Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2021; 10(1): 1988-1998
Received: 13-11-2020
Accepted: 15-12-2020

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In vitro assessment of quantitative analysis of phytochemicals, iron (II) chelating activity and antimicrobial efficacy of methanolic solvent extract of citrus maxima leaf

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Abstract
Herein, we report the quantitative analysis of phytochemicals, iron (II) chelating activity, and antimicrobial efficacy of *Citrus maxima* (Pomelo) leaves in methanol extract using Folin-Ciocalteu's colorimetric assay, aluminium chloride colorimetric assay, Ferrous-ferrozine complex inhibition assay and agar disc diffusion method. The total phenol contents extracted as 117.68±0.63 mg of GAE/g of dry extract and 14.39±0.09 mg of CE/g of dry extract and for total flavonoid it was 28.55±0.39598 mg of CE/g of dry extract. The inhibitory concentration (IC_{50}) of ferrous ion chelating activity was recorded as 323.79 µg/mL. Also, this chelating activity increases with the extract concentrations. On the other hand, this leaf extracts displaced a concentration dependent inhibition of microorganisms, the eleven different cultures responded to samples, by virtue of which we obtained in the range of 5.5-12 mm zone of inhibition. Especially, this leaf extract shows a satisfactory antibacterial efficacy on *Salmonella paratyphi, Salmonella typhi* and *V. cholerae C6706*. The outcomes of the study corroborate the extract samples, and can even be a brand-new source for several drug discoveries.

Keywords: *Citrus maxima*, phytochemicals, total phenolic, total flavonoid, iron (II) chelating, antimicrobial efficacy

Introduction
In recent years, Research has been going on to find out the relationships between bio-activity and prevention of several diseases including Tuberculosis, Cholera, Salmonellosis, Cardiovascular disease, Cancer, Diabetes, Thalassemia, *etc*., which has been increasing sharply [1]. Free radicals which are responsible for severe diseases, such as cancer and cardiovascular diseases by cell degeneration can be generated and acquired by the body's normal function and the environment [2]. Cellular and metabolic injury associate with oxygen radicals can accelerate aging, cancer, cardiovascular diseases, neurodegenerative diseases, and inflammation [3, 4]. Several diseases (like cancer, diabetes, and cardiovascular diseases) are potentially protected by the food containing phytochemicals with antioxidants, which are strongly suggested by Epidemiological and *in vitro* studies [5]. Synthetic antioxidants such as butylated hydroxyl anisole and butylated hydroxyl toluene, which have been suspected of being responsible for liver damage and carcinogenesis. Because of that, synthetic antioxidants have restricted use in food [5, 10]. However, dietary antioxidants can prevent or limit the oxidative damage [2]. The major compounds such as phenolics, flavonoids, vitamins, and carotenoids, are considered as beneficial for human health and decreasing the risk of degenerative diseases by reduction of oxidative stress, which also play a major role in contributing the antioxidant activity [11, 19].
However, endothelial dysfunction, is a condition which is evident in adults suffering from various cardiovascular diseases including thalassemia, which is ultimately happened by the Oxidative stress [20, 21]. To improve both quality of life and survival, thalassemia patients always require blood transfusions but human cannot eliminate excess iron from the body, which is further deposited as ferritin and hemosiderin in the spleen, liver, myocardium and endocrine organs. Tissue damage is happened by the accumulation of toxic quantities of iron, which leads to complications such as heart failure, endocrine abnormalities like hypothyroidism, diabetes, liver failure and ultimately early death [22, 24]. Several conditions like anaemia, iron overload, potentiating of ROS and damage to major organs (especially the cardiovascular system) characterize thalassemia major [25].

Iron chelators can form soluble and stable complexes with tissue irons, which are then excreted by the urine or feces. Additionally, the dysregulation of brain iron and its association with amyloid precursor protein plaque formation, that is involved in Alzheimer's Disease (AD) pathology. Because of that, iron chelation can be considered as a rational therapeutic strategy for AD [26]. In reducing iron-related complications, Chelation therapy can be used and which will improve quality of life, and overall survival but its lower oral bio-availability, short plasma half-life and severe side effects are still not optimal [20, 21, 27, 30].

Very few reasons for physical disabilities, health problems and mortalities around the world are as major as microbial infection. Human and plant pathogenic bacteria, fungi, and viruses can be contested without any side effects and environmental hazards by the potent plant components. Searching antimicrobial properties in plant products has recently intensified, along with phytochemicals are used as antimicrobial agents in the medical field right way [31, 32]. Compared to commercial antibiotics, natural phytochemicals are used as an alternative remedy for the treatment of various diseases due to their more effective and lower side effects. Our plant extracts were conducted to investigate the antibacterial activity against selected pathogenic microbes, that cause human skin disorders, and diarrhoea.

As a food and medicine, Pomelo (*Citrus maxima*) or Jambura (in Bangladesh) has long been used [33], that's known as by completely different names since it grows in tropical regions. In the treatment of Epilepsy, chorea, Convulsive cough and hemorrhage disease, the leaf of pomelo has been reported to use as a drug, and Oil from fresh leaves is used as anti-dermatophytic and Fungicidal properties [34]. In Indian subcontinent, it has been used as a medicinal leaf, however there has been very little info regarding quantitative analysis of phytochemicals, Iron (II) chelating, antioxidant and antimicrobial activities of it.

