

***In Vitro* Antioxidant Activity and Cytotoxicity of The Ethanolic Extracts of Satkara (*Citrus Macroptera*) Fruit Peels at Different Maturity Stages**

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Abstract: There is a growing interest in the study of natural products, especially in the field of drug screening, development and biological research. Plants are the potential source of biological products and pathogenic agents. The present study was undertaken to extract the bioactive compounds from *Citrus macroptera* in its maturity stages and to clarify that the plant extracts has great potential value to combat against different diseases. In this study, antioxidant activity and cytotoxicity of three varieties of satkara (*Citrus macroptera*) fruit peels (BARI, Advance Line and Local) at different maturity stages were evaluated. The antioxidant activity seemed to increase with the change of maturity. DPPH activity was seemed higher in the local variety comparing to the other varieties. Total phenolic compounds was found highest in the ripen stage of local variety (129.23±4.57 mg/gm). Lowest amount was recorded in the mature stage of BARI (79.74±2.74 mg/gm). Amount of total flavonoids was found highest in the mature stage of BARI (27.43±4.27 mg/gm) and lowest amount was recorded in the ripen stage of local variety (17.16±1.33 mg/gm). Cytotoxicity assay of satkara (*Citrus macroptera*) fruit peels ethanol extracts using brine shrimp lethality bioassay showed very high toxicity. Highest toxicity was seen in the BARI at ripen stage having LC50 value of 6.11 µg and lowest toxicity was observed in local variety at mature stage having LC50 value of 19.46 µg. Toxicity was seemed to increase significantly with the change of maturity.

Key words: *Citrus macroptera*, ethanolic extracts, antioxidant activity, and cytotoxicity

INTRODUCTION

Infectious diseases are considered as a major threat to human health, because of the unavailability of vaccines or limited chemotherapy. Those diseases were considered for approximately one half of all deaths in tropical countries [1]. Traditional medicines play an important role in health services around the world. About three-quarters of the world population relies on plants and plant extracts for healthcare. The rational design of novel drugs from traditional medicine offers new prospects in modern healthcare. Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural resources. Traditional medicine is an important source of potentially useful new compounds for the development of chemotherapeutic agents [2]. In addition, high cost and adverse side effects are

commonly associated with popular synthetic antibiotics such as hypersensitivity, allergic reactions, immune-suppression and are major burning global issues in treating infectious diseases [3]. Although pharmacological industries had produced considerable number of commercial antibiotics time to time but resistance in pathogens towards these drugs has increased at high rate and multi-drug resistant microorganisms have exacerbated the situation [4]. In the present scenario, there is an urgent and continuous need of exploration and development of cheaper, effective new plant based drugs with better bioactive potential and least side effects. Hence, recent attention has been paid to biologically active extracts and compounds from plant species used in herbal medicines [5]. Plant extracts have been proved effective in the treatment of infectious diseases

simultaneously mitigating many of the side effects which are often associated with synthetic antibiotics [1].

Citrus macroptera Montr., a semi wild species of citrus genus, is known as 'Satkara' in Bangladesh. It may be mentioned here that the English meaning of Satkara is 'Wild orange'. The maximum height of the tree of this fruit is 5 meter. The diameter of the fruit is 6–7 cm. The fruit becomes yellow when it ripens and its rind is thick. As the pulp of this fruit is somewhat dry, it does not have enough juice, which is very sour and a bit bitter. The antioxidant activities of crude extracts of the stem bark of *Citrus macroptera* and isolated lupeol and stigmasterol was reported [6]. The anti-microbial activity of essential oil of *Citrus macroptera* against five bacteria and five fungi strains was reported previously [7]. Besides, the author found the presence of beta-pinene as major component. Essential oils obtained by hydro-distillation from the fresh peels of *Citrus macroptera* contained limonene, beta-caryophyllene and geranial as main compounds [8]. Gaillard et al. isolated edulinine, ribalinine and isoplatydesmine and five aromatic compounds [9]. The people of Bangladesh eat this fruit as a vegetable. The fruit is used as ingredient in cooking different kinds of meat and chicken. Nowadays many Bangladeshi and Indian restaurants offer meat and chicken curries cooked with Satkara. Traditionally, this fruit is used as appetite stimulant and in treatment of fever. In spite of the diverse uses of this fruit, there seems to be a dearth of information about the antioxidant, cytotoxic activity as well as medicinal values of this fruit in its different stages of maturation. Therefore, we have designed our study protocol to evaluate the medicinal (viz. antioxidant activity and cytotoxicity) values of the ethanolic extracts of fruit peels.

