

Quantitative Phytochemical Estimation and Evaluation of Antioxidant and Antibacterial Activity of Methanol and Ethanol Extracts of *Heliconia rostrata*

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Moonmun, et al.: Antioxidant and Antibacterial Activity of *Heliconia rostrata*

Rhizomes of *Heliconia rostrata*, (family: Heliconiaceae) have been well known for antiophidic property. Ethnobotanically, rhizome of this plant has also been used to cure jaundice, intestinal pain and hypertension. This study aimed at evaluating the antioxidant and antimicrobial potency of two extracts of rhizomes of *H. rostrata* to find out the one, which exhibited better activity. Methanol and ethanol extract of the dried and defatted rhizomes were prepared and were subjected to qualitative and quantitative estimation of phytoconstituents, antioxidant assay and antibacterial evaluation. The methanol extract was found to give better yield than the ethanol extract. All phytoconstituents detected in the methanol extract were found in the ethanol extract also except for the glycoside content, which was found only in the methanol extract. Total phenolic and flavonoid content was higher in the ethanol extract compared to the methanol extract, whereas total tannin content was higher in the methanol extract. Both ethanol and methanol extracts exhibited antioxidant and antibacterial potency. The antioxidant activities of both extracts were found to be equal, while the ethanol extract exerted significantly higher antibacterial activity compared to the methanol extract.

Key words: Heliconiaceae, rhizome, phytoconstituents, antioxidant, antimicrobial

Medicinal plants offer alternative therapies with spectacular opportunities^[1]. *Heliconia rostrata*, popularly known as false bird of paradise and hanging lobster claw (family: Heliconiaceae) is a shrub that is reported to contain calcium, nitrogen, potassium, magnesium, iron, phosphorous, chlorine, sulphur, sugar, carbohydrate, starch and protein^[2]. This plant is reported to be well known for antiophidic property^[3]. Ethnobotanically, the traditional people of Malaysia used these rhizomes to cure jaundice, intestinal pain and hypertension^[4]. However, not much of experimental evidence was documented.

Oxygen, the most important component for the survival of living being is highly reactive and can do serious damage to healthy cells of the body as a free radical. Oxidation produces free radicals, which can start chain reactions in the cell leading to cell damage and even cell death^[5]. An antioxidant terminates these radical reactions by removing free radical intermediates, which causes ageing and disease like atherosclerosis, cancer, cardiovascular diseases and CNS disorders^[6] thus, play a vital role in inhibiting or delaying the oxidation at the cellular level^[7]. Synthetic antioxidants, butylatedhydroxytoluene

(BHT), butylatedhydroxyanisole (BHA) and tert-butyl hydroquinone (TBHQ) are being evaluated in many countries for their potential health hazard and have been found to promote liver damage and causing cancer to laboratory animals^[8]. In comparison, the plant sources prove to have less or no side effect^[6], thus channelizing the interest towards the nature for antioxidant potency.

Researchers now are focusing on the natural products to develop new molecules for the treatment of microbial diseases, since current trend shows the failure of the existing antimicrobials in treating the infectious diseases^[9,10]. Thus, there exists requirement of some novel antimicrobials for the obliteration of new opportunistic pathogens^[11]. To identify the possible medicinal potency of any plant, the *in vitro* antibacterial studies and the antioxidant activity assay have to be carried out as the first step^[12]. Present study

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aimed at evaluating the antioxidant and antimicrobial potency of two extracts of rhizomes of *H. rostrata* to find out the extract, which produced the better activity to carry out further studies.

MATERIALS AND METHODS

Plant of *H. rostrata* was collected from and authenticated at the Regional Plant Resource Centre (RPRC), Bhubaneswar (Authentication Field Number, MDHR-1). These plants were cultivated in the house garden for 6 mon before using them for the experimental purpose and were harvested in the early morning of first week of September. All the chemicals and solvents used in the study were of analytical grade procured from the Merck Specialities Private Limited.

Fresh rhizomes of *H. rostrata* were collected, washed in tap water to get rid of any contaminants^[13] and then dried to remove water from the surface of the rhizomes. The rhizomes were cut into small pieces and dried under shade at room temperature for 7 d. The dried rhizomes were ground to a coarse powder^[14] and defatted using petroleum ether. Equal quantities of defatted dried rhizome of *H. rostrata* were subjected to maceration with methanol and ethanol for 72 h each. It was then filtered and concentrated (rotary-evaporator) to obtain dried extracts rhizome methanol extract (RME) and rhizome ethanol extract (REE). The % yield was calculated using the following Eqn.: yield (%) = (weight of extract in g/weight of defatted dried rhizomes in g) × 100.

Phytochemical screening:

The extracts were screened for the presence of alkaloids, glycosides, steroids and triterpenoids, flavonoids, proteins and amino acids, carbohydrates and reducing sugars, tannins and saponins using standard methods^[15-17].

Test for glycosides:

Half a gram of powdered extract was diluted to 5 ml with water followed by the addition of 2 ml of glacial acetic acid and a drop of ferric chloride solution. To this, 1 ml of concentrated sulphuric acid was added very slowly. Presence of glycosides would result appearance of a brown ring at the interface^[15].

Test for alkaloids:

To 0.5 g of powdered RME and REE, 1.5 ml of ammonia solution was added. This was allowed to stand for some time and then 5 ml of chloroform was

added, shaken well and then filtered through a Whatman filter paper. The filtrate was warmed on a water bath to evaporate chloroform. One millilitre of Mayer's reagent was added to it. Formation of a cream colour precipitate indicated the presence of alkaloids^[16]. To 0.5 g of extract 5 ml of 1% of aqueous HCl was added, warmed on a water bath for a few minutes and filtered. To the filtrate few drops of Dragendorff's reagent was added. Turbidity or precipitation indicates the presence of alkaloids^[17].