The concept of nanotechnology is not new; it was started over forty years ago and it is one of the most active areas of research in modern materials science and technology. Research on Nano materials is growing day-to-day with increasing demand because metals in nanometer size will display marked properties that differs and is more superior from bulk metals[35]. Nanoparticles show a higher surface-to-volume ratio with reducing size of nanoparticles. Specific surface area is relevant to display their catalytic reactivity and other associated properties like antimicrobial activity in silver nanoparticles. In the sector of plant-mediated nanotechnology, for the synthesis of silver nanoparticles various plant extracts of specific parts such as root, bark, stem, leaves, seed, fruit, peel, and flower have been used [36, 38]. Antimicrobial activity of silver is well known. From ancient time Silver has been used for treatment of several diseases [39]. Synthesized silver nanoparticles by various methods were extensively tested against number of pathogenic bacteria with proof of strong antimicrobial activity against a broad-spectrum bacteria including both Gram-negative and Gram-positive. AgNPs are more effectual against Gram-negative bacteria have been revealed by some researchers [40-42], while opposite results have also been found [43]. The difference in sensitivity of Gram-positive and Gram-negative bacteria against AgNPs may result from the variation in the thickness and molecular composition of the membranes.

During the ancient civilization, Plant extracts were used to the treatment of various ailments. Even today, these materials stay a vital resource for combating diseases. Because of that, the present study was conducted to investigate the quantitative analysis of phenolic and flavonoid compounds and to evaluate antimicrobial and Iron (II) chelating activities, by using the several *in vitro* methods.

Materials and Methods

Collection of Plant Materials

The leaves of *Citrus maxima* (Pomelo) were collected from the local area (Santosh, Tangail district in Dhaka province of Bangladesh).

Chemicals and Reagents

Folin-Ciocalteu's phenol reagent [Merck KGaA, Germany], Gallic acid, Catechin hydrate [Japan], Sodium carbonate anhydrous, Aluminium chloride [Mumbai, India], Sodium nitrite [China], Sodium hydroxide [Mumbai, India], Methanol [Merck KGaA, Germany], Mueller Hinton Agar (MHA) [Becton, Dickinson and Company, USA], Mueller Hinton Broth (MHB) [Himedia, India], Standard antibiotic disc (Ampicillin, Azithromycin, Kanamycin) [Himedia, India], Ferrozine monosodium [India], Ferrous (II) sulphate [Mumbai, India], Ethylenediaminetetraacetic acid (EDTA), Ethanol [Merck KGaA, Germany], deionized distilled water and Dimethyl Sulphoxide (DMSO) [Merck KGaA, Germany] used for the assessment of total phenolic and flavonoid contents, iron (II) chelating activity and antimicrobial efficacy. All of these, were used as analytical grade.

Preparation of leaf Extracts

Preparation of powder: Considering the uniform size and colour of leaves, pomelo (CM) leaves were chosen, which were manually removed from the stem and washed in tap water, and then dried under the shade at RT for 15 days. Then, the dried samples were crushed by using an electric blender. To an average particle size of 107 µm, crushing samples were sieved, and stored in an airtight container prior to the extraction process and kept under normal RT until required.

Preparation of leaf extracts in methanol solvents

Each 40 g of CM powder was subjected to solid-liquid extraction by using 100% methanol solvent, in where they were maintained with solid to solvent ratio 1: 10 (w/v) and kept at RT for 1 day. After 1 day, the solvent extracts were continuously swirled at 150 rpm in orbital shaker for 2 hours at RT, and then transferred into falcon tube for good rupturing the cell wall of cells, and well dissolved the components into the solvents, by centrifuge machine at 8000 rpm for 15 minutes. Using What man filter paper (120 mm) number 1, the sample extracts were filtered, and further re-filtered twice following the same procedure. The collected filtrates were concentrated by rotary evaporator, removing the entire methanol solvent at 64 °C temperature. Finally, the crude extracts were formed and then stored at − 20 °C until further analysis.

Quantitative Analysis of Phytochemicals

***Determination of total phenolic content in the extracts*:** Singleton and Rossi described Folin Ciocalteu's (FC) reagent method [44], which was used to evaluate the total phenolic contents of crude extracts. Firstly, the stock solutions of plant extract and standard sample were prepared in methanol solvent (2 mg/ml). To get the concentration of 40 µg/ ml, CM leave extracts were diluted with methanol. Then, 1 mL of diluted MeCM sample was added to 15 mL test tube. To that

5 ml of FC reagent (10%) and 4 ml of 7% Na_2CO_3 were added to get a total of 10 ml solution. The mixture showed blue colour that was shaken vigorously and then incubated for 30 minutes at 40 ºC in a water bath. Finally, the absorbance was measured at 760 nm against blank (containing all reagents except sample or standard). The total phenolic contents were calculated by using the standard calibration curve of Gallic acid and Catechin. All the reaction was conducted in three times. The results were expressed as mg of GAE (Gallic equivalents)/g of dry extract or mg of CE (Catechin equivalents) /g of dry extract (mg/g).

Determination of total flavonoids content in the extracts: Acharya P. described aluminium chloride colorimetric assay [45], which was used to determine the total flavonoid contents of crude extracts. Firstly, the stock solutions of plant extract and standard sample were prepared in methanol solvent (2 mg/mL). To get the concentration of 300 µg/ mL, CM leave extracts were diluted with methanol. Then, 1 mL of diluted MeCM sample was added to 10 mL test tube (containing 4 ml of double distilled water). At the zero time, 0.3 mL, 5% sodium nitrite was added to the flask. After 5 minutes, 0.3 ml of 10% $AlCl_3$ was added to the flask. At 6 minutes, 2 mL of 1M sodium hydroxide was added to the mixture. Immediately, the total volume of the mixture was made up to 10 mL by the addition of 2.4 mL double distilled water, and mix thoroughly. The mixture showed pink colour that was shaken smoothly. Finally, the absorbance was measured at 510 nm against blank (containing all reagents except sample or standard). The total phenolic contents were calculated using of standard calibration curve of Catechin. All the reaction was conducted in three times. The results were expressed as mg of CE (Catechin equivalents) /g of dry extract (mg/g).

