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Antioxidant, Brine Shrimp Lethality and Antimicrobial Activities of Methanol and Ethyl-Acetate Extracts of *Citrus macroptera* Montr. Fruit Using *In vitro* Assay Models

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Authors' contributions

This work was carried out in collaboration among all authors. Author NU made the final copy. Authors NU, MRH and MMH performed the experimental study. Authors NU and MMH wrote the protocol. Author NU performed the statistical analysis of the study. Authors NU and AF designed the study. Authors AR and TI collected and extracted the fruit. Author MSH managed the literature searches. Author MSR finalized the drafting of the manuscript. All authors read and approved of the final manuscript.

Original Research Article

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ABSTRACT

Aims: To study and evaluate *In vitro* antioxidant, brine shrimp lethality and antimicrobial activities of both methanol and ethyl-acetate extracts of *Citrus macroptera* Montr. Fruit (Family-Rutaceae).
Study Design: *In vitro* antioxidant, brine shrimp lethality and antimicrobial activities.
Place and Duration of Study: Department of Pharmacy, Jahangirnagar University, Savar, Dhaka-1342. The study was carried out from November 2013 to January 2014.
Methodology: *In vitro* antioxidant activities of the extracts were studied using DPPH radical scavenging assay, NO scavenging assay, total phenol, total flavonoid content, total antioxidant capacity, total tannin content, lipid peroxidation by TBA, lipid peroxidation in human erythrocyte cell, reducing power capacity and cupric reducing capacity assays. Lethality bioassay was performed on *Artemia salina* Leach nauplii. Antimicrobial activity was investigated by disc diffusion technique.

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Results: Methanol extract showed better activity than ethyl acetate extract in DPPH, NO, lipid peroxidation by TBA, reducing power capacity assay, total phenol, total flavonoid and total antioxidant capacity assays while ethyl –acetate extract showed more potency than methanol extract in total tannin content, cupric reducing capacity and lipid peroxidation in human erythrocyte assays. In brine shrimp bioassay both extracts showed promising lethal activity but methanol extract was found to be more potent than ethyl acetate extract ($\chi 2$=39.874, P<0.0001). In disc diffusion technique among six bacterial species, ethyl acetate extract showed broad spectrum antimicrobial activity against two gram positive *Bacillus sublitis* and *Staphylococcus aureus* and one gram negative *Escherichia coli*.
Conclusion: The results demonstrate that methanol and ethyl-acetate extracts of *C. macroptera* fruit can be used as potential antioxidant, cytotoxic and antimicrobial agents. That is why extensive researches are necessary to search for active principles responsible for these activities.

Keywords: Citrus macroptera; antioxidant; brine shrimp lethality; antimicrobial.

1. INTRODUCTION

Citrus macroptera Montr. commonly called 'Satkara' (wild orange) is a semi-wild species of *citrus* genus. The tree which has thorns can reach 5m in height. Its fruit is about 6–7cm in diameter. It has a fairly smooth, moderately thick rind, and is yellow when ripe. The pulp of the fruit is greenish yellow and dry (does not produce much juice). The juice is very sour, and somewhat bitter. In Bangladesh the fruit of *Citrus macroptera* is eaten as a vegetable. It has a unique taste and aroma. The thick rind is sliced into small pieces and cooked with beef, mutton, and fish curries. Curries cooked with satkara and beef or mutton are served in many restaurants in the UK. This plant is used medicinally in Assam. Traditionally this fruit is used as appetite stimulant and in the treatment of fever [1]. In the previous study researchers reported hypoglycemic potential of the fruit [2]. To the best of our knowledge no sporadic attempts have been taken to investigate antioxidant, brine shrimp lethality and antimicrobial properties of the pulp of this fruit. That is why we have designed our research project to explore the possible aforementioned properties by using *In vitro* assay models.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Folin-Ciocalteu reagent, Methanol, Sodium Phosphate (Na_3PO_4) and Ammonium molybdate were purchased from Merck, Germany. Sodium carbonate, Potassium Acetate and Concentrated H_2SO_4 (98%) were purchased from Merck (India) Limited. Gallic acid, Quercetin and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemicals, USA. Aluminium Chloride and Ascorbic acid were purchased from SD Fine Chem. Ltd., Biosar, India. Vincristine sulphate was obtained from Techno Drugs Ltd., Bangladesh. All chemicals and reagents which were used were of analytical grade.