Test for steroids and triterpenoids:

Liebermann Burchard reaction was performed by adding to 5 ml of the extract solution, 2 ml of acetic anhydride followed by 2 ml of concentrated sulphuric acid. Change of violet colour to blue indicated the presence of steroids^[17]. To 5 mg of extract, 2 ml of chloroform was added followed by the addition of 1 ml of acetic anhydride and 1 ml of concentrated sulphuric acid. Formation of reddish violet colour indicates the presence of triterpenoids^[16].

Test for flavonoids:

Five millilitres of dilute ammonia was added to 0.5 g of extract dissolved in water. One millilitre of concentrated sulphuric acid was added to it. Disappearance of yellow colour on standing indicated the presence of flavonoids^[15]. Formation of yellow colour on addition of few drops of 1% aluminium chloride indicates the presence of flavonoids^[15].

Test for proteins and amino acids:

Biuret test was performed by adding to 3 ml of extract, 4% sodium hydroxide and a few drops of 1% copper sulphate solution. Presence of proteins or amino acids is indicated by the formation of a violet or pink colour^[17].

Test for carbohydrates and reducing sugars:

Molisch test was performed by the addition to 5 ml of aqueous extract, 2 drops of alcoholic alpha naphthol solution and shaking the mixture well. To this mixture concentrated sulphuric acid was added through the walls of the test tube. Appearance of a violet ring indicates the presence of carbohydrates^[17]. Fehling test was done by adding to 1 ml of Fehling A solution, 1 ml of Fehling B solution and boiling the mixture for a minute. Then equal amount of extract solution was added and heated in a water bath for 5 to 10 min. Appearance of a brick red precipitate indicated the presence of reducing sugars in the extract^[17].

Test for tannins:

Five millilitres of n-butanol HCl solution was added to 2 ml of extract solution in a test tube. This mixture was warmed on a water bath at 95° for an hour. Presence of tannin is indicated by the appearance of a red colour^[15]. Five millilitres of chloroform followed by 1 ml of acetic anhydride was added to 0.5 ml of extract solution in a test tube. Then 1 ml of concentrated sulphuric acid was added through the walls of the test tube. Appearance of a green colour indicated the presence of tannin^[16].

Test for saponins:

Five millilitres of distilled water was added to 0.5 g of dry extract and shaken vigorously. Stable foam indicated the presence of saponins. Further, formation of an emulsion on addition of 3 drops of olive oil and vigorous shaking also indicated the saponin presence in the extract^[15].

Quantitative estimation of total phenolic content (TPC):

TPC was estimated using Folin-Ciocalteu reagent (FCR) with gallic acid as the standard^[18]. FCR being sensitive to reducing compounds including polyphenols produce blue colour upon reaction. Absorbance of each was checked at 760 nm spectrophotometrically. Standard curve was prepared with different dilutions of gallic acid in ethanol and methanol. All the determinations were done in triplicate. The TPC is expressed in mg gallic acid equivalent (GAE)/g dry extract.

Quantitative estimation of total tannin content (TTC):

TTC was estimated using Folin-Denis reagent (FDR) and taking tannic acid as the standard^[19]. The solution was mixed properly and the absorbance of each was checked at 700 nm spectrophotometrically. Standard curve was prepared with standard dilutions of tannic acid in ethanol and methanol. All the determinations were done in triplicate. The TTC is expressed in mg tannic acid equivalent (TAE) per gram dry extract.

Quantitative estimation of total flavonoid content (TFC):

TFC was determined taking quercetin as the standard by aluminium chloride method^[20]. Absorbance was determined spectrophotometrically at 510 nm. Standard curve was prepared with quercetin solution prepared in ethanol and methanol. All the determinations were done in triplicate. The TFC is expressed in mg quercetin equivalent/g dry extract.

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay^[21]:

DPPH radical scavenging assay is one of the common methods of determining the antioxidant activity of plant extracts. A working solution of DPPH was prepared by diluting stock solution of DPPH (0.24 mg/ml) using methanol to achieve an absorbance of 0.980 ± 0.020 at 517 nm. Three millilitres of working solution of DPPH was mixed with 100 μ l of dilution of extracts and kept undisturbed at room temperature for 15 min. Absorbance was taken at 517 nm. Ascorbic acid was used as the standard. Percent scavenging of DPPH (% inhibition) was calculated for different concentration (50-250 μ g/ml) of extract and standard using the following Eqn., $\text{DPPH \% scavenging} = (A_0 - A_1 / A_0) \times 100$, where, A_0 is the absorbance of the control at 15 min and A_1 is the absorbance of the sample at 15 min. The concentration of extract at which 50% inhibition is observed (IC_{50}) is calculated in μ g/ml.

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt) radical scavenging assay^[22]:

ABTS radical scavenging assay is another method of determining the antioxidant capacity of plant extracts. A total of 14 mmol/l ABTS solution and 4.9 mmol/l potassium persulphate solution was mixed and kept in dark at room temperature for 16 h to produce ABTS⁺ cation radicals. This solution was diluted with ethanol to attain an absorbance of 0.700 ± 0.020 at 745 nm. Three millilitres of ABTS working solution was mixed with 100 μ l of dilution of extracts, mixed well and kept at room temperature for 5 min. Absorbance was measured spectrophotometrically at 745 nm. Ascorbic acid was used as the standard. Percent scavenging of ABTS radical (% inhibition) was calculated for different concentrations (50-250 μ g/ml) of extract and standard using the following Eqn., $\text{ABTS \% scavenging} = (A_0 - A_1 / A_0) \times 100$, where, A_0 is the absorbance of the control at 5 min and A_1 is the absorbance of the sample at 5 min. The concentration of extract at which 50% inhibition is observed (IC_{50}) is calculated in μ g/ml.