Determination of Iron (II) chelating activity

The iron (II)– ferrozine complex method was used to evaluate the ferrous ion chelating activity of crude extracts at 562 nm [46, 47]. At first solution of 0.125mM and 0.3125 mM ferrozine were prepared. The stock solutions of plant extract and standard EDTA were prepared in the concentration of 0.50 mg/ml in volumetric flask. To get the concentration of 25-150 µg/ mL, CM leave extracts were diluted with methanol. Then, 1 ml of diluted MeCM sample was added to 10 ml test tube, and then it was with 1 mL of 0.125 mM $FeSO_4$ and 1 mL of 0.3125 mM ferrozine. Then, the mixture that was shaken vigorously, and then incubated for 10 minutes at RT. Finally, the absorbance was measured at 562 nm against a blank (containing the same components as stated above but the extracts were replaced with deionized distilled water). Under the same conditions as the test samples, the blank was incubated. EDTA was used as positive control. All the reaction was conducted in three times. The percentage inhibitions of ferrozine (Fe^{2+}) by the extracts & standard were determined using the following expression.

$$\text{Chelating Effect (\%) or Rate} = \left(\frac{\text{A control} - \text{B sample}}{\text{A control}}\right) \times 100$$

Green synthesis of AgNPs from *Citrus maxima*

Preparation of $AgNO_3$ Solution. At first, 50 mM $AgNO_3$ stock solution was prepared. Then two different concentrations of (5mM and 1mM) $AgNO_3$ solutions were prepared by diluting with sterile deionized water and stored in foil coated bottle in cool and dry place.

Synthesis of AgNPs from Citrus maxima

Different concentrations of (5mM and 1mM) $AgNO_3$ solutions were mixed with leaf extracts of *Citrus maxima* in a ratio 1:1.5 to get different volume such as 138.3mL (5mM CM), 137.5 mL (1mM CM). It was mixed properly and kept at room temperature in the dark place to avoid photo activation of $AgNO_3$ for 24 h until dark brown was formed with slight modification [48]. Then the mixture was subjected to centrifuge at 9000 rpm for 15 minutes for the separation of silver nanoparticles. Then crude sample was mixed with 1mL sterile deionized water to prepare diluted 1mL of AgNP solution with the help of vortex machine. Finally, the samples of crude mixture were prepared by diluting 1 mL of mixture (collected at the end of reaction) in 9 mL of sterile deionized water with the help of vortex for 15 minutes & the sample was then stored at 4 ºC for further use.

Characterization of synthesized nanoparticles

Development of silver nanoparticles with colour change: Reduction of Ag+ to Ag0 was confirmed by the colour change of solution from colourless to brown yellow solution. That brown-yellow solution was indicating the formation of silver (AgNPs) nanoparticles. Its formation was also confirmed by using UV-Visible spectroscopy.

UV–vis absorbance spectroscopy analysis: Reduction of silver ions in silver nanoparticles was monitored by UV-visible spectroscopy at the wavelength range of 200-800 nm [49]. The dilutes samples for the ultraviolet (UV) measurements of crude mixture were well prepared by diluting 1.2 ml of silver Nano particles aliquots which was diluted with 2.8 ml of distilled water with the help of vortex for 15 min and then the absorbance was recorded [50].

Antimicrobial susceptibility assay of synthesized AgNPs.

Test microorganism: In antimicrobial efficacy, the crude extract of MeCM was nominated and tested individually against eleven microorganisms (Table 1), which were gram positive and the remaining were gram negative.

Preparation of fresh culture and Test plates: 100 µL of each test organisms (previously prepared) was transferred to different MHB slants (containing 2 ml) in aseptic condition, and then were incubated with 150 rpm at RT for 24 h to assure the growth of test organisms, which were latterly used for the Antimicrobial Susceptibility Assay. To give a uniform depth of approximately, the agar plates were prepared, which contained 30 mL of MHA medium.

Preparation of sample: Different concentration of sample stock was prepared previously. Then stock solution was mixed with the vortex machine. Finally, the stock solution was diluted with sterile deionized water for different concentrations (800µg/mL and 850 µg/mL) of sample extract.

Preparation of discs: Three types of discs were prepared for antibacterial screening. These are-

a) **Sample discs:** Sterilized (BBL, USA) filter paper discs (5 mm in diameter) were prepared with the help of punch machine and were taken in a blank petri dish. Sample solution of desired concentration was applied on the discs with the help of a micropipette in an aseptic condition.

b) Standard discs: These were used to compare the antibacterial activity of test material.

c) Solvent control discs: These were prepared using same filter paper (5 mm diameter) and 25 µl of sterile deionized water,1mM $AgNO_3$ & 5mM $AgNO_3$ as solvent without sample following the same process and condition.

Disc diffusion method: The antibacterial activities of synthesized AgNPs were tested using disc diffusion bioassay. In disc diffusion method [51], bacteria were cultured in MHB overnight and spread with a sterile cotton swap into petri plates containing 30 ml of MHA.

Sterile filter paper discs (5mm in diameter) impregnated with the plant extract were placed on the cultured plates and incubated at 37 °C for 24 hrs. Sterile deionized water was used as solvent. Each of discs contained 25 µL of sample or Sterile deionized water or 1mM $AgNO_3$ & 5mM $AgNO_3$. Kanamycin, azithromycin, rifampicin & Erythromycin were applied as standard antibiotic. Diameters of inhibition zone formed were measured in millimetre(mm) using measuring scale.

