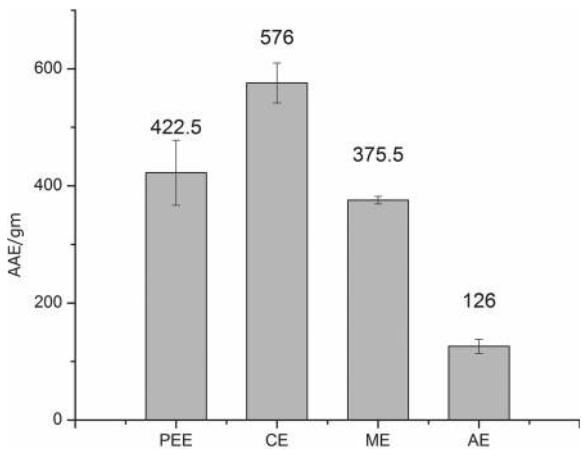

Fig. 4 Total antioxidant activity of extracts of *C. pallida* stem. All tests were done three times. PEE-Petroleum ether extract, CE- Chloroform extract, ME-Methanol extract, AE-Aqueous extract

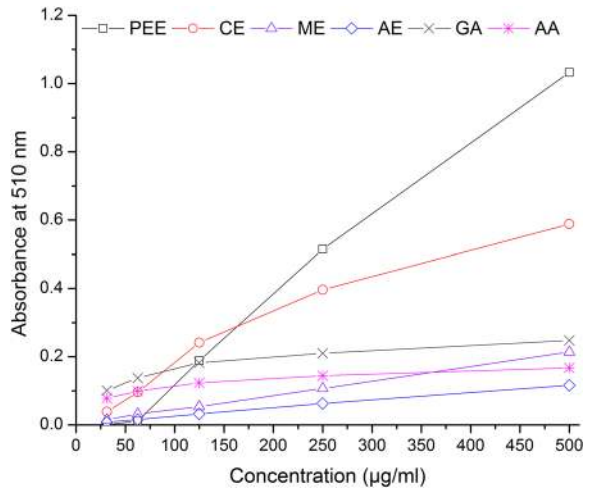

Fig. 5 Reduction of Fe^{3+} ions of different extracts of *C. pallida* stem and standard antioxidants. All tests were done three times. PEE-Petroleum ether extract, CE- Chloroform extract, ME-Methanol extract, AE-Aqueous extract, AA-Ascorbic acid, GA-Galic acid

absorbance. Therefore, the ability of a compound to transfer electron is a significant indicator of its potential as an antioxidant [28].

Antibacterial activity

The antibacterial activities of PEE, CE and ME of *C. pallida* stem were observed against two Gram-positive bacteria, the *S. aureus*, *B. cereus*, and two Gram-negative bacteria, the *E. coli*, *P. aeruginosa*. Imipenem was used as a positive control. The results of antibacterial assay are shown in Fig. 6. PEE and CE of *C. pallida* stem showed inhibitory effect against all of the bacteria used in this study except *B. cereus*. The ranges of zone were from 12 to 20 mm. In this study, *E. coli* showed highest sensitivity against PEE with the zone of inhibition of 20 mm whereas *P. aeruginosa* showed lowest sensitivity against CE with the zone of inhibition of 12 mm. *B. cereus* showed no sensitivity against any of the tested extracts. The reasons behind the difference in efficacy of different solvents extracts are yet to study, however; it might be due to varying degrees of solubility of the active constituents with the solvents. The antagonized effects of the solvents with the constituents of *C. pallida* stem extract might be responsible for no effect against bacteria [29]. A lot of mechanism is available in which tannins and flavonoids usually form complex with bacterial cell, bind with protein and may inhibit the enzyme resulting in kill of bacteria. Research indicates that most pathogenic bacteria such as *S. aureus* and *E. coli* isolated from the hands of health workers are resistant to many antimicrobial agents [30]. But this plant extract shown to be active moderate to highly against such antimicrobial agents.

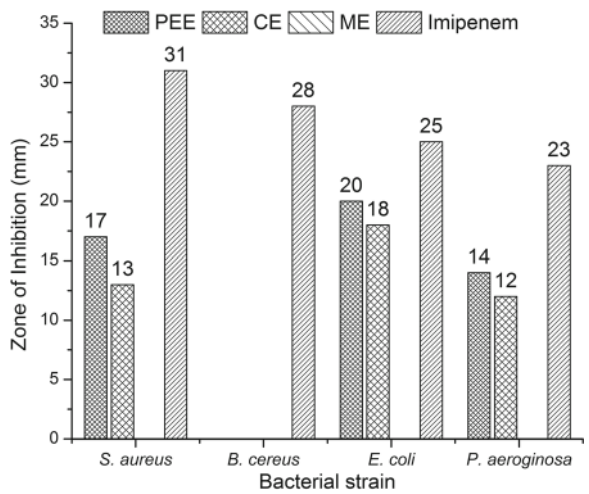

Fig. 6 Zone of inhibition of the *C. pallida* stem extracts against four bacterial strains. All tests were done two times. PEE-Petroleum ether extract, CE-Chloroform extract, ME-Methanol extract

Conclusion

It was evident from the above discussion that these extracts did show reductive potential and could serve as electron and/or proton or free radical donors terminating the radical chain reaction, as well as exhibited antibacterial activity towards both Gram-positive, and Gram-negative bacteria. Further study, however, needs to be done to identify and characterize the active compounds responsible for the observed properties. Thus, it could be concluded that the extracts of the *C. pallida* stem possessed imperative pharmacological properties, which will be potential to develop natural compounds based pharmaceutical products.

Abbreviations

AA: Ascorbic acid; AAE: Ascorbic acid equivalent; AE: Aqueous extract; ATCC: American type culture collection; BHT: Butylated hydroxytoluene; CE: Chloroform extract; DPPH: 2,2-diphenyl-1-picrylhydrazyl; GA: Gallic acid; GAE: Gallic acid equivalent; ME: Methanol extract; PEE:Petroleum ether extract; TAC: Total antioxidant capacity; TPC: Total phenolic content; UV-Vis: Ultraviolet-visible

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Authors' contributions

Sample collection, all laboratory experiments and data collection were done by MZI except antibacterial activity. FH performed antibacterial activity. Study design and supervision, data analysis, graphical representation, interpretation and drafting manuscript were done by MTH. MZI, FH, SKM and NS helped to draft the manuscript. SCP helped to revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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