Hydrogen peroxide (H_2O_2) assay^[23]:

H_2O_2 assay was performed with ascorbic acid as the standard. A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (0.2 mol/l, pH 7.4). One hundred microlitres of different concentrations (50-250 μ g/ml) of the extracts were added to 3 ml of H_2O_2 solution and kept for 10 min. The absorbance was measured

spectrophotometrically at 230 nm after 10 min of reaction time. H_2O_2 %scavenging (%inhibition) was calculated for different concentration of extract and standard using the following Eqn., H_2O_2 %scavenging = $(A_0 - A_1/A_0) \times 100$, where, A_0 is the absorbance of the control after 10 min and A_1 is the absorbance of the sample at 10 min. The concentration of extract at which 50% inhibition is observed (IC_{50}) is calculated in $\mu\text{g/ml}$.

Phosphomolybdate assay^[18]:

Total antioxidant activity (TAC) of REE and RME was determined using ascorbic acid as the standard. Phosphomolybdate reagent was prepared by mixing 100 ml of 28 mM sodium phosphate, 100 ml of 0.6 M sulphuric acid and 100 ml of 4 mM ammonium molybdate solutions. To 3 ml of phosphomolybdate reagent, 300 μl of different concentrations (50-250 $\mu\text{g/ml}$) of extract solution were added and incubated in the dark for 90 min at 95° . The absorbance was measured spectrophotometrically at 765 nm. TAC %scavenging = $(A_0 - A_1/A_0) \times 100$, where, A_0 is the absorbance of the control after 10 min and A_1 is the absorbance of the sample at 10 min. The concentration of extract at which 50% inhibition is observed (IC_{50}) is calculated in $\mu\text{g/ml}$.

Reducing power assay^[24]:

Equal volume of 0.2 M, pH 6.6 phosphate buffer and 1% ferrocyanate were mixed with different concentration (50-250 $\mu\text{g/ml}$) of extracts and standard (ascorbic acid) and incubated for 20 min at 50° . To 5 ml of the mixture 2.5 ml of 10% trichloroacetic acid was added and centrifuged at 3000 rpm for 10 min. To the supernatant 2.5 ml of distilled water and 0.5 ml of 1% ferric chloride was added. Absorbance was measured at 700 nm. Higher reducing power is indicated by high absorbance. Reducing power is a reflection of antioxidant activity of any compound. These compounds have the ability to donate electrons, thus reducing the oxidized intermediates and acting as antioxidants^[25].

Antibacterial study:

The antibacterial activity of the plant extracts was tested against three Gram-positive bacteria, *Bacillus stercorophilus* (American Type Culture Collection (ATCC) 7953), *B. subtilis* (Microbial Type Culture Collection (MTCC) 441) and *Staphylococcus aureus* (ATCC 29737) and six Gram-negative bacteria, *Escherichia coli* (ATCC 11229),

Klebsiella pneumoniae (MTCC 9401), *Salmonella enterica* (MTCC 9844), *Pseudomonas aeruginosa* (ATCC 9027), *Vibrio cholera* (ATCC 51395) and *Acinetobacter baumannii* (ATCC 17978). All the microorganisms with ATCC Number were obtained from the Laboratory of Microbiology, NICU, SUM Hospital, Bhubaneswar. All the microorganisms with MTCC Number were obtained from MTCC and Gene Bank, Institute of Microbial Technology, Chandigarh.

Well diffusion method for determination of zone of inhibition:

The antibacterial activity evaluation of RME and REE was performed using the well diffusion method^[26,27]. The inoculum of the microorganism was prepared from bacterial cultures. Fifteen millilitres of nutrient agar (HiMedia) medium was poured in clean sterilized Petri-plates and allowed to cool and solidify. Hundred microlitres of broth of bacterial stain was pipetted out and spread over the medium evenly with a spreading rod till it dried properly. Wells of 6 mm in diameter were bored using a sterile cork borer. Solutions of both the extracts (10 mg/ml) in dimethyl sulphoxide (DMSO) were prepared. Hundred microlitres of plant extract solutions was added to the wells. The Petri-plates were incubated at 37° for 24 h. Streptomycin (1 mg/ml) was used as a positive control and DMSO was taken as negative control. Antibacterial activity was evaluated by measuring the diameters of the zones of inhibition (ZI). All the determinations were performed in triplicate.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)^[28]:

Stock solutions of the strength 100 mg/ml of REE and RME were prepared in DMSO. Sterilized nutrient broth was cooled and supplemented with 0.05% 2,3,5-triphenyltetrazolium chloride. Nutrient broth, extract solution and 100 μl of bacterial inoculum were added to each well to achieve concentrations ranging 10 mg/ml to 1 mg/ml of each extract. The plates were incubated at 37° , in aerobic conditions for 24 h. The 11th well was kept as the negative control without any added extract. Growth of the bacteria was indicated by the formation of pink colour in the wells. MIC was the lowest concentration of REE/RME that inhibited the formation of pink colour, thereby inhibiting the growth of the bacteria in the well. For the determination of MBC, bacteria from each inhibited well was sub-cultured on nutrient agar plate and incubated for further 24 h at 37° under aerobic conditions. The minimum

dilution at which no bacterial growth was observed was considered as the MBC of the extracts.

Statistical analysis:

Statistical analysis was performed using SPSS version 20. One-way analysis of variance (ANOVA) was used to detect the significant difference between the extracts in antioxidant studies and antimicrobial study. A probability value $P \leq 0.05$ is considered to represent a statistically significance difference.

RESULT AND DISCUSSION

Extraction of defatted rhizomes of *H. rostrata* was carried out using methanol and ethanol as liquid extractor to yield dried RME (blackish brown powder) and REE (reddish brown powder). Defatting of plant parts is a pre-extraction process, which is expected to remove undesirable fatty substances from the plant powder. This process also enhanced the polarity of phenolic compounds thus increasing their yield in the extract^[29]. Percent yield of RME (5.38%) was found to be more than that of REE (2.25%) as shown in fig. 1. Phytochemical screening results of REE and RME have been presented in Table 1, which demonstrated the absence of alkaloids and steroids in both these extracts. Both REE and RME contained flavonoids, proteins and amino acids, carbohydrates and reducing sugars, tannins and saponins. Glycosides were found to be present in RME only. Absence of steroids and triterpenoids might be due to the defatting prior to the extraction of rhizomes with ethanol and methanol.