Measurement of the zone of inhibition: After incubation, the antibacterial activities of the test samples were determined by measuring the diameter of inhibitory zones in term of millimeter with a transparent scale.

Statistical Analysis

Using Microsoft Excel 2016, the statistical mean was calculated with ± SD and regression analysis was performed to calculate the dose response relation between the extracts. Then, to find out the Pearson's correlation coefficient, Linear regression analysis was performed. Finally, Statistical significance of the assessments was evaluated the based on 95% of the confidence limits employing P value, which was declared at $p< 0.05$.

Table 1. Name of the microorganisms used in the experiment.

Name of the test bacteria	
Gram positive bacteria	**Gram negative bacteria**
1. *Streptococcus mutans*	1. *Salmonella typhi*
2. *Staphylococcus epidermidis*	2. *Salmonella paratyphi*
3. *Staphylococcus aureus*	3. *Pseudomonas aeruginosa*
	4. *Klebsiella pneumoniae*
	5. *Vibrio cholerae* N-16961
	6. *Vibrio cholerae* C6706
	7. *Enterotoxigenic Escherichia coli (ETEC)*
	8. *Enteropathogenic Escherichia coli (EPEC)*

Results and Discussion

Quantitative Analysis of Phytochemicals

Total Phenolic Contents

The leaves of *Citrus maxima* display the total phytochemical constituents like phenolic and flavonoid, which was carried out by our research work. Folin-Ciocalteu's colorimetric method used to determine the total phenolic contents in plant extract, in where standard calibration curve of Gallic acid and Catechin (Figure 1 and 2) used for getting the concentration value of the extract. The results of total phenolic contents of leaf extracts are shown in Table 2.

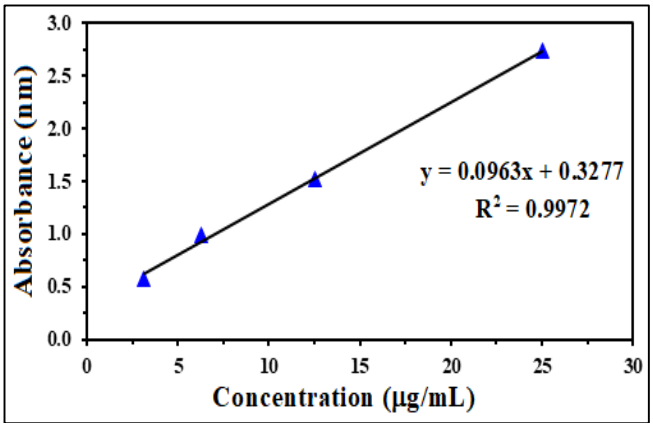

Fig 1: Standard calibration curve of Gallic acid for the assessment of total phenolic. R^2 values represented mean data (n = 3) at concentrations of 3.125 - 25 µg/mL. Means were significantly different at the level of P [0.00086] < 0.001.

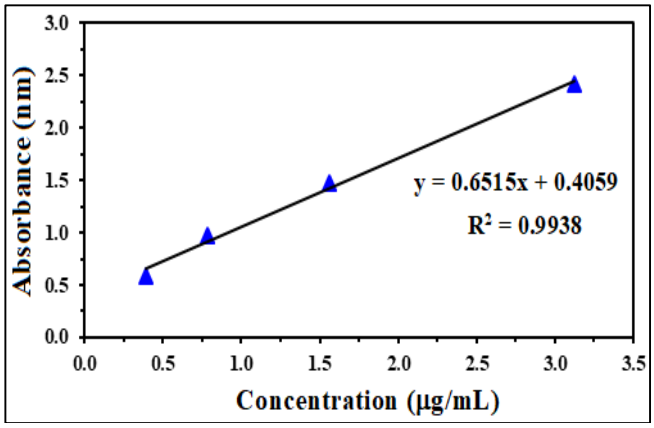

Fig 2: Standard calibration curve of Catechin for the assessment of total phenolic. R^2 values represented mean data (n = 3) at concentrations of 0.391-3.125 µg/mL. Means were significantly different at the level of P [0.0024] < 0.005.

From the regression equation Y=0.0963x+0.3277, R^2=0.9972 of Gallic acid and Y= 0.6515x+0.4059, R^2=0.9938 of Catechin, the concentration of phenolic contents in MeCM leaves were calculated individually. The total phenolic contents were calculated using the following expression and expressed as mg Gallic acid equivalents (GAE) per g of extract (in mg/g) and mg Catechin equivalents (CE) per g of extract (in mg/g).

$$TPC = \frac{C \times V}{M}$$

Here, TPC= total phenolic contents in mg/g (mg GAE/ g or in mg CE /g). C = concentration of sample extracts established from the calibration curve in mg/mL V= the volume of extract in mL & M= the weight of plant extract in g. Finally, the TPCs of MeCM are found as117.68±0.63 mg of GAE/g of dry extract & 14.39±0.09 mg of CE/g of dry extract. From the previous study it was revealed that TPC in ethanolic extracts of leaves, cortex, and peel extracts of pomelo (*C. maxima*) were found 2.99, 13.7, and 6.88 g GAE/100 g, respectively. This study was analogous to the study from West Java-Indonesia [52] regarding citrus leaves extracts, which exposed that TPC in ethanolic leaves extract of *C. reticulata*, *C. maxima*, *C. limon*, *C. hystrix*, and *C. aurantifolia* were 5.30, 4.55, 3.31, 3.66, and 6.33 g GAE/100 g, respectively [53].

