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# QUALITATIVE PHYTOCHEMICAL SCREENING AND EVALUATION OF ANTIOXIDANT, TOXICITY, ANTIBACTERIAL AND ALPHA-AMYLASE INHIBITORY ACTIVITIES OF *CITRUS MACROPTERA*

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## Abstract

This study was designed to evaluate antioxidant, toxicity, antibacterial and α-amylase inhibitory activities of ethanolic peel extract of *Citrus macroptera* Montr. fruit by DPPH (1, 1-diphenyl-2picrylhydrazyl) free radical scavenging assay, nitric oxide scavenging capacity assay, cupric reducing antioxidant capacity, total phenol content, lipid peroxidation assay; brine shrimp lethality bioassay; disc diffusion technique; modified starch iodine protocol. The results showed that the extract possessed antioxidant properties. In brine shrimp lethality bioassay, the extract demonstrated 26.685 µg/mL of $LC_{50}$ value. It showed highest zone of inhibition against *Staphylococcus aureus* (13.5 ± 0.707 mm). In α-amylase inhibitory test, the extract presented 4.874±0.035 mg/mL of $IC_{50}$ value. The present findings suggest that *Citrus macroptera* Montr. fruit may be the possible sources of antioxidant, toxicity, antibacterial and antidiabetic agents. However, further investigation is required for the confirmation of their activities.

**Key Words:** Antioxidant, toxicity, antibacterial, antidiabetic, *Citrus macroptera.*

## Introduction

*Citrus macroptera* Montr. commonly called 'Satkara' (wild orange) is a semi-wild species of *Citrus* genus. The tree which has thorns can reach 5 m in height. Its fruit is about 6–7 cm 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. 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 is served in many restaurants in the UK. In Bangladesh the fruit of *Citrus macroptera* is eaten as a vegetable. This plant is used medicinally in Assam. Traditionally the fruit of this plant is used as appetite stimulant and in the treatment of fever [1]. In the previous study, researchers reported hypoglycemic potential of the pulp of this fruit through *in vitro* α-amylase inhibitory assay and *in vivo* oral glucose tolerance test and hypoglycemic effect in normal rats assay [2]. Moreover, antioxidant activity, brine shrimp lethality and antibacterial activity of the pulp of same fruit were also reported [3]. The peel part of this fruit may also possess these activities. That is why, this research was designed to explore the above mentioned properties of ethanolic peel extract of *Citrus macroptera* Montr. fruit (EPECM).

## Materials and Methods

### *Chemicals and Reagents*

Folin-Ciocalteu reagent and ethanol were purchased from Merck, Germany. Sodium carbonate was purchased from Merck (India) Limited. Gallic acid, quercetin and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemicals, USA. Ascorbic acid was purchased from SD Fine Chem. Ltd., Biosar, India. Vincristine sulphate was obtained from Techno Drugs Ltd. Bangladesh. Acarbose tablet was purchased from local market, manufactured by Pacific Pharmaceuticals Ltd., Bangladesh. Starch was purchased from local scientific market, Motijheel, Dhaka. All chemicals and reagents which were used were of analytical grade.

### *Plant Materials*

*Citrus macroptera* fruits 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.

### *Preparation of Plant Extract*

At first pulps were removed. Peels without pulp 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 50°C) to make suitable for extraction process. After that the dried peel parts were grinded to coarse powders using high capacity grinding mill. 200 g powdered materials were treated with 2 L ethanol following cold extraction with occasional shaking for 4-5 days at room temperature. The extract was 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. The extraction yield was 10 % (w/w).

### *Phytochemical Screening*

EPECM underwent phytochemical screening to detect presence of phytoconstituents like alkaloids, flavonoids, saponins, tannins, carbohydrates, glycosides, glucosides, fat and fixed oils, steroids, proteins and amino acids and terpenoids [4].

### *Antioxidant Activity Evaluation*

### *DPPH Free Radical Scavenging Assay* [5]

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

### *Nitric Oxide Scavenging Capacity Assay* [6]

4 mL of EPECM and standard (Ascorbic Acid) of different concentrations (200, 100, 50, 25 and 5 µg/mL) solutions were taken in different test tubes and 1 mL of sodium nitroprusside (5 mM) solution was added to these test tubes. The test tubes were incubated for 2 h at 30°C to complete the reaction. 2 mL 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 was calculated using linear regression analysis.

### *Cupric Reducing Antioxidant Capacity* [7]

500 µL of EPECM and standard (Ascorbic Acid) of

different concentrations (200, 100, 50, 25 and 5 µg/mL) were taken in different test tubes. 1 mL of 0.01 M $CuCl_2.2H_2O$ solution, 1 mL of ammonium acetate buffer (pH 7), 1 mL of 0.0075 M of neocaproin solution and distilled water (600 µL) were added and the final volume of the mixture was adjusted to 4.1 mL. The total mixtures were incubated for 1 h at room temperature. The absorbance of these 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.