Literature review on this plant collected from different regions of the world show some contradicting results. The 70% ethanol extract of rhizomes of *H. rostrata* collected from Areado city, Brazil, displayed the presence of flavonoids and absence of alkaloids, tannin and saponin^[30]. Meanwhile, rhizomes of *H. rostrata* collected from Columbia showed the presence of terpenoids, proteins, phenolic compounds and carbohydrates^[3,31], when percolated with ethanol without defatting. This demonstrated that with the change in the solvent and method of extraction, different phytochemical constituents get extracted. Influence of geographical region from which the plant is collected also a likely factor contributing to differences in phytoconstituents.

The results of quantitative estimation of TPC, TTC and TFC along with the standard curves plotted have been depicted in fig. 2A, 2B, 2C, 2D, 2E and 2F. It is clearly evident from the results that TPC and TFC

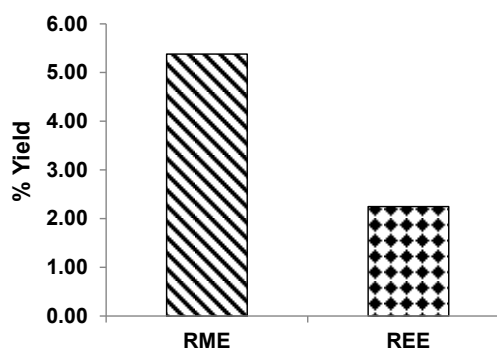


Fig. 1: % Yield of extracts

RME: rhizome methanol extract, REE: rhizome ethanol extract

TABLE 1: PHYTOCONSTITUENTS IN RME AND REE

Phytoconstituents	RME	REE
Alkaloid	-	-
Glycoside	+	-
Steroids and triterpenoids	-	-
Flavonoids	+	+
Proteins and amino acids	+	+
Carbohydrates and reducing sugar	+	+
Tannin	+	+
Saponin	+	+

‘+’ denotes presence and ‘-’ denotes absence of phytoconstituents

in REE were greater than the TPC and TFC of RME. RME contained TTC more than that in REE.

Antioxidant activity of any extract could possibly be attributed to numerous mechanisms. Binding of transition metal ion catalyst, radical scavenging, decomposition of peroxide, reducing capacity, prevention of continued hydrogen abstraction and prevention of chain initiation are some of these mechanisms. DPPH is a stable free radical with an absorption band at 515 nm. When reduced by an antioxidant to form DPPH, the natural deep violet colour of DPPH changes to pale yellow^[32]. The change in the colour would be proportional to the strength of the antioxidants and a significant decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the test material. ABTS radical scavenging assay, unlike DPPH radical scavenging assay can be used at a different pH level, thus making this method useful in studying the antioxidant activity at varying pH levels. Moreover ABTS model can evaluate the scavenging activity of both polar and non-polar samples^[22]. The reaction time of ABTS is lower than that of DPPH. Bluish green coloured ABTS radical was produced by reacting ABTS with the oxidising agent, potassium persulfate overnight. Reduction of ABTS radical by hydrogen donating antioxidant is measured spectrophotometrically^[33]. Results from the DPPH and ABTS radical scavenging assays were shown in fig. 3A