2.2 Plant Materials

Fruits of *Citrus macroptera* were collected from Sylhet, Bangladesh and authenticated by Md. Abdur Rahim, Technical Officer, Department of Botany, Jahangirnagar University. A

voucher specimen (No. 38619) was deposited in the herbarium for future reference. The fruits were used for further processing.

2.3 Extracts Preparation

At first peels were removed. Fruits without peel were cut into pieces, sun-dried separately and then, dried in a hot air oven (Size 1, Gallen kamp) at reduced temperature (not more than 50ºC) to make suitable for extraction process. Fruits were then stored in air-tight container with necessary markings for identification and kept in cool, dark and dry place for further investigation.

2.3.1 Extraction procedure

The dried fruits were treated with sufficient amount of pure ethyl acetate and methanol following cold extraction for 3-4 days at room temperature respectively with occasional shaking. The extracts were filtered through a cotton plug followed by Whatman No. 1 filter paper. The filtrate was then evaporated under reduced pressure to give a dark green viscous mass and stored at 4ºC. % of yield was 18.3% for methanol extract and 16.06% for ethyl acetate extract.

2.4 Antioxidant Activity Evaluation

2.4.1 DPPH free radical scavenging assay [3]

Different concentrations (500, 200, 100, 50, 25 and 5 µg/ml) of fruit extracts and standard were taken in test tube which contained 1ml of each concentration and was properly marked. Then 2 ml of 0.004% DPPH solution in the solvent was added to each test tube to make the final volume 3 ml. The mixture incubated at room temperature for 30 minutes in a dark place. Then the absorbance was measured at 517 nm. IC_{50} value was calculated using linear regression analysis.

2.4.2 Nitric oxide scavenging capacity assay [4]

4.0 ml of each fruit extracts and standard of different concentration (200, 100, 50, 25 and 5 µg/ml) solutions were taken in different test tubes and 1.0 ml of Sodium nitroprusside, (5mM) solution was added to the test tubes. The test tubes were incubated for 2 hours at 30^0C to complete the reaction. 2.0ml solution was withdrawn from the mixture and mixed with 1.2 ml of griess reagent and the absorbance of the solutions was measured at 550 nm using a spectrophotometer (UV-1601PC, Shimadzu) against blank. A typical blank solution contained the distilled water. The percentage (%) inhibition activity was calculated using linear regression analysis.

2.4.3 Reducing power capacity assessment [5]

2.0ml of each fruit extracts and standard of different concentration solutions (200, 100, 50, 25 and 5µg/ml) were taken in different test tubes and 2.5 ml of Potassium ferricyanide [$K_3Fe(CN)_6$], 1% solution was added to each of test tubes. The test tubes were incubated for 10 minutes at 50ºC to complete the reaction and 2.5 ml of Trichloro Acetic acid, 10% solution was added to each of the test tubes. The total mixtures were centrifuged at 3000 rpm for 10 min. 2.5ml supernatant solution was withdrawn from each of the mixtures and

mixed with water (2.5 ml). 0.5ml of Ferric chloride ($FeCl_3$), 0.1% solution was added to each of the test tubes. The absorbance of the solution was measured at 700nm using a spectrophotometer against distilled water. A typical blank solution contained the same solution mixture without fruit extracts or standard and it was incubated under the same conditions as the rest of the samples solution. The absorbance of the blank solution was measured at 700 nm against the solvent used in solution preparation. Increased absorbance of the reaction mixture indicated increase in reducing power.

2.4.4 Cupric reducing antioxidant capacity [6]

500µl of each fruit extracts or standard of different concentration solutions (200, 100, 50, 25 and 5 µg/ml) were taken in different test tubes. 1.0 ml of 0.01M $CuCl_2.2H_2O$ solution, 1.0ml of ammonium acetate buffer, pH 7.0, 1.0ml of 0.0075 ml of neocaproin solution and distilled water (600µl) were added and the final volume of the mixture was adjusted to 4.1ml. The total mixtures were incubated for 1 hour at room temperature. The absorbance of the solutions was measured at 450 nm using a spectrophotometer against blank. A typical blank solution contained the reagent mixture without extract or standard and treated as same.