***Determination of Total Phenol Content*** [8]

1 mL of EPECM or standard (Gallic acid) of different concentrations (200, 100, 50, 25, 12.5 and 6.25 µg/mL) were taken in test tubes. 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 min at 20°C to complete the reaction but the test tubes containing extract solution were incubated for 1 h at 20°C 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 peel extract. The total content of phenolic compounds in EPECM was expressed in mg/g gallic acid equivalent (GAE).

***Lipid Peroxidation by Thiobarbituric Acid (TBA) Assay*** [9]

1mL of different concentrations (200, 100, 50, 25, 12.5 and 6.25 µg/mL) of EPECM or standard (BHT, Butylated hydroxytoluene) was added to pre-labeled test tubes. 1 mL of 0.15 M KCl was added to each test tube and 0.5 mL of liver homogenate was added to it later and mixed well. After that, 100 µL of 0.2 mM $FeCl_3$ solution was added to initiate peroxidation. The total mixture was incubated at 37°C for 30 min. After the incubation, the reaction was terminated with the addition of 2 mL of ice-cold 0.25 N HCl containing 15% TCA, 0.38% TBA and 0.5% BHT. The reaction mixture was heated at 80°C 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. $IC_{50}$ value was calculated by linear regression method.

***Brine Shrimp Lethality Bioassay (BSLB)*** [10, 11]

Brine shrimp eggs (*Artemia salina* leach) were hatched in simulated seawater to get nauplii. Sample solutions were prepared by dissolving the test materials in pre-calculated amount of DMSO (Di-methyl sulphoxide). Ten nauplii were taken in vials containing simulated seawater. The samples of different concentrations were added and the volume was adjusted up to 5 mL. Survivors were counted after 24 hours. Vincristine sulphate was used as the reference cytotoxic drug. The percentage of mortality of brine shrimp nauplii was calculated for each concentration of the extract and then corrected using Abott formula [12]. After correcting the % mortality, $LC_{50}$ value was calculated using Fenny probit analysis [13].

***Antibacterial Activity***

***Microorganisms***

One Gram positive bacteria *Staphylococcus aureus* and four Gram negative bacteria *Salmonella typhi*, *Salmonella paratyphi*, *Salmonella* grb and *Shigella sonni* were used for the study.

***Experimental Procedure***

Antibacterial activity of EPECM was investigated by disc diffusion technique [14]. Subcultures prepared from pure cultures of five 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 5 mL of nutrient broth contained in screw-capped test tubes using a transfer loop and then incubated for 24 h at 37 °C for their optimum growth of $5x10^5$ cfu/mL. Peel extract of 400 µg/disc disc was used for this investigation. Standard disc of Chloramphenicol (30 µg/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, standard discs and control discs were placed gently on the previously marked zones in the nutrient agar plates. The plates were kept in an incubator at 37 °C for 48 hours to facilitate bacterial growth. After incubation, the antibacterial activity of the test materials was determined by measuring the diameter of the zone of inhibition in millimeter with a transparent scale.

***α-Amylase Inhibitory Activity***

This study was performed by a modified starch iodine protocol [15]. In short, 1 mL of peel extract or

standard of different concentrations (2, 1, 0.5 mg/mL) was taken in pre-labeled test tubes. A volume of 20 µL of α-amylase was added to each test tube and incubated for 10 min at 37 °C. After the incubation 200 µL of 1% starch solution was added to each test tube and the mixture was re-incubated for 1 h at 37 °C. Then 200 µL of 1% iodine solution was added to each test tube and 10 mL distilled water was added to it. Absorbance of the mixture was taken at 565 nm. Sample, substrate and α-amylase blank were undertaken under the same conditions. Each experiment was done in triplicate. $IC_{50}$ value was calculated by using regression analysis.

% α-amylase inhibition= [1- (SA-SBB)-SMB/AAB] X100
SA=Sample absorbance, SMB=Sample blank, SBB=Substrate blank and AAB=α-amylase blank.

***Statistical Analysis***

Values are presented as mean ± SEM (Standard error of mean) and mean ± SD (Standard deviation). Unpaired t test, Pearson correlation analysis and probit analysis were performed to analyze different data sets in these experiments. $P < 0.01$ and $P < 0.0001$ were considered statistically significant. Statistical programs which were used were Graph Pad Prism version 6 (Graph Pad Software Inc., San Diego, CA, USA) and SPSS version 16 (IBM Corporation, New York, USA).