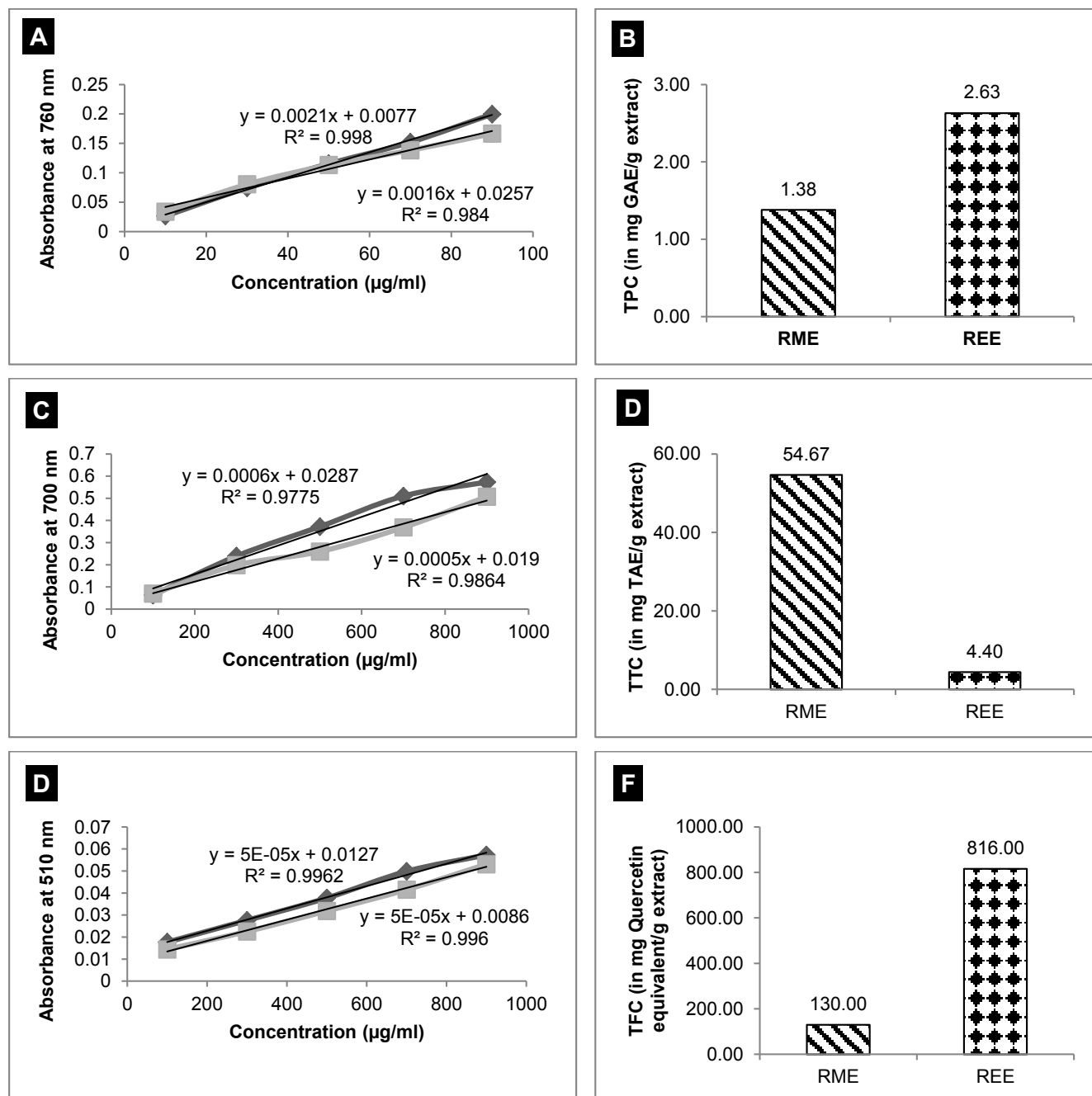


Fig. 2: Quantitative estimation of phytoconstituents

Standard curve of gallic acid (A), tannic acid (C) and quercetin (E) in both methanol and ethanol. Comparison by graphical representation of the total phenolic content (B), total tannin content (D) and total flavonoid content (F) in RME: rhizome methanol extract, REE: rhizome ethanol extract. A- ◆— gallic acid in methanol, —◆— gallic acid in ethanol; C- ◆— tannic acid in methanol, —◆— tannic acid in ethanol; E- ◆— quercetin in methanol, —◆— quercetin in ethanol

and 3B. H_2O_2 scavenging activity of the plant extracts was measured spectrophotometrically, which indicated the disappearance of H_2O_2 at 230 nm^[34]. H_2O_2 forms a hydroxyl radical in the cells, which can be toxic to them. Thus, removal of H_2O_2 is very important^[35]. Results of H_2O_2 scavenging activity have been shown in fig. 3C. Phosphomolybdate assay measures the capacity of an extract to destroy a free radical by transferring an electron to the later. Antioxidants present in the extract reduce molybdate (VI) to molybdate (V) and this can

be measured spectrophotometrically at 700 nm^[36]. Result of phosphomolybdate assay was illustrated in fig. 3 D.

The IC_{50} values of REE and RME were depicted in Table 2. The IC_{50} value of REE is lower than RME, which could be attributed to the fact that REE possessed better antioxidant property than RME. The antioxidant activity demonstrated a positive correlation with TPC^[1]. Moreover, flavonoids also act as scavengers to various

oxidising species^[37]. Therefore, it was quite sensible to determine the TPC and TFC in the plant extract. TPC and TFC of REE have been found to be more than that of RME as evident from fig. 3B and 3D, respectively, and this could possibly explain why the IC₅₀ of REE is lower than that of RME.

Depending on the reducing power of the testing compound, the yellow colour of the Fe(III) changes to Fe(II), Perl's Prussian blue which can be measured spectrophotometrically at 700 nm^[21]. The reductive capacity of REE and RME was compared to that of ascorbic acid in fig. 4. It is observed that there existed a positive correlation between the antioxidant activity and reducing power of REE and RME. Like the antioxidant activity study, reducing power of REE and

RME also increased with increasing concentrations of the extract. Reductions, which contribute to the antioxidant activity by breaking the free radical chain and donating a hydrogen atom, are also responsible for the reducing properties^[38]. Since all the tests performed for *in vitro* antioxidant activity demonstrated significant activity as shown in the Table 3, there is no significant difference between the results obtained from the antioxidant activity determination and reducing power study of REE and RME.

In the present study, the antibacterial potential of REE and RME have been tested against three Gram-positive and six Gram-negative bacteria. The well diffusion method of testing antibacterial activity has been widely considered to be better and is

TABLE 2: IC₅₀ VALUES OF RME AND REE

Testing samples	IC ₅₀ (µg/ml)			
	DPPH radical scavenging assay	ABTS radical scavenging assay	Hydrogen peroxide assay	Phosphomolybdate assay
Ascorbic acid	57.39	53.22	46.68	53.83
REE	127.84	125.01	117.05	125.77
RME	147.70	137.30	132.27	178.79