Materials and Methods

Sample collection

Two different stages of *Citrus macroptera* fruit (mature and ripened) of two varieties- BARI-Satkara and Advanced line were collected from the Citrus Research Center, Jointapur, Sylhet at different times September (mature) and December (ripened). Mature and ripened stage of local Satkara was also collected from local market. After collecting the sample, fruits were thoroughly washed to remove dirt and other materials. Ethanol extracts of the satkara fruit peels were prepared as described previously [10].

Measurement of anti-oxidant activity

Estimation of DPPH (2, 2-diphenyl-1-picrylhydrazyl) scavenging assay

Free radical scavenging activity of the ethanol extract of *Citrus macroptera*, based on the scavenging activity of the stable free radical DPPH (2, 2-diphenyl-1-

picrylhydrazyl) was determined by the method described by Braca and co-workers [11]. DPPH (2, 2-diphenyl-1-picrylhydrazyl) is a dark-violate colored crystalline powder composed of stable free-radical molecules which gives a deep violate colored solution in water. It becomes colorless or pale yellow when neutralized by the chemical reaction.

Reducing power assay

Reducing power was determined following the method as described previously [12]. The different concentrations of extract (1000, 500, 250, 125 and 62.5 µg/mL) in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide $K_3Fe(CN)_6$ (2.5 mL, 1% w/v). The mixture was incubated at 50°C for 20 min. after that, 2.5 mL of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1% w/v). After that, the absorbance of the solution was measured at 700 nm. The increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as the reference and phosphate buffer (pH 6.6) was used as blank solution.

Determination of total phenol contents

Total phenol content was determined using Folin-Ciocalteu reagent (FCR) [13]. Extract was mixed with FCR reagent and 7.5% sodium carbonate. The mixture was incubated for 20 minutes at 25°C. Then the absorbance at 765 nm was taken. The concentration of total phenol content in the extract was determined as mg of gallic acid equivalent by using an equation that was obtained from the standard gallic acid graph.

Determination of total flavonoids contents

The total flavonoids content was determined by following the method described by Kumaran et al. [14]. using quercetin as a reference compound. 1 mL of the extract was mixed with 200µl of aluminium trichloride in 3 mL methanol and 200µl of potassium acetate was added to it. After that, 5.6 mL of distilled water was added to the solution and incubated for 30 minutes at 25°C. The absorption at 420 nm was taken. Blank samples were prepared from 1 mL of distilled water instead of extract. The total flavonoid content was determined by using the standard curve of quercetin and expressed as mg of quercetin equivalent (QE/mg of extract).

Assessment of cytotoxicity using brine shrimp lethality bioassay

The cytotoxicity assay was performed on brine shrimp nauplii (*Artemia salina*) by following the method described by Meyer et al. [15] that also concurred with

the method used by Hossain et al. [16]. The brine shrimp lethality bioassay was used to determine the cytotoxicity of compounds using simple zoological organism *Artemia salina* as a convenient monitor for the screening.

The eggs of Brine shrimp (*Artemia salina* Leach) were collected and hatched in a tank at 25°C with constant oxygen supply. The nauplii were hatched for about 48 hours. Sample extract solution was prepared by dissolving required amount of extracts in specific volume of pure dimethyl sulfoxide (DMSO) and sea water. The nauplii were taken in separate test tubes containing 5 mL dimethyl sulfoxide (DMSO) and sea water mix (10 nauplii per test tube). Then specific volumes of sample were introduced to the test tubes to get final sample concentrations of 5, 10, 20, 40, 80 and 100 µg/mL. After incubating for 24 hours at 37°C, the test tubes were observed and the number of surviving nauplii in each test tube was counted. From this, the percentage of mortality of brine shrimp nauplii was calculated for each concentration of the extract. The percentage of mortality of the brine shrimp was calculated for each concentration by using the following formula: % Mortality = $N_t/N_0 \times 100$ %.