2.4.5 Determination of total phenol content [7]

1ml of fruit extracts or standard of different concentrations were taken in a test tube. 0. 5 ml of Folin- Ciocalteu (Diluted 10 fold) reagent solution was added to the test tubes. 7.5% Sodium carbonate solution (4 ml) was added to the same test tubes and mixed well. Test tubes containing standard solutions were incubated for 30 minutes at 20ºC to complete the reaction but the test tubes containing extract solution were incubated for 1 hour at 20ºC to complete the reaction. Then the absorbance of the solution was measured at 765 nm using a spectrophotometer against blank. A typical blank solution contained the solvent used to dissolve the plant extract. The Total content of phenolic compounds in fruit extracts was expressed in mg/g gallic acid equivalents (GAE).

2.4.6 Determination of total flavonoid content [8]

1ml of fruit extracts or standard of different concentrations was taken in a test tube and 3 ml of methanol was added. Then 200µl of 10% aluminium chloride solution was added into the same test tube followed by the addition of 200µl of 1M potassium acetate. Finally, 5.6 ml of distilled water was mixed with the reaction mixture. The reaction mixture was then incubated for 30 minutes at room temperature to complete the reaction. Then the absorbance of the solution was measured at 415 nm using a spectra photometer against blank. Methanol served as blank. The Total content of flavonoid compounds in fruit extracts was expressed in mg/g quercetin equivalent (QE).

2.4.7 Determination of total antioxidant capacity [9]

300µl of each fruit extracts or standard of different concentration solutions were taken into different test tubes and 3 ml of reagent solution (3.3ml of concentrated H2SO4 (98%), 0.381 gm sodium phosphate and 0.494gm of ammonium molybdate were taken into three separate 100ml volumetric flasks and the volumes were adjusted with distilled water) was added into each of the test tubes. The test tubes were incubated at 95ºC for 90 minutes to complete the reaction. The absorbance of the solutions was measured at 695 nm using a spectrophotometer against blank after being cooled in room temperature. A typical blank

solution contained 3 ml of reagent solution and the appropriate volume (300µl) of the same solvent was used for the sample and incubated under the same conditions as the rest of the samples solution. The antioxidant activity was expressed as the number of equivalents mg/g of ascorbic acid (AAE).

2.4.8 Total tannin content [10]

0.1 ml of the sample extract is added with 7.5ml of distilled water in a test tube. 0.5 ml of Folin Phenol reagent was added. Then 1ml of 35% sodium carbonate solution was added. The volume was adjusted upto 10mL with distilled water. The mixture was shaken well, kept at room temperature for 30 min and absorbance was measured at 725nm. Blank was prepared with water instead of the sample. A set of standard solutions of tannic acid was treated in the same manner as described earlier and read against a blank. The results of tannins were expressed in terms of mg/g tannic acid equivalent (TAE).

2.4.9 Lipid peroxidation by thiobarbituric acid (TBA) assay [11]

1ml of different concentrations (200, 100, 50, 25, 12.5 and 6.25µg/ml) of fruit extracts or standard was added to pre-labeled test tubes. 1 ml of 0.15M KCl was added to each test tube and then 0.5 ml of liver homogenate was added to it and mixed well. After that 100µl of 0.2mM $FeCl_3$ solution was added to initiate peroxidation. The total mixture was incubated at 37°C for 30min. After the incubation the reaction was terminated with the addition of 2 ml of ice-cold 0.25N HCl containing 15% TCA, 0.38% TBA and 0.5% BHT. The reaction mixture was heated at 80ºC for 60 min. The sample was cooled and centrifuged, and the absorbance of the supernatant was measured at 532 nm. An identical experiment (Control) was performed to determine the amount of lipid peroxidation obtained in the presence of inducing agents without any extract or standard. IC_{50} is the concentration at which 50% inhibition of lipid peroxidation occurred and can be determined by linear regression method.

2.4.10 Inhibition of erythrocyte lipid peroxidation [12]

Venous blood was collected from a healthy volunteer and delivered into heparinized tubes. The whole blood was centrifuged at 4000 rpm for 10 minutes, washed three times with desired phosphate buffered saline (pH 7.4) and resuspended in the same buffer to obtain desired hematocrit level. A portion of 200µL erythrocyte was delivered in a test tube followed by 100µL hydrogen peroxide (100µM) to induce lipid peroxidation. The test samples 200µL were then added. The contents were incubated for 1hour at 37˚C. The reaction was stopped by thiobarbituric acid stock reagent (0.375% TBA, 15%TCA, 0.2M HCl). After cooling the solution was centrifuzed at 3000 rpm for 5 min. The absorbance of the supernatent was measured at 532 nm. IC_{50} value was calculated using linear regression method.