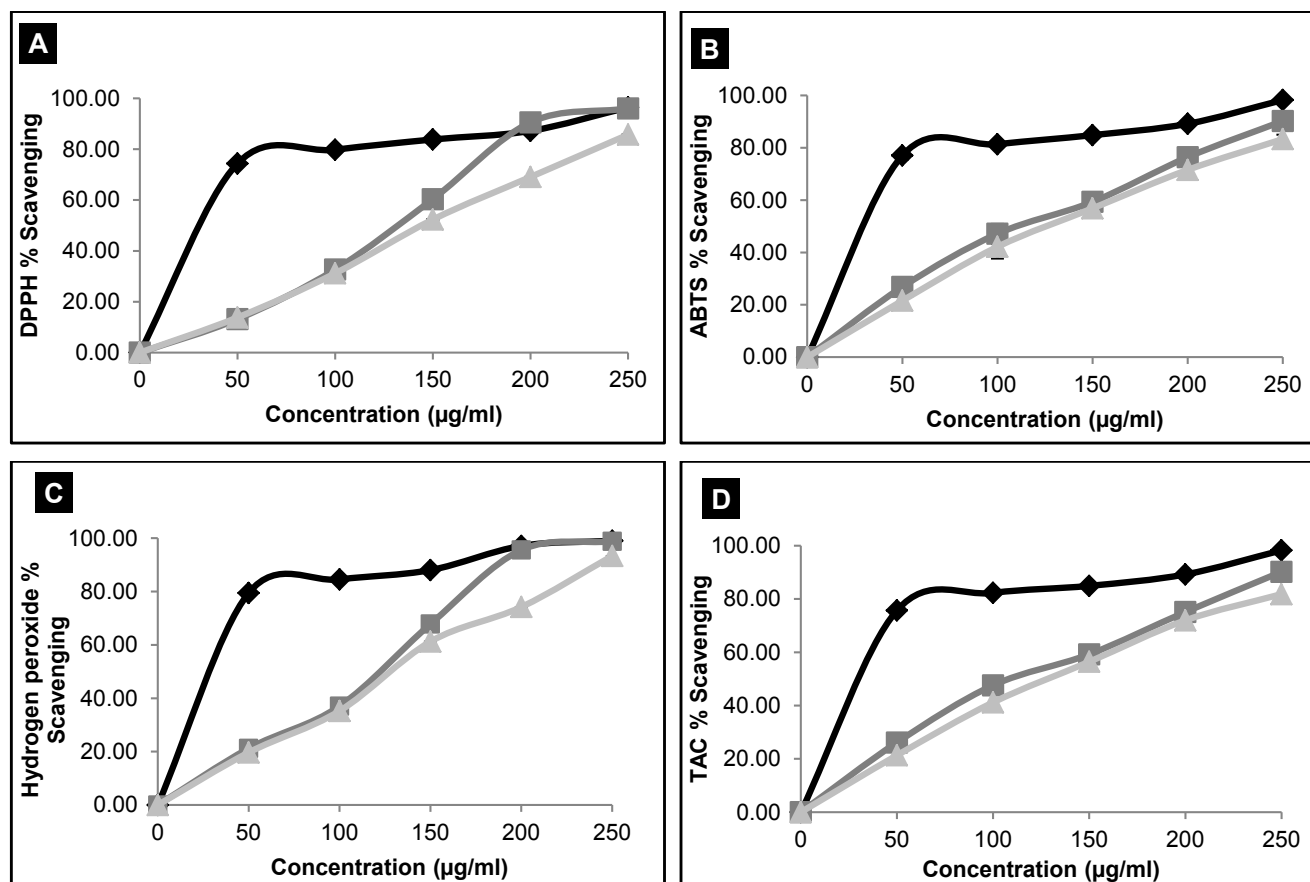


Fig. 3: Antioxidant activity study of ascorbic acid REE and RME using (A) DPPH radical scavenging assay, (B) ABTS radical scavenging assay, (C) hydrogen peroxide assay and (D) phosphomolybdate assay. All the results are mean±SD (n=3). —◆— Ascorbic acid; —■— (REE) rhizome ethanol extract; —▲— (RME) rhizome methanol extract

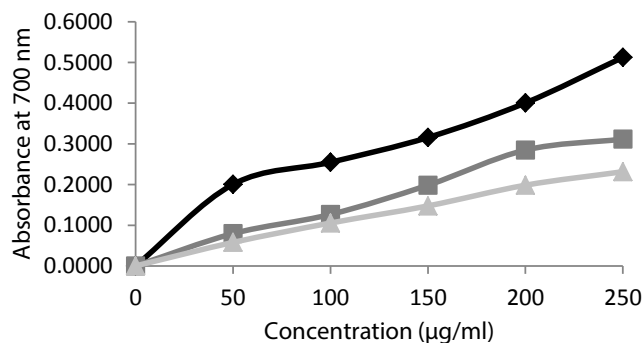


Fig. 4: Reducing power assay

All the results are mean±SD (n=3), —◆— ascorbic acid; —■— (REE) rhizome ethanol extract; —▲— (RME) rhizome methanol extract

TABLE 3: STATISTICAL EVALUATION (ONE-WAY ANOVA) OF THE EXTRACTS (REE AND RME) WITH DIFFERENT ANTIOXIDANT ASSAY METHODS. (SIGNIFICANCE LEVEL, A=0.05)

Method	F-value	P-value
DPPH radical scavenging assay	0.547	0.466
ABTS radical scavenging assay	0.347	0.561
H ₂ O ₂ radical scavenging assay	0.457	0.505
Phosphomolybdate assay	0.384	0.541
Reducing power	3.207	0.084

related to the carrier solvent, DMSO, which diffused effortlessly across the medium^[10]. Here, DMSO is used as a solvent for dissolving REE and RME in the antibacterial study. DMSO is a dipolar aprotic solvent that has the ability to dissolve both polar and nonpolar compounds. DMSO is used as a cryoprotectant for subzero temperature fractionation and long term low temperature conservation of various biomaterials^[39] and thus considered to have no effect on the growth of microorganisms. Though the literature supports the use of DMSO as solvent for antibacterial studies, a negative control of DMSO is also included in each plate to be extra sure.

In the present scenario rising resistance by many organisms worldwide towards the antibacterial usually in use^[40], it is very important that the actual ingredients having antibacterial potential needs to be extracted. The present study is helpful in finding the extract which showed better antibacterial activity. Literature survey revealed the fact that *H. rostrata* when extracted with 70% w/v ethanol showed no antibacterial activity at a concentration of 10 mg/ml^[30]. Chavasco *et al.* reasoned that this could be due to the solvent chosen for extraction such as 70% w/v ethanol, which failed to extract the active constituents responsible for antibacterial activity. In this study, 70% ethanol was replaced with absolute ethanol and methanol as solvents for extraction. This study also compared the antibacterial activity of ethanol