From the Earlier research stated that TPC in methanolic peel extract of white tambun pomelo showed higher TPC (406.65 mg GAE/100 g) compared to its peel and pulp extracts of pink tambun pome [54]. The microwave-assisted extraction research [55] presented that TPC in methanolic peel extract of *C. reticulata* was 17.5 mg GAE/100 g and by ultrasound-assisted extraction method TPC in methanolic peel extracts of *C. sinensis* and *C. reticulata* were 6.64 and 5.87 g GAE/100 g, respectively. The similar results were reported in previous studies which represented that TPC in ethanolic peel extract of *C. hystrix* was 4.4 g GAE/100 g [56]; TPC in methanolic peel extracts of *C. sinensis* var. Washington Navel, *C. sinensis* var. Sungin, *C. sinensis* var. Valencia were 16, 15.4, and 13.3 g GAE/100 g, respectively [57]; TPC in ethanolic peel extracts of *C. sinensis* from Kintamani, Jember, and Banyuwangi were 10.08, 8.85, and 9.54 g GAE/100 g, respectively [58], and methanolic peel extract of *C. limon* was 13.1 g GAE/100 g [57]. Research by Pichaiyongvongdee [59] presented that TPC in ethanolic seed extract of pomelo cultivar Thong Dee had the highest TPC (495 mg/100 g) among different parts of fruit from seven cultivars of pomelo, whereas Duan [60] figured that methanolic small fruit extract of *C. grandis* gave the highest TPC (105 mg GAE/g) compared to its medium fruit, large fruit, flower, leaves, and branch.

Table 2. Total phenolic and flavonoid contents in the extracts

Plant parts	Total Phenolic contents		Total Flavonoid Contents
	100% methanol mg GAE/ g of dry extract (Mean TPC± S.D)	100% methanol mg CAE/ g of dry extract (Mean TPC± S.D)	100% methanol mg CAE/ g of dry extract (Mean TFC± S.D)
Leaves extract of MeCM	117.68±0.63	14.39±0.09	28.55±0.39598
Values are mean ± S.D, n=3			

Total Flavonoid Contents: Aluminium chloride colorimetric assay used to determine the total flavonoid contents in plant extract, in where standard calibration curve of Catechin (Figure 3) used for getting the concentration value of extract. The results of total flavonoid contents of leaf extracts are shown in Table 2.

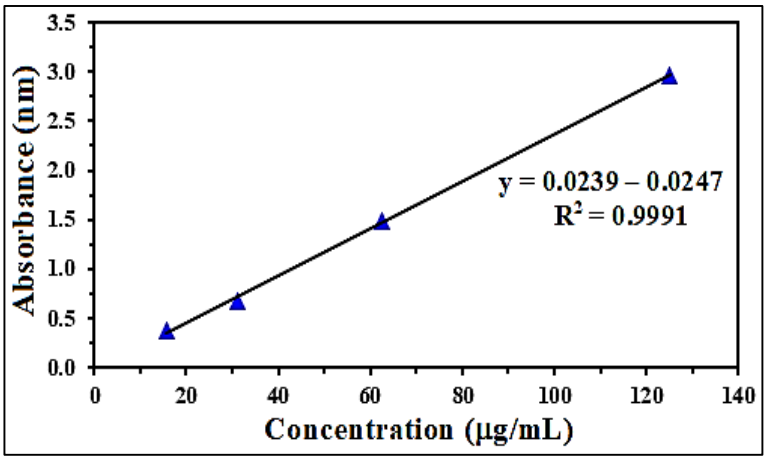

Fig 3: Standard calibration curve of Catechin for the assessment of total flavonoids. R² values represented mean data (n = 3) at concentrations of 15.63-125 µg/mL. Means were significantly different at the level of P< 0.001.

From the regression equation Y=0.0239x-0.0247, R^2 =0.9991 of Catechin, the concentration of flavonoid contents in MeCM leaves were calculated individually. The total flavonoid contents were calculated using the following expression and expressed as mg Catechin equivalents (CE) per g of extract (in mg/g) as we mentioned earlier.

$$TFC=\frac{C\times V}{M}$$

The TFCs of MeCM are observed as 28.55±0.39598 mg of CE/g of dry extract. Figure 4 displayed the TPC and TFC values.

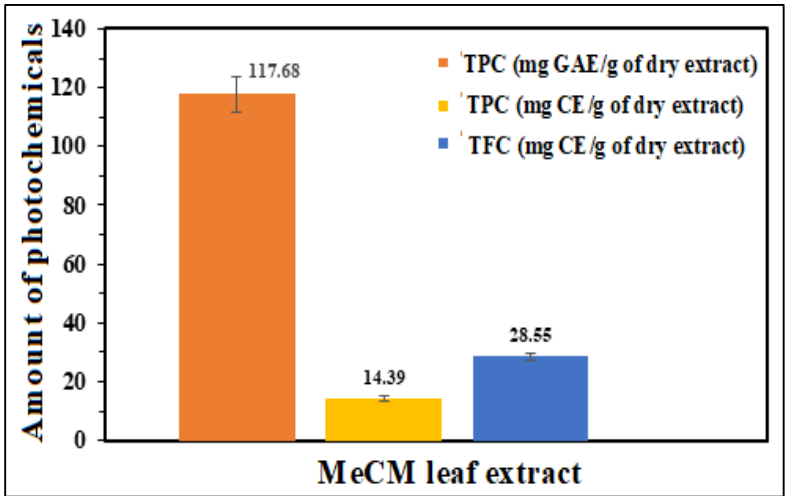

Fig 4: Comparison of total phytochemical constituents of MeCM leaf extract. Values are expressed as mean with standard error (n = 3) which were significantly different at the level of $P < 0.05$.