2.5 Brine Shrimp Lethality Bioassay [13,14]

The brine shrimp lethality bioassay is very useful tool for the isolation of bioactive compounds from plant extracts. The eggs of Brine shrimp (*Artemia salina* Leach) were collected and hatched in a tank at a temperature “around 25ºC” with constant oxygen supply. Two days were allowed to hatch and mature the nauplii. Stock solutions of the samples were prepared by dissolving required amount of extracts in specific volume of pure dimethyl sulfoxide (DMSO) and sea water. Then specific volumes of sample were transferred from the stock solution to the test tubes to get final sample concentrations of 1, 5, 10, 20, 50, 100, 200 and 500µg/ml. In the control tubes same volumes of DMSO (as in the sample tubes)

were taken. With the help of a Pasteur pipette 10 living nauplii were put to each of the test tubes. Vincristine sulfate was used as positive control and evaluated at very low concentration (10, 5, 1, 0.5, 0.25, 0.125 and 0.06µg/ml). After 24 hours the test tubes were observed and the number of nauplii survived in each test tube was counted. From this, the percentage of mortality of brine shrimp nauplii was calculated for each concentration of the extract and then corrected using Abott formula [15]. After correcting the % mortality, probit analysis was performed and found out LC_{50} value calculated using Fenny probit analysis [16].

2.6 Antimicrobial Activity

2.6.1 Microorganisms

Two Gram positive bacteria *Bacillus subtilis*, *Staphylococcus aureus* and four Gram negative bacteria *Escherichia coli*, *Salmonella typhi*, *Salmonella abony* and *Pseudomonas aeruginosa* were used for the study.

2.6.2 Experimental procedure

Antimicrobial activity of the plant extracts was investigated by disc diffusion technique described by Bauer et al. [17]. Subcultures prepared from pure cultures of six microorganisms were used for the sensitivity test. In an aseptic condition under laminar air hood cabinet, the test organisms were transferred from the subculture to 5mL of nutrient broth contained in screw-capped test tubes using a transfer loop and then incubated for 24 hours at 37ºC for their optimum growth $5x10^5$cfu/mL. Fruit extracts of 400µg/disc disc were used for this investigation. Standard disc of Azithromycin (30µg/disc) and blank discs (impregnated with solvents followed by evaporation) were used as positive and negative control respectively. Bacterial cell suspension was spread throughout the plates by using sterile 'L' shape spreader. The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the nutrient agar plates. The plates were kept in an incubator at 37ºC for 48 hours to facilitate bacterial growth. After incubation, the antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

2.7 Statistical Analysis

Values are presented as mean± SEM (Standard error of mean) and mean ±SD (Standard deviation). One was analysis of variance followed by Bonferroni multiple comparison, student's t test, pearson product moment correlation analysis and probit analysis were performed to analyze different data sets in these experiments. $P<0.05$, $P<0.01$, $P<0.001$ and $P<0.0001$ were considered statistically significant. Statistical programs which were used were Sigma Plot (version 12.0, Systat Software Inc., San Jose, California, USA) and BIOSTAT 2009 (Analyst Soft Inc.).

3. RESULTS

3.1 *In vitro* Antioxidant Assays

Antioxidant potential was evaluated using DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay, NO (Nitric Oxide) radical scavenging assay, reducing power capacity