## Results

The phytoconstituents found in EPECM include carbohydrates, glycosides, steroids, glucosides, terpenoids and saponins. In DPPH and LPO assays, significant $IC_{50}$ values of the EPECM were 751.518 ± 11.162 µg/mL ($^{\Delta}P < 0.0001$, *vs* Ascorbic acid) and 421.625 ± 1.641 µg/mL ($^{\Delta}P < 0.0001$, *vs* BHT) respectively (Table 1). Again, in NO assay, EPECM presented significant ($P<0.0001$, *vs.* BHT) $IC_{50}$ value of 1751.311±13.197 µg/mL (Table 1). We noticed significant positive correlation between two variables (absorbance and concentration) for both EPECM and standard (ascorbic acid) in case of cupric reducing antioxidant capacity. For EPECM ($r= 0.96$ and $P < 0.01$) and standard ($r= 0.98$ and $P < 0.01$) (Figure 1). Total phenol content of EPECM was 78.75 ± 1.286 mg/g GAE (Table 1). In BSLB, EPECM was found to be toxic against brine shrimp nauplii with $LC_{50}$ of 26.685 µg/mL ($\chi 2$ value = 19.163 and it's $P < 0.01$) whereas anticancer drug vincristine sulphate showed $LC_{50}$ value of 1.891 µg/mL (Table 2).

Chloramphenicol showed zone of inhibition against all the tested pathogens which ranged from 7.00 ± 0.00 mm to 28.5 ± 0.707 mm. The highest antibacterial activity of EPECM was observed against *Staphylococcus aureus* (zone of inhibition of 13.5±0.707 mm) (Table 3). In α-amylase inhibitory assay, EPECM showed significant ($p < 0.0001$, *vs* acarbose) $IC_{50}$ value of 4.874 ± 0.035 mg/mL (Table 4).

## Discussion

In this experiment, EPECM was found to show antioxidant potential. Formerly, it was noted that the hot methanol extract of stem bark of *C. macroptera* showed potential antioxidant activity ($IC_{50}$ of 178.96 µg/mL). On the other hand, cold methanol and dichloromethane extracts of stem bark showed $IC_{50}$ values of 242.78 µg/mL and 255.78 µg/mL respectively. n-hexane extract of the stem bark of *C. macroptera* was found to possess $IC_{50}$ of 422.94 µg/mL. Besides, stigmasterol and lupeol were isolated from the crude extracts of the stem bark of *Citrus macroptera*. These terpenoids type compounds may account for free radical scavenging activity [16]. In this study, EPECM contained carbohydrates, steroids, saponins, terpenoids, glycosides and glucosides which may contribute to its antioxidant activity. In addition, the total phenol content was noted 78.75±1.286 mg/g GAE for EPECM. Phenolic compounds 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 [17]. Phenolic compounds have been shown to block LDL oxidation, decrease the formation of atherosclerotic plaques and reduce arterial stiffness, leaving arteries more responsive to endogenous stimuli of vasodilation [18, 19]. This ability is believed to be mainly due to their redox properties [20], which play an important role in adsorbing and neutralizing free radicals. The above mentioned research findings provide strong rationale for the phenolic compounds as potential antioxidant agent [21, 22]. Therefore, presence of different phytoconstituents may account for antioxidant potential of EPECM. The brine shrimp bioassay is a safe, practical and economic method for the determination of bioactivities of synthetic compound as well as natural products [23, 10]. Extracts derived from natural resources having $LC_{50} \leq 1000$ µg/mL using brine shrimp bioassay were claimed to contain bioactive principles [10].

Compound's or plant extract's $LC_{50}$ value above 1000 µg/mL are non-toxic, between 500 & 1000 µg/mL are weak toxic, and below 500 µg/mL are toxic [24]. In this study, EPECM showed $LC_{50}$ value of 26.685 µg/mL. According to the National Cancer Institute (NCI), the criterion of cytotoxic activity for the crude extracts is $LC_{50}$ value of ≤ 20 µg/mL which is considered to be very cytotoxic [25]. Therefore, the extract may be considered as toxic. It was reported that toxicity of plant extracts is attributed to different types of secondary metabolites such as saponins, terpenoids, steroids, tannins, alkaloids etc. [26]. Therefore, the presence of phytoconstituents in EPECM may contribute to this toxicity. In disc diffusion technique, EPECM showed the antibacterial activity against *Staphylococcus aureus* and *Salmonella* grb. Antibacterial activities were reported from different polyphenols. Antimicrobial activity of saponins [27, 28, 29] and steroids [30, 31, 32] were reported in different research studies. And the inhibitory capacity of phenolic compounds on microorganisms may occur for iron deprivation or hydrogen bounding with vital proteins such as microbial enzymes [33]. Our study has somewhat matched with the earliar findings about *C. macroptera*. Waikedre et al., 2010 proved that *C. macroptera* leaf oil exhibited pronounced activity against Trichophyton mentagrophytes var. interdigitale, with a minimal-inhibitory concentration (MIC) of 12.5 µg/mL [34]. Therefore, the presence of saponins, steroids, terpenoids, glycosides and glucosides type polyphenols may contribute to the antibacterial activity of EPECM.