Ferrous ion chelating activity of the extracts

Chelation of Iron by the extract was assessed by Dinis *et al.* [61]. Ferrozine monosodium can form complex with Fe^{2+} in quantitatively but the complex formation is disrupted in the presence of chelating agents, as a result, the red colour of the complex is decreased. The estimation of the chelating activity of the co-existing chelator is allowed by the measurement of colour reduction. The transition metal ion (such as Fe^{2+}), which has ability to move single electron, that can start with relatively non-reactive radicals, and latterly allow the formation and propagation of many radical reactions [62]. To evaluate the chelating effects of MeCM leaf extracts, the chelating rate was investigated [46]. The results of Iron (II) chelating activities standard EDTA and MeCM leaf extract are shown in (Figure 5, and Table 3). MeCM leaf extract quenched $FeSO_4$ (Table 3) in a dose dependent manner: [R^2= 0.9798] [P(0.0002) < 0.001].

Table 3: Chelating activity of methanol solvent extracts of the leaf sample of CM.

Specimen	Solvent	Concentration (µg/mL)	Inhibition % (Mean±SD)	Linear Equation (Y=mx+C, R^2)	IC_{50}(µg/mL)
Sample	MeCM	25	24.85±0.24	Y = 0.0861x+ 22.122, R^2=0.9798	323.79
		50	26.35±0.49		
		75	28.34±0.14		
		100	29.84±0.28		
		125	32.83±0.51		
		150	35.73±0.37		

Values are mean ± S.D, n=3. Means which were significantly different at the level of P [0.0002] < 0.001

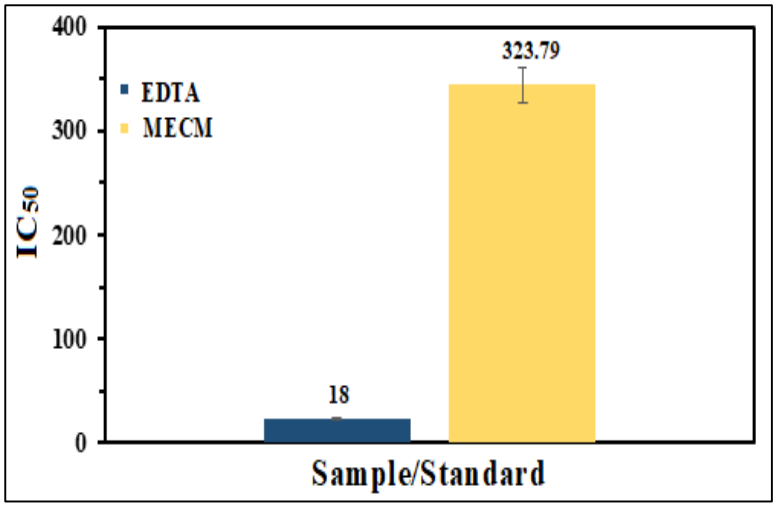

Fig 5: Comparison of IC_{50} of MeCM leaf extract and standard EDTA. Results expressed as the mean with standard error (n = 3) at concentrations of 20- 200 µg/mL.

The IC_{50} values revealed that MeCM is the active extracts interfered with the formation of ferrous and ferrozine complex, and captures ferrous ion as an iron chelator (Table 3). The IC_{50} value for chelating activity of the extract is lower than the positive standard EDTA (Figure 5). Also, this chelating activity increases with the extract concentrations.

Development of silver nanoparticles with colour change

Reduction of Ag^{+} to Ag^{0} was confirmed by the colour change of solution from colourless to black or brownish-yellow solution, indicating the formation of silver (AgNPs) nanoparticles.

UV spectrum of green synthesized silver nanoparticles from *Citrus maxima*

The UV-vis spectra recorded from the reaction medium as a function of reaction time using *Citrus maxima* leaf together with the spectra of pure silver nanoparticles (Figure 6). It was observed that the maximum absorbance of pure silver occurs at 380 nm, respectively. While the increase in intensity of the silver surface *Citrus maxima* band for the Ag solution is quite distinct with some shift to 400 nm in high intensity, a well-defined band corresponding to silver nanoparticles is not observed. Similar spectra for the Au/Ag bimetallic solution using Neem leaf broth and mentioned that the silver particles did not form a uniform layer around the gold nanoparticles, leading to considerable damping of a distinct silver plasmon vibration band at ca. 400nm [63].

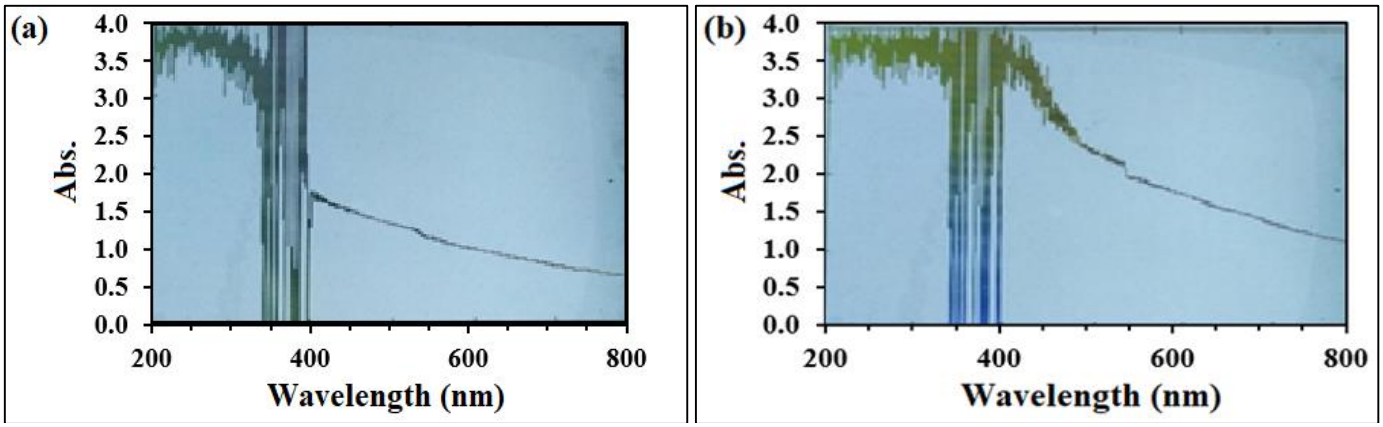

Fig 6: UV Spectrum of synthesized AgNPs from (a) 1mM *Citrus maxima* (b) 5mM *Citrus maxima*