assay, cupric reducing capacity assay, lipid peroxidation assay by TBA method, lipid peroxidation method in human erythrocyte cells, total antioxidant capacity assay, total phenol content assay, total flavonoid content determination and total tannin content determination assay. In DPPH assay between two extracts, methanol extract was found to show moderate IC_{50} value 772.763±11.162µg/ml, whereas ascorbic was found to exhibit very good IC_{50} value of 17.663±1.310µg/ml (Table 1). Graphical representation of qualitative DPPH scavenging is presented in Fig. 1. In NO radical scavenging method, between the two extracts the highest NO radical scavenging was demonstrated by methanol extract with IC_{50} value of 535.875±13.19µg/ml whereas IC_{50} value of standard ascorbic acid was 89.135±3.645µg/ml (Table 1). In comparison between two extracts, only methanol extract showed moderate potential. On the other hand, ethyl acetate extract exhibited poor DPPH and NO radical scavenging activities. In reducing power capacity assessment, the methanol extract exhibited the slightly higher reducing power than ethyl acetate extract (correlation coefficient r=0.993 and P<0.001) whereas ascorbic acid was found to possess the highest reducing capacity (r=0.999 and P<0.0001) (Fig. 2). In case of CUPRAC assay the ethyl acetate extract showed maximum reducing capacity (r=0.95 and P<0.05) and this extract has moderate reducing capacity comparing with ascorbic acid (r=0.95 and P<0.05) (Fig. 2). The standard ascorbic acid showed the highest reducing capacity. In case of total phenol, total flavonoid and total antioxidant content assays methanol extract was found to exhibit better content than ethyl acetate extract (46.421±1.286mg/gm Gallic Acid Equivalent, 14.1±1.153mg/gm Quercetin Equivalent and 78.576±0.277mg/gm Ascorbic Acid Equivalent respectively). However in total tannin content assay, ethyl acetate showed more capacity than methanol extract (19.583±7.217mg/gm Tannic Acid Equivalent). For the aforementioned properties the values do not differ significantly (P<0.05) (Table 1). In lipid peroxidation by TBA assay, methanol extract presented maximum potential (IC_{50} = 129.430±1.641µg/ml) but ethyl acetate extract exhibited maximum activity (IC_{50}= 5.862±0.155mg/ml) in erythrocyte lipid peroxidation method (Table 1).

3.2 Brine Shrimp Lethality Bioassay

In Brine shrimp lethality bioassay, methanol extract was found to be the most toxic to brine shrimp nauplii, with LC_{50} of 14.583µg/ml ($\chi 2$=39.8746, P<0.0001) whereas anticancer drug vincristine sulphate showed LC_{50} value of 1.891µg/ml ($\chi 2$=14.198, P<0.01). Ethyl acetate extract also showed potent toxicity ($\chi 2$=83.414, P<0.0001). Data sets are illustrated in Table 2.

3.3 Antimicrobial Activity

In antimicrobial study ethyl acetate extract proved efficacy against maximum number of microbes (*Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli*). The first two bacteria are gram positive and the last one is gram negative. Between two extracts the ethyl acetate extract showed the highest zone of inhibition against *E. coli* (7.5±0.707mm). The standard, Azithromycin, exhibited good zone of inhibition against all tested pathogenic organisms. All results are presented in Table 3.

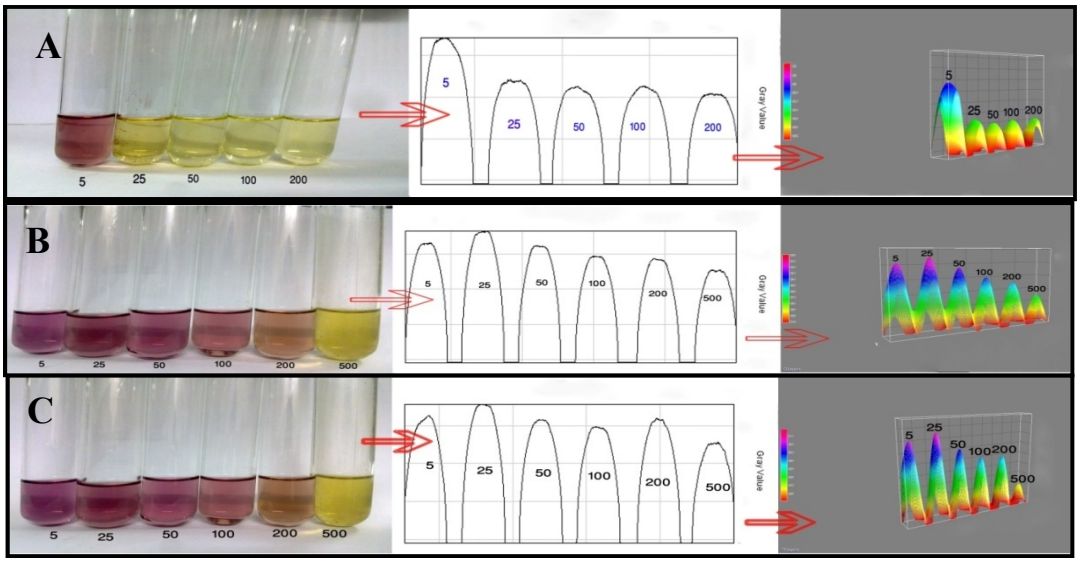

Fig. 1. A) Ascorbic acid B) Methanol extract C) Ethyl acetate extract. Here 5, 25, 50,100,200 and 500 means different concentrations (µg/mL). Increase in scavenging activity means when concentration increases, intensity of deep purple color becomes pale yellow and the height of color peak for each concentration decreases which is presented by both plot profile and interactive 3D surface plot. Image J program was used to analyze this picture