Where, N_t = Number of dead nauplii after 24 h of incubation, N_0 =Number of total nauplii transferred, i.e. 10. After that, using the percentage of mortality rate, the LC50 (medium lethal concentration) value of the extract solution was calculated using probit analysis.

Results

Anti-oxidant activity

To determine anti-oxidant activity- DPPH activity test, reducing power test, total phenol content and total flavonoid content was determined.

DPPH activity

DPPH activity seemed to increase as the stages of maturity increases. Thus, the ripen stages had more activity than the mature stages. In both the stages, local variety had the highest activity (Figure 1 and 2). BARI seemed to have the least activity among the mature stages (Figure 1) and advance line had the least activity in the ripen stages (Figure 2).

Table 1: Total phenol content in Satkara extracts

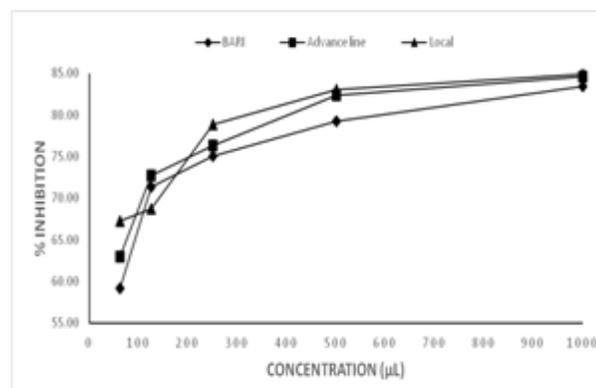


Figure 1: DPPH activity at mature stages

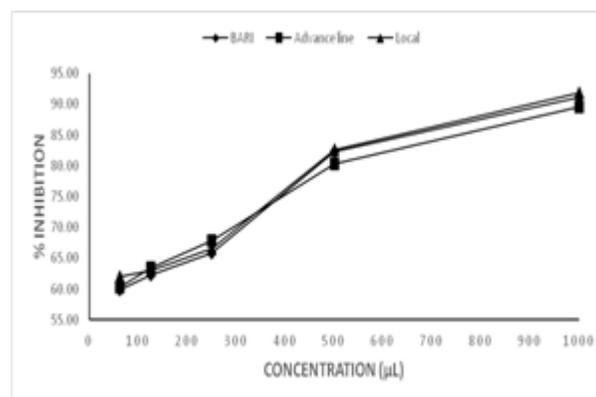


Figure 2: DPPH activity at ripen stages

Total phenol contents

The local variety at both ripen and mature stages had the highest amount of phenol (Table 1) and BARI had the lowest amount. At the mature stages, highest amount was recorded 97.44 mg/mL in Local variety and lowest was 79.74 mg/mL in BARI (Table 1). In ripen stages, highest content was recorded 129.23 mg/mL in local and lowest was 115.90 mg/mL in BARI (Table 1).

Stages	Mature			Ripen		
Variety	BARI	Advance line	Local	BARI	Advance line	Local
Content (mg/gm)	79.74	87.43	97.44	115.90	120.26	129.23

Total flavonoid contents

Total flavonoid content (TFC) was calculated as mg quercetin equivalent (QE)/100 gm sample dry weight. Total flavonoid content seemed to be higher in the mature stages comparing the ripen stages (Table 2).

Among the mature stages, highest amount of total flavonoid was recorded in BARI (27.43 mg/gm) and lowest in Local variety (17.61 mg/gm). At the ripen stages, highest amount was 19.08 mg/ml in BARI and lowest was recorded 17.16 mg/gm in Local variety.

Table 2: Total flavonoid content in Satkara fruit peel extracts

Stages	Mature			Ripen		
Variety	BARI	Advance line	Local	BARI	Advance line	Local
Content (mg/gm)	27.43	20.18	17.61	19.08	18.53	17.16

Cytotoxicity assay using brine shrimp napuli

Cytotoxicity test seemed to increase significantly with maturity to ripen stages. Highest toxicity was observed in ripen stages of BARI (LC50 - 6.11µg/mL) and lowest in local variety at mature stages (LC- 19.46 µg/mL) (Figure 1 and 2). 100% mortality was seen at 80 µg/mL concentration for most of the ripen varieties except for Local variety (90% mortality). At 100 µg/mL concentration, almost all variety showed 100% mortality at both mature and ripen stages (except for Local that showed 90% mortality).