and methanol extracts of rhizome of *H. rostrata*, REE and RME, respectively at the same concentration of 10 mg/ml. Results presented in figs. 5 and 6 demonstrated that both REE and RME at the concentration 10 mg/ml exerted antibacterial activity against Gram-positive and Gram-negative bacteria. DMSO showed no antibacterial activity. The antibacterial activity of REE and RME might be attributed to the TPC, TTC and TFC in REE and RME. The very high levels of TFC and TPC in REE compared to RME might have resulted in the greater antibacterial potency of REE, since flavonoids and phenolic compounds are believed to be playing a role protecting plants against microbial attack^[37]. Tannins also act as astringents and have the ability to bind, precipitate or shrink proteins^[41]. This ability of tannins could directly damage the bacterial cell wall, precipitate bacterial proteins leading to the death of bacteria^[42,43]. The presence of tannin in RME could be the reason for the antibacterial activity of RME. The ZI obtained in this investigation have been shown in Table 4 and from the figs. 5 and 6, which compares the ZI formed by REE and RME against different bacteria, it would be possible to state that REE exhibited greater antibacterial activity when compared to RME. Both REE and RME failed to show any ZI against *K. pneumonia*. Concentrations higher than 10 mg/ml might be effective against this bacterium.

MIC is the lowest concentration of extract that prevented the visible microbial growth^[44]. 2,3,5-Triphenyl tetrazolium chloride is used as a growth indicator in the MIC determinations. 2,3,5-Triphenyl tetrazolium chloride is a white crystalline compound. It is a redox indicator, which is used to distinguish between metabolically active and sedentary tissues. The white coloured 2,3,5-triphenyl tetrazolium chloride reduced to pink/red coloured 1,3,5-triphenyl formazan (TPF) in the presence of living tissues due to the activity of various enzymes important in cellular metabolism^[45,46]. 2,3,5-Triphenyl tetrazolium chloride at concentration >0.125% did not show any inhibitory effect on the bacterial growth and concentrations <0.003% did not exhibit a change in colour^[47]. Thus, MIC is taken to be the lowest concentration in which the pink colouration is not observed. MIC values of REE were lower than or equal to those of RME against all bacteria tested. MBC is the lowest concentration of the extract at which no bacterial growth is observed after sub-culture on to an antimicrobial free medium. The MBC of REE and RME against all the bacteria except *P. aeruginosa* were found well within 10 mg/ml. MIC and MBC of

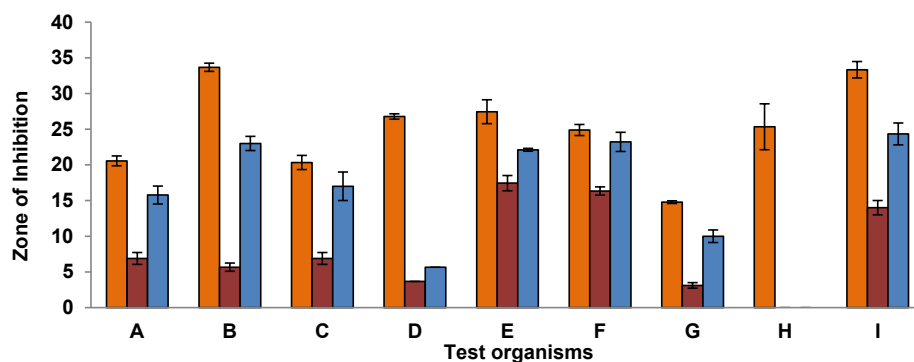


Fig. 5: Zone of inhibition of various bacteria for positive control

RME and REE where, (A) *Staphylococcus aureus*, (B) *Bacillus stercorothermophilus*, (C) *Bacillus subtilis*, (D) *Pseudomonas aeruginosa*, (E) *Vibrio cholera*, (F) *Escherichia coli*, (G) *Acinetobacter baumannii*, (H) *Klebsiella Pneumoniae* and (I) *Salmonella enterica*. All the results are mean \pm SD (n=3). ■ Positive control ■ (RME) rhizome methanol extract ■ (REE) rhizome ethanol extract