Table 1. Antioxidant potential of two different extracts of *Citrus macroptera* fruit

***In vitro* antioxidant models**	**Methanol extract**	**Ethyl-acetate extract**	**Ascorbic acid**	**BHT**
DPPH(IC_{50})	772.763±11.162[b]	1350.830±18.229[c]	17.663±1.310[a]	-
NO (IC_{50})	535.875±13.197[b]	1375.740±56.111[c]	89.135±3.645[a]	-
Total phenol (mg/g GAE)	46.421±1.286[a]	44.681±0.428[a]	-	-
Total flavonoid (mg/g QE)	14.1±1.153[a]	14.06±1.101[a]	-	-
Total antioxidant (mg/g AAE)	78.576±0.277[a]	77.213±1.606[a]	-	-
Total Tannin (mg/g TAE)	15.417±7.217[a]	19.583±7.217[a]	-	-
LPO by TBA (IC_{50})	129.430±1.641[b]	152.431±23.237[b]	-	8.941±0.940[a]
LPO in Human erythrocyte(IC_{50})	7.945±0.419[c]	5.862±0.155[b]	1.713± 0.143[a]	

A) Values are presented as mean±SEM (n=3). For DPPH, NO, LPO by TBA and LPO in Human erythrocyte, one way ANOVA followed by Bonferroni multiple comparison was performed. Values in the same row with different superscripts are significantly different from one another (P<0.05). B) Values are presented as mean±SD (n=3). For total phenol, total flavonoid, total antioxidant and total tannin assays, student's t test was performed. Values in same row with different superscripts are significantly different from each other (P<0.05)

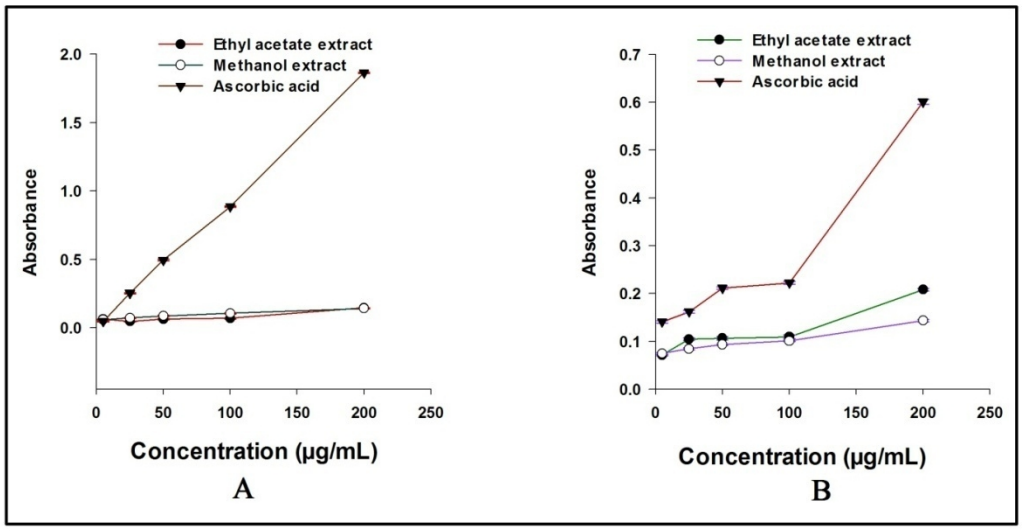

Fig. 2. A) Reducing power capacity assessment of two different fruit extracts and standard: *Values are presented as mean±S.E.M (n=3). Pearson Product Moment Correlation analysis was performed between different concentrations and absorbance of each fruit extract and standard. For methanol extract, correlation coefficient r= 0.993 and P<0.001; for ethyl acetate extract, r= 0.925 and P<0.05; for ascorbic acid, r= 0.999 and P<0.0001*.