Antimicrobial activity of Synthesized AgNPs

The antimicrobial activity of 1mM AgNPs of CM and 5mM AgNPs of CM was tested against 11 bacteria at concentrations 20 µg/ disc. Standard antibiotic disc Kanamycin (30µg/disc), Azithromycin (30µg/disc) & Rifampicin (5µg/disc) were used for comparison. The obtained results are demonstrated in Table 4 and Figure 7. From Table 4, the produced zone of inhibition for 5mM AgNPs of CM against gram positive bacteria (*Streptococcus mutans, Staphylococcus aureus, Staphylococcus epidermidis) & gram negative bacteria (S. paratyphi, S. typhi, V. cholerae(N-16961), V. cholerae(C6706), ETEC, EPEC, K. pneumonia, P. aeruginosa)* were 8±0, 9±0, 9.5±0.5, 9.5±0, 12±0, 8.5±0.5, 8±0, 8.5±0.5, 8.5±0.5, 10.5±0.5, 11±0 mm at 20 µg/disc, respectively, whereas 1mM AgNPs of CM at 20 µg/ disc dose the produced zone of inhibition against the same bacteria were 9.5±0.5, 7.5±0.5, 8±0, 6.5±0.5, 9.5±0.5, 6.5±0.5, 7±0, 7±0, 7.5±0.5, 9±0, 8.5±0.5 mm respectively. And, the zone of inhibition for MeCM against gram negative bacteria *S. paratyphi, S. typhi, V. cholerae C6706, EPEC, K. pneumonia, P. aeruginosa* are observed 9.5, 12, 8,8.5, 10.5 and 11 mm respectively.

Table 4: *In vitro* antimicrobial activity of 5mM AgNPs of CM & control & standard antibiotics against gram negative and gram positive pathogens.

Name of Bacterial Strain	Zone of inhibition of sample extract/ standard antibiotic disc (mm)				
	AgNPs of *CM*	Control		Standard Antibiotics	
	Conc. (20 µg/disc)	Sterilized deionized water	5mM $AgNO_3$	K (30µg/disc)	RIF (5µg/disc)
ETEC	8.5±0.5	5	6	16.5±.0.5	Resistant
EPEC	8.5±0.5	5	6	15.5±0.5	Resistant
P. aeruginosa	11±0	5	5.5	19.5±0.5 (AZM)	Resistant
K. pneumoniae	10.5±0.5	5	6	18.5±0.5 (AZM)	Resistant
S.typhi	12±0	5	6	18±0	Resistant
S.paratyphi	9.5±0	5	5.5	15.5±0.5	Resistant
V. cholerae (N-16961)	8.5±0.5	5	5.5	15.5±0.5	Resistant
V. cholerae (C6706)	8±0	5	5.5	16±0	Resistant
S. aureus	9±0	5	5.5	19±1	Resistant
S.epidermidis	9.5±0.5	5	5.5	20.5±0.5	Resistant
S. mutans	8±0	5	5.5	21.5±0.5	Resistant