Amylase inhibitors are also known as starch blockers because they prevent dietary starch from being absorbed by the body and thereby lower the postprandial glucose levels. Slowing the digestion and breakdown of starch may have beneficial effects on insulin resistance and glycemic index control in people with diabetes [35, 36, 37]. Formerly it was reported that natural polyphenols inhibit the activity of carbohydrate hydrolyzing enzymes like α-amylase and α-glucosidase [36]. In the previous studies saponins have also been found to be a probable α-amylase inhibitor [38, 39]. Therefore, the observed α-amylase inhibitory activity of EPECM may be due to the presence of polyphenolic phytoconstituents like saponins, steroids and terpenoids.

## Conclusion

Coordinated and well-structured studies are required to isolate the bioactive compounds responsible for these activities and to determine their underlying molecular mechanism of action to find out novel lead candidates.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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**Table 1.** Antioxidant potential of EPECM.

| Antioxidant Models | EPECM | Ascorbic Acid | BHT |
|---|---|---|---|
| DPPH($IC_{50}$) | 751.518 ± 11.162$^{\Delta}$ | 17.663±1.310 | - |
| NO ($IC_{50}$) | 1751.311±13.197$^{\Delta}$ | 89.135±3.645 | - |
| Total phenol (mg/g GAE) | 78.75±1.286 | - | - |
| LPO by TBA ($IC_{50}$) | 421.625±1.641$^{\Delta}$ | - | 8.941±0.940 |

Values are presented as mean ± SEM (n=3) for DPPH, NO and LPO assays and mean ± SD (n=3) for total phenol content. Unpaired t test was performed to analyze the data set. $^{\Delta}P <$ 0.0001, *vs* Ascorbic acid; $^{\Delta}P < 0.0001$, *vs* BHT.

**Table 2.** Brine shrimp lethality of EPECM.

| Extract/Standard | $LC_{50}$ (µg/mL) | CI | χ2 | P value |
|---|---|---|---|---|
| EPECM | 26.685 | 17.863-40.08 | 19.163 | <0.01 |
| Vincristine sulphate | 1.891 | 0.626-8.611 | 14.198 | <0.01 |

The experiment was done in triplicate (n=3). Fenny probit analysis was performed to find out $LC_{50}$, CI, χ2 and P values.

**Table 3.** Antibacterial activity of EPECM in disc diffusion method.

| Test Organisms | Zone of inhibition (mm) | |
|---|---|---|
| | Chloramphenicol | EPECM |
| *Salmonella typhi* | 15.00±0.00 | 0.00±0.00$^{\Delta}$ |
| *Staphylococcus aureus* | 25.5±0.707 | 13.5±0.707$^{\Delta}$ |
| *Shiggla sonni* | 28.5±0.707 | 0.00±0.00$^{\Delta}$ |
| *Salmonella paratyphi* | 13.00±1.414 | 0.00±0.00$^{\Delta}$ |
| *Salmonella* grb | 7.00±0.00 | 7.00±0.00 |

Values are presented as mean ± SD (n=3). Unpaired t test was performed to analyze this data set. $^{\Delta}P < 0.0001$, *vs* Chloramphenicol.

**Table 4:** $IC_{50}$ values for EPECM and acarbose in α-amylase inhibitory assay.

| Extract/Standard | $IC_{50}$ value ( mg/mL) |
|---|---|
| EPECM | 4.874±0.035$^{\Delta}$ |
| Acarbose | 0.917+0.002 |

Values are presented as mean ± SEM (n=3). Unpaired t test was performed to analyze this data set. . $^{\Delta}P < 0.0001$, *vs* Acarbose.

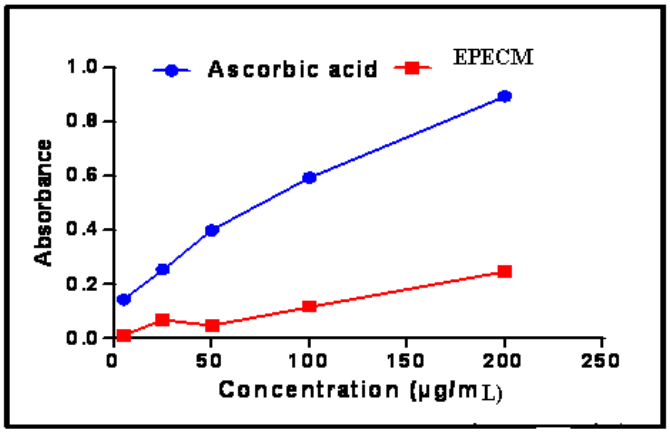


**Figure 1.** Cupric reducing antioxidant capacity of EPECM and standard (ascorbic acid). Values are presented as mean ± SEM (n=3).