B) Cuprac reducing capacity assessment of two different fruit extracts and standard: *Values are presented as mean±S.E.M (n=3). Pearson Product Moment Correlation analysis was performed between different concentrations and absorbance of each fruit extract and standard. For methanol extract, correlation coefficient r= 0.989 and P<0.01; for ethyl acetate extract, r= 0.95 and P<0.05; for ascorbic acid, r= 0.95 and P<0.05.*

Table 2. Brine shrimp lethality of different extracts of *C. macroptera* fruit

Extracts/Standard	LC_{50} (µg/ml)	CI	$\chi 2$	*P* value
Methanol	14.583	4.9295-43.142	39.8746	<0.0001
Ethyl -acetate	19.134	5.587-68.876	83.414	<0.0001
Vincristine sulphate	1.891	0.626-8.611	14.198	<0.01

CI= Confidence Interval, $\chi 2$= Chi square; the experiments were done in triplicate (n=3). Fenny probit analysis was performed to findout LC_{50} values, confidence interval limit, chi square and P value

Table 3. Antimicrobial activity of Methanol and Ethyl acetate extracts of *C. macroptera* fruit in disc diffusion method

Test organisms	Zone of inhibition (mm)		
	Azithromycin	Methanol extract	Ethyl-acetate extract
Bacillus subtilis	15.5 ± 0.707^{c}	0.00 ± 0.00^{a}	7.25 ± 0.353^{b}
Staphylococcus aureus	27.5 ± 0.707^{c}	0.00 ± 0.00^{a}	6.75 ± 0.353^{b}
Escherichia coli	25.5 ± 0.707^{c}	6.25 ± 0.353^{a}	7.5 ± 0.707^{b}
Salmonella typhi	27.5 ± 0.707^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Salmonella abony	27.75 ± 0.353^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Pseudomonas aeruginosa	17 ± 1.414^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

Values are presented as mean± SD (n=2). Values with different superscript in each row are significantly different from one another (P<0.05). One way ANOVA followed by Bonferroni multiple comparison was performed to analyze this data set

4. DISCUSSION

In DPPH and NO assays methanol extract showed more potential than ethyl acetate extract. Moreover, their results also varied significantly (P<0.05). Formally, it was noted that when DPPH accepted an electron which was donated by an antioxidant compound, the DPPH became decolorized. This was quantitatively measured due to the changes in absorbance. Chowdhury et al. [18] found that the hot methanol extract of stem bark of *C. macroptera* showed potential antioxidant activity (IC_{50}: 178.96 µg/ml): On the other hand cold methanol and dichloromethane extracts of stem bark showed moderate activity having IC_{50} value of 242.78µg/ml and 255.78µg/ml respectively. N-hexane extract of the stem bark of *Citrus macroptera* was found to possess mild antioxidant activity (IC_{50}: 422.94µg/ml). Besides, the author isolated Stigmasterol and Lupeol from the crude extracts of the stem bark of *Citrus macroptera.* These terpenoids type compounds may account for this free radical scavenging activity [18]. Uddin et al. [2] detected the presence of terpenoids in the methanol extract. These findings further strengthen the potentiality of methanol extract [2]. Nitric oxide (NO) is a physiologically important chemical mediator generated by endothelial cells, macrophages, neurons and involved in the regulation of various biochemical processes. Excess generation and accumulation of nitric oxide are implicated in the cytotoxic effects observed in several disorders like cancer, AIDS, arthritis etc. [19]. Over production of NO can mediate toxic effects such as DNA fragmentation, cell damage and neuronal cell death [20]. Several reports have pointed out the role of phenolic compounds in NO scavenging activity [21,22]. The fruit extracts may have capacity to undermine the effects produced by NO formation as well as can prevent the chain of reactions that is caused by excess NO generation. Polyphenols have antioxidative properties due to their high reactivity as hydrogen or electron donors which can stabilize and delocalize the unpaired electron (known as chain-breaking function). In addition, they present their antioxidant potential by chelating metal ions and terminating Fenton reaction [23]. Polyphenols have been shown to exert anticarcinogenic effects by modulating enzyme systems that metabolize carcinogens or pro-carcinogens to genotoxins by converting them to less reactive compounds before they react with DNA. Polyphenols have been shown to inhibit the Cytochrome P_{450} superfamily of enzymes that metabolizes many pro-carcinogens to reactive compounds before they react with DNA and induce malignant transformation, thus reducing the formation of reactive intermediates [24]. In this study both extracts have been found to possess considerable amount of gallic acid equivalent substances (polyphenolic compounds). Phenolic compounds or polyphenols are major areas of research, because they are considered as potent antioxidant, anti-cancer, anti-bacterial, anti-inflammatory and antiviral agents [25,26]. The total antioxidant assay is used to quantify vitamin E in seeds and this protocol is simple and independent of other antioxidant measurement methods [9]. In this study both extracts possess considerable antioxidant constituents. We have noticed moderate reducing activity of both extracts in reducing power and cupric reducing capacity assays. The extracts may possess polyphenolic compounds that usually show reducing power. The reducing ability of a compound is normally dependent on the presence of polyphenolic reductants [27], which exhibit antioxidant activity by breaking the free radical chain via donating a hydrogen atom [28]. They also prevent the formation of peroxide by reacting with some of its precursors. These types of effects of the reductants in the sample indicate their contribution in the antioxidant effect [29]. Now, it can be speculated that the presence of reductants (i.e. antioxidants) in *C. macroptera* fruit extracts may account for this reducing capacity. The consequences of lipid peroxidation (LPO) are destabilization and disintegration of the cell membrane thus leading to liver injury, arteriosclerosis and kidney damage [30]. Peroxy radicals are known to mediate lipid peroxidation process and thereby damage cell membrane. Hydrogen peroxide when comes in contact with hemoglobin causes the