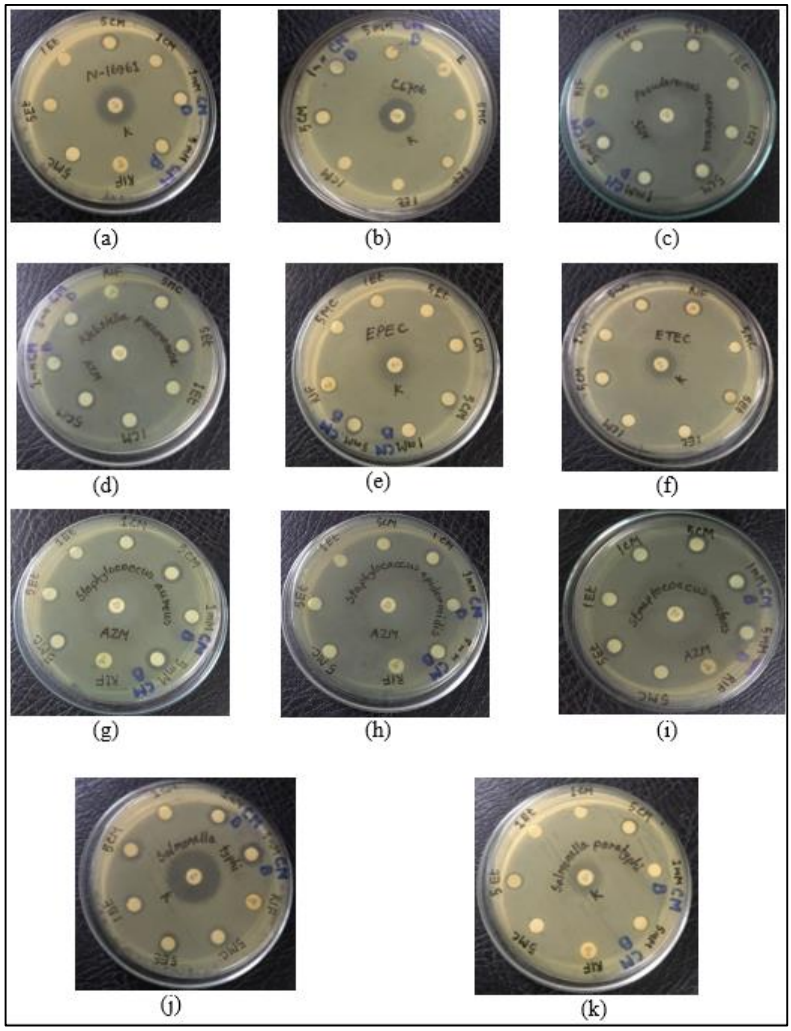

Fig 7: *In vitro* antimicrobial activity of 1mM & 5mM AgNPs of CM & standard antibiotics against gram positive and gram negative pathogens. Here, (a) *V. cholera (N-16961* (b) *V. cholera (C6706)* (c) *P. aeruginosa* (d) *K. pneumonia* (e) EPEC (f) ETEC (g) *Staphylococcus aureus* (h) *Staphylococcus epidermidis* (i)*Streptococcus mutants* (j) *Salmonella typhi* (k) *Salmonella paratyphi*

The antibacterial activities of *Citrus maxima* against S. aureus but not against E.coli was reported [64]. The present study showed antibacterial activities of the extracts of *Citrus maxima* against S. aureus and also showed antibacterial activities against *Streptococcus mutans* and K. pneumoniae. However, there is no significance antibacterial activity shown against *Staphylococcus epidermidis*. Das *et al*. revealed the antibacterial activity of the ethanolic extracts of *Citrus maxima* leaves against *E. coli* and *Pseudomonas aeruginosa* [65]. Present findings of the antibacterial activity of methanolic leaf extracts of *Citrus maxima* are compared to the reported one but there used *Citrus aurantium* [66].

The anti-bacterial activity of the plant extracts can be attributed to the different phytochemicals present in the Citrus plant parts. The present study can pave way to locate compounds in Citrus plants to develop safer antimicrobial agents in this era of anti-microbial resistance. The MeCM leaf extract shows a satisfactory antibacterial efficacy on *Salmonella paratyphi* and *Salmonella typhi*. Table 4 indicates that, the different cultures responds to MeCM leaf extract in a variable manner, resulting in zones of inhibition of 5.5–12 mm.

On the other hand, the standard antibiotic disc of Kanamycin shows more susceptible to *Salmonella typhi, Salmonella paratyphi, V. cholerae N-16961, V. cholerae C6706, ETEC, EPEC*. Likewise, Azithromycin shows more susceptible to *S. aureus, S. epidermidis, S. mutans, P. aeruginosa and K. pneumonia*. However, Ampicillin is resistant to those bacteria species.

Although the antibacterial effect of silver nanoparticles has been widely studied, there are some factors affecting the antimicrobial properties of AgNPs, such as shape, size, and concentration of nanoparticles and capping agents [67]. Nakkala *et al*. [68] analyzed AgNPs with the average size of 21 nm, and the size distribution was found to be 1–69 nm prepared by medicinal plant *Ficus religiosa*. These nanoparticles showed excellent antibacterial activity in *P. fluorescens, S. typhi, B. subtilis*, and *E. coli*. Bacterial cells exposed to lower concentration of AgNPs exhibited delays of growth which may be due to the bacteriostatic effect, while at the higher concentration (of 60 and 100 µg), the AgNPs were found to exhibit bactericidal effect as no growth was observed. The smaller particles with a larger surface-to-volume ratio were able to reach bacterial proximity most easily and display the highest microbicidal effects than larger particles [69]. Normally, a high concentration leads to more effective antimicrobial activity, but particles of small sizes can kill bacteria at a lower concentration.

Furthermore, apart from size and concentration, shape Green-Synthesized Silver Nanoparticles and Their Potential for Antibacterial Applications also influences the interaction with the Gram-negative organism *E. coli* [70]. Pal *et al*. discussed about depending of nanoparticles' shape and size on antibacterial activity against Gram-negative bacteria E. coli.

They found that observed interaction between nanoparticles of silver with various shapes and E. coli was similar, and the inhibition results were variable. They speculated about the fact that AgNPs with the same surface areas, but different shapes, may have unequal effective surface areas in terms of active facets [71]. Also different antimicrobial effects of nanosilver shapes (nanoparticles, nanorods, and nanoplates) for *S. aureus* and *E. coli* are reported [72] found SEM analysis indicated that both strains were damaged and extensively inhibited by Ag-nanoplates due to the increasing surface area in AgNPs.

Conclusion

The presence of significant amount of phytochemical constituents, chelating activity, and antibacterial efficacy of MeCM leaf extract is indicated by the results of the present study. These results also permit us to aggregate that Citrus maxima leaf is a good natural source of phenolic, flavonoid, antibacterial, and iron (II) chelator. Thus, the phenolic contents of this leaf may be a good substitute for synthetic ones. Pomelo blossom do exhibit some significant antimicrobial activity against few tested microorganism culture including *S. paratyphi*, *S. typhi* and *V. cholerae C6706* so, it can act as antimicrobial agents to some selected microorganism. In the indigenous people to treat and heal number of infections and diseases, the use of Pomelo leaves is explained by all this probable property. To isolate and elucidate the structures of active compounds, further investigation of these extracts are required.

Acknowledgements

The authors are grateful to Mawlana Bhashani Science and Technology University (MBSTU) for financial support (3631108). We wish to profoundly acknowledge the entire staff of the Department of Biochemistry and Molecular Biology, MBSTU for the understanding while we had to be away for the research purpose. I.M.J. express his appreciations to the icddr,b (International Centre for Diarrhoeal Disease Research, Bangladesh) for providing the bacterial strain.

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