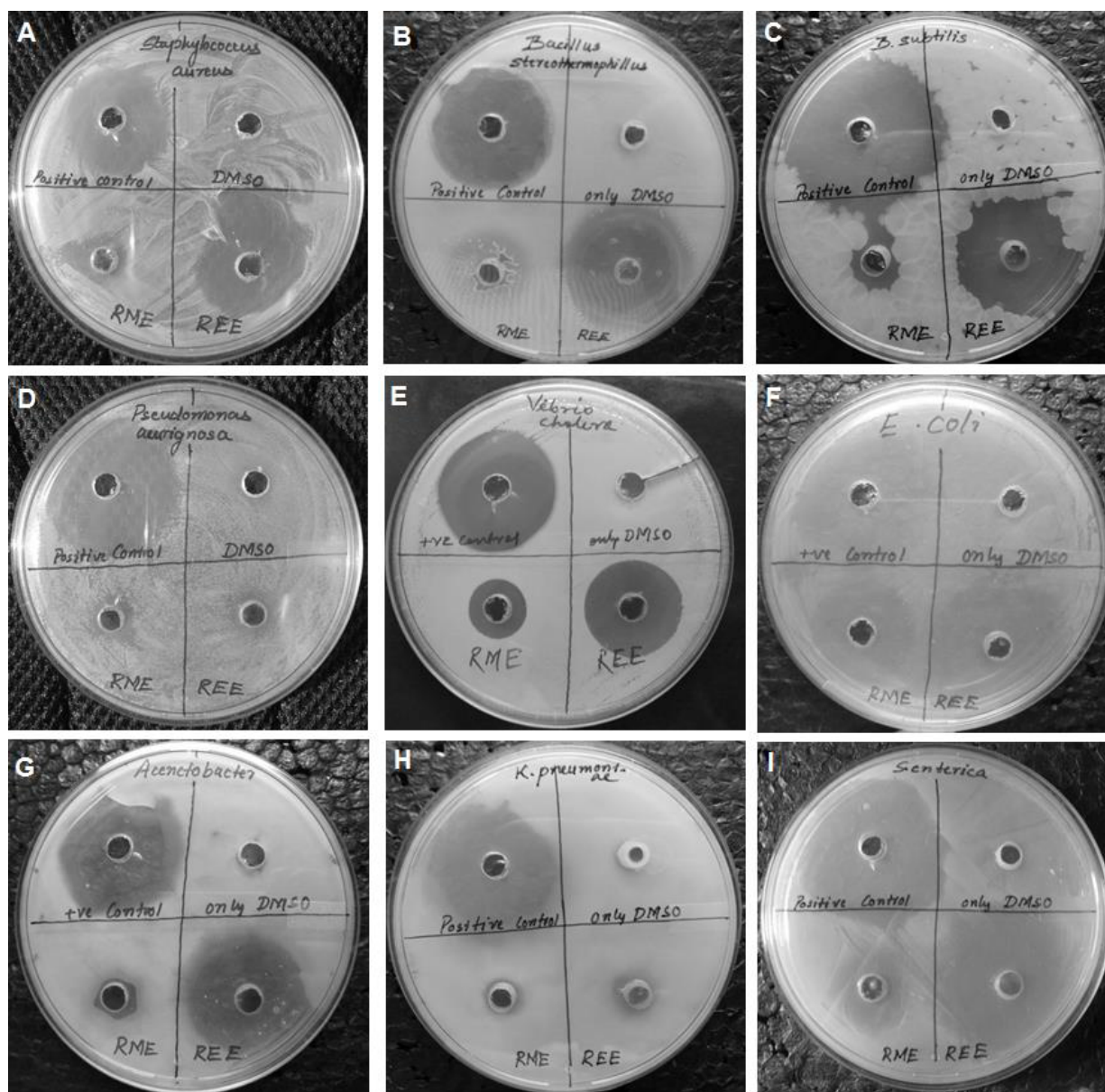


Fig. 6: Antibacterial study

RME and REE against (A) *Staphylococcus aureus*, (B) *Bacillus stercorothermophilus*, (C) *Bacillus subtilis*, (D) *Pseudomonas aeruginosa*, (E) *Vibrio cholera*, (F) *Escherichia coli*, (G) *Acinetobacter baumannii*, (H) *Klebsiella Pneumoniae* and (I) *Salmonella enterica*

TABLE 4: ZONE OF INHIBITION (ZI), MINIMUM INHIBITORY CONCENTRATION (MIC), MINIMUM BACTERICIDAL CONCENTRATION (MBC)

Test organism	Test sample	ZI±SD (mm)	MIC	MBC	F-value	P-value
<i>S. aureus</i> *	DMSO	0		ND		
	Positive control	20.56±0.69				
	REE	15.78±1.26	<4 mg/ml	<4 mg/ml	103.22	0.001
	RME	6.89±0.84	<4 mg/ml	<4 mg/ml		
<i>B. subtilis</i> *	DMSO	0		ND		
	Positive control	33.67±0.58				
	REE	23.00±1.00	<2 mg/ml	<2 mg/ml	676.00	0.000
	RME	5.67±0.58	<1 mg/ml	<4 mg/ml		
<i>B. stereothermophilus</i> *	DMSO	0		ND		
	Positive control	20.33±1.00				
	REE	17.00±2.00	<1 mg/ml	<1 mg/ml	65.21	0.001
	RME	6.89±0.84	<1 mg/ml	<2 mg/ml		
<i>P. aeruginosa</i>	DMSO	0		ND		
	Positive control	26.78±0.38				
	REE	5.67±0.00	<4 mg/ml	>10 mg/ml	NF	NF
	RME	3.67±0.00	<8 mg/ml	>10 mg/ml		
<i>V. cholerae</i>	DMSO	0		ND		
	Positive control	27.44±1.68				
	REE	22.11±0.19	<1 mg/ml	<1 mg/ml	55.13	0.002
	RME	17.44±1.07	<1 mg/ml	<1 mg/ml		
<i>E. coli</i>	DMSO	0		ND		
	Positive control	24.89±0.77				
	REE	23.22±1.35	<1 mg/ml	<1 mg/ml	66.27	0.001
	RME	16.33±0.58	<2 mg/ml	<2 mg/ml		
<i>A. baumannii</i>	DMSO	0		ND		
	Positive control	14.78±0.19				
	REE	10.00±0.88	<1 mg/ml	<1 mg/ml	165.37	0.000
	RME	3.11±0.38	<1 mg/ml	<1 mg/ml		
<i>K. pneumonia</i>	DMSO	0		ND		
	Positive control	25.33±3.21				
	REE	0	NF	NF	NF	NF
	RME	0	NF	NF		
<i>S. enterica</i>	DMSO	0		ND		
	Positive control	33.33±1.15				
	REE	24.33±1.53	<1 mg/ml	<1 mg/ml	96.10	0.001
	RME	14.00±1.00	<1 mg/ml	<4 mg/ml		

Statistical evaluation of the extracts (REE and RME) against different Gram-positive and Gram-negative bacteria. All the results are mean±SD (n=3). (Significance level, $\alpha=0.05$). Marked bacteria are Gram-positives and rest are Gram-negatives. ND=Not Done, NF=Not found.

REE and RME were not determined for *K. pneumonia*, since no ZI could be observed at 10 mg/ml. Results presented in figs. 5 and 6 and Table 4 indicated that REE showed significantly higher antibacterial activity compared to that of RME.

Rhizomes of *H. rostrata* were extracted to obtain REE and RME. Both REE and RME were subjected to phytochemical screening and were found to contain flavonoids, proteins and amino acids, carbohydrates and reducing sugars, tannins and saponins except for glycosides, which were found only in RME. TPC and TFC is more in REE while TTC was more in RME. Both REE and RME showed no significant difference

in their antioxidant potential while, REE was found to have greater antibacterial activity.

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Conflict of interest:

There are no conflicts of interest.

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