degradation of hem, releases Fe ions and thus initiates Fenton reaction to produce free radicals. This process consequently causes lipid peroxidation which is a mechanism for cell deterioration and death [31,32]. In LPO models both extracts showed potential. We can correlate this activity with antioxidant effect of both extracts. Both extracts can scavenge the free radical and stop peroxidation process.

The brine shrimp bioassay has been established as a safe, practical and economic method for the determination of bioactivities of synthetic compound as well as plant products [33,13]. The relationship between the brine shrimp bioassay and growth inhibition of human solid tumor cell lines (*In vitro*) demonstrated by the national Cancer Institute (NCI, USA) is significant because it exhibits the importance of this lethality bioassay as a pre-screening tool for anti-cancer drug research [34]. According to Meyer et al. [13] extracts derived from natural resources which have $LC_{50} \leq 1000\mu g/ml$ using brine shrimp bioassay were claimed to contain bioactive principles [13]. Criteria of brine shrimp toxicity for compound or plant extract which were established as LC_{50} values above 1000µg/ml are non-toxic, between 500 & 1000µg/ml are weak toxic, and below 500µg/ml are toxic [35]. In this study methanol and ethyl-acetate extracts showed very good LC_{50} values of 14.583 and 19.134µg/mL respectively. According to the National Cancer Institute (NCI), the criterion of cytotoxic activity for the crude extracts is LC_{50} value of ≤ 20µg/mli which is considered to be very cytotoxic [36]. Therefore both extracts are considered very toxic. Between two extracts the highest lethality activity was found for methanol extract. It was reported that toxicity of plant extracts is attributed to different types of secondary metabolites such as saponins, terpenoids, steroids, tannins, alkaloids etc. [37]. Therefore, the presence of steroids, saponins and terpenoids in the methanol extract may contribute to this toxicity [2].

In comparison between the results of two extracts, ethyl-acetate extract has more moderate antimicrobial activity than methanol extract. The antimicrobial activity of ethyl-acetate extract may be due to cell lysis, inhibiting DNA synthesis, having astringent property that can induce complexation with microbial enzymes or substrates, hydrogen bonding or nonspecific interaction with microbial enzymes, iron deprivation, toxic action on microbial cell membranes and complexation of metal ions [38-42]. Our study has somewhat matched with the earliar findings about *C. macroptera*. Waikedre et al. [43] proved that *C. macroptera* leaf oil exhibited pronounced activity against Trichophytonmentagrophytes var. interdigitale, with a minimal-inhibitory concentration (MIC) of 12.5µg/ml [43]. It can be assumed that ethyl acetate may have broad spectrum antimicrobial property because it showed activity against both gram positive and gram negative bacteria.

5. CONCLUSION

We are still not sure about how the fruit extracts exerted the aforementioned therapeutic activities. There is every possibility to suggest that activity may be due to the presence of different phytochemicals. However, further co-ordinated and well-structured studies would be required to isolate the bioactive compounds responsible for these activities and determine their underlying molecular mechanism action to find out novel lead candidates.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

In this research work the authors declare that they have no interest in competing with other researchers.

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