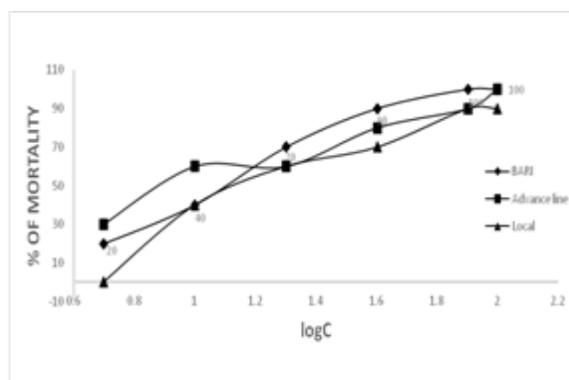


Figure 3: Mortality rate in Satkara fruit peel extracts at mature stages

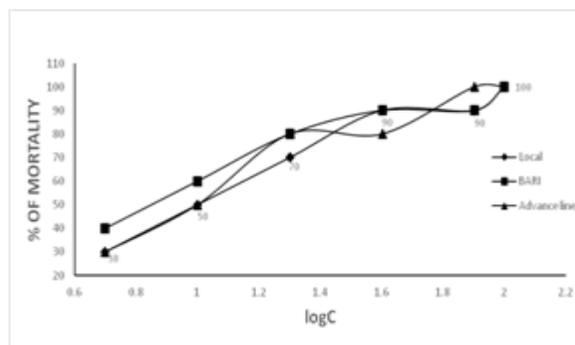


Figure 4: Mortality rate in Satkara fruit peel extracts at ripen stages

DISCUSSION

In vitro antioxidant assays are widely carried to screen various plant which contain phenolic and flavanoids compounds. The antioxidant assay showed that the antioxidant activity in satkara (*Citrus macroptera*) peels has stronger potential comparing to the mature stages. Also, it was noted that the local variety had the strongest activity comparing to the other varieties (BARI and Advance line). The brine shrimp lethality bioassay was established as a safe, practical and economic method for the determination of bioactivities of synthetic compound as well as plant products. The relationship between the brine shrimp bioassay and

growth inhibition of human *in vitro* tumor cell lines was described by the National Cancer Institute (NCI, USA) is significant because it exhibits the importance of the lethality bioassay as a pre-screening tool for anti-cancer drug research. According to previous report, extracts that were derived from natural resources having LC50 ≤ 1000 µg/mL using brine shrimp bioassay claimed to contain bioactive principles [15]. The criteria for brine shrimp toxicity of plant extract were established as- a) LC50 value above 1000 µg/mL are considered non-toxic, b) LC50 value having the range between 500 and 1000 µg/mL are considered as weak toxic and c) value below 500

µg/mL are toxic [17]. Considering the above conditions, the Satkara (*Citrus macroptera*) fruit peels extracts have very high toxic effects. Toxicity increased with the change of maturity. BARI showed highest toxic effects among the three varieties and the local variety had the least toxic effects. It was reported that plant extracts had cytotoxic attribute because of the presence of different types of secondary metabolites such as saponins, steroids, terpenoids, tannins and alkaloids etc. Therefore, the presence of steroids, saponins and terpenoids in the satkara extracts may result in the toxicity.

CONCLUSION

From the above study it can be concluded that the natural products extracted from satkara (*Citrus macroptera*) would be a potential candidate as future therapeutic agent. Also it is a good source of antioxidants. Resistances to antibiotics of some infectious organisms have become a major concern to the modern world. Natural extracts from satkara (*Citrus macroptera*) may have some role in it. At present scientists are giving their best regard in developing a more potent and site specific drug for the treatment of cancer. Satkara (*Citrus macroptera*) could be a potential source of natural products that could contribute in developing an anticancer agent.

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