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In vitro antioxidant, anti-diabetic, cholinesterase and tyrosinase inhibitory potential of fresh juice from *Citrus hystrix* and *C. maxima* fruits

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Abstract

In the present study, antioxidant potential, α -amylase, α -glucosidase, cholinesterase and tyrosinase inhibitory activity of fresh juice from indigenous fruits of *Citrus hystrix* and *C. maxima* (Red & White var.) were investigated using an *in vitro* model. The contents of total phenolics, tannins, and total flavonoids ranged between 836.90 and 909.52 mg gallic acid equivalent (GAE)/L, 333.33 and 523.21 mg gallic acid equivalent (GAE)/L and 224.88 and 262.22 mg rutin equivalent/L, respectively. The antioxidant activity of fresh juice was evaluated by employing different *in vitro* assays such as reducing power assay, DPPH[•], ABTS^{•+} and [•]OH radical scavenging capacities, peroxidation inhibition activity, antihemolytic assay. In addition, 75.55%–79.75% of α -amylase and 70.68%–72.83% of α -glucosidase enzyme inhibition characteristics were found under *in vitro* starch digestion bioassay. Also, all the juice samples exhibited excellent tyrosinase (76.95%–80.79%), acetylcholinesterase (75.71%–79.74%) and β -glucuronidase inhibitory activity (68.13%–69.38%). These results indicated that fresh juice of *C. hystrix* and *C. maxima* (Red & White var.) could be used as a source of antioxidant agents, functional food and nutraceuticals.

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Keywords: Citrus fruits; Fresh juice; Antioxidant potential; Enzymatic inhibitory activity; Nutraceuticals

1. Introduction

Natural antioxidants from fruits juices offer an alternative source of dietary ingredients to promote healthy life. For example, α -amylase, α -glucosidase inhibitors are considered as one of the effective measures for regulating type II diabetes by controlling glucose uptake, and acetylcholinesterase inhibitors are used to treat the Alzheimer's disease and tyrosinase inhibitors are clinically useful for the treatment of Parkinson disease [1–3]. In this research health relevant functionality of juice from indigenous fruit of *Citrus hystrix* and *C. maxima* (Red & White variety) is explored. Citrus fruits belong to the family of *Rutaceae* which is one of the most important fruits crops of the world and it is

consumed mostly as fresh produce or juice because of its nutritional value and pleasant flavor. Citrus is an excellent source of many nutrients and phytoconstituents, and is able to supply a healthy diet. Phenolic acids and flavanones are the two main groups of phenolic compounds in citrus fruit juices [4]. Generally, citrus juices are a rich source of antioxidant compounds, especially phenol, flavonoids and ascorbic acid [5]. The phenolic compounds have a wide range of biological activities such as antioxidant activity, protection against coronary heart diseases, anti-inflammatory, anticancer and antimicrobial activities [6].

C. hystrix DC (commonly known as Kaffir lime or wild lime) and *C. maxima* L. are giant citrus (commonly known as Pummelo) that are originated from South East Asia, India and cultivated throughout the tropical and temperate regions. *C. hystrix* is pear-shaped, bumpy, greenish yellow fruit with acidic flavor. It has a very thorny bush with aromatic leaves and fruits. The whole fruit is used in traditional medicine for headache, flu, fever, sore throats, bad breath and indigestion [7]. These fruits are rich in phenolic compounds including flavonoids, glyceroglycolipids, α -tocopherol, limonoids, furanocoumarins, benzenoid derivative and quinolinone alkaloids with potential health-promoting properties [8,9]. These compounds have been proposed as important contributors to the radical scavenging activity. They showed variety of pharmaceutical effects such as anti-tumor, antimicrobial, anti-inflammation and antioxidant activities [9,10].

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C. maxima fruit is the largest of all citrus varieties. It is globose, pear-shaped with 11–14 segments. The pulp is white or pinkish red, spindle-shaped juice sacks that may separate easily from one another with sweetish-acidic flavor. Its fruits are used in traditional medicine as cardiac stimulant and stomach tonic [11]. Its fruits provide a range of key nutrients as well as many non-nutrient phytochemicals with antioxidant properties. The antioxidant and anti-radical activities of pummelo fruits are mainly due to the presence of carotenoids, lycopene, polyphenols, flavonoids, limonoids, pectin, fiber and vitamin C, leading to their protective effects against chronic diseases such as hyperglycemic and hypercholesterolemic [12–15]. Fruit, leaves and juice of pummelo are reported to contain flavanone glycoside (naringin), narirutin, prunigen, hesperidin and neohesperidin [16,17].

Due to their important health-promoting properties, there is a growing interest to incorporate these compounds into food products, either to create functional foods or to simply replace commonly used synthetic food additives such as butylated hydroxytoluene and butylated hydroxyanisole in order to prevent side effects. Nutritional value and pharmacological properties of different parts of indigenous fruit of *C. hystrix* and *C. maxima* have been greatly reported, but there has been very little information on antioxidant and medicinal properties of juices. Hence, the present study attempts to investigate the total phenolics content, antioxidant, cholinesterase, tyrosinase, glucuronidase and type II diabetic related enzyme inhibition properties of fresh juice from the fruit of *C. hystrix* and *C. maxima* (Red & White var.).

2. Materials and methods

2.1. Chemicals

All the chemicals used in this study were of analytical grade. 2,2-azino bis (3-ethyl benzothiazoline-6-sulfonic acid) diammonium salt (ABTS), butylated hydroxy anisole (BHA), 2,2'-diphenyl-1-picryl-hydrazyl (DPPH), β -carotene, linoleic acid, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), α -amylase, α -glucosidase, acetylthiocholine iodide, tyrosinase and β -glucuronidase enzyme were purchased from Sigma Chemicals Co (St. Louis, MO, USA). All the other chemicals were obtained from HiMedia Laboratories (Mumbai, Maharashtra, India).

2.2. Fruit collection, juice preparation and yield (%)

The fruits of *Citrus hystrix* & *Citrus maxima* (Red and White) were collected from Mayiladuthurai, Nagai district, Tamil Nadu. Fresh fruits were collected during April 2010. Citrus fruits samples were peeled and squeezed by hand and then juices were collected in a separate container and stored under -20°C for further analysis. The yield of juice was calculated using the following formula:

$$\text{Juice yield/\%} = (\text{Juice weight/fruit weight}) \times 100$$

2.3. Determination of total phenolics and tannin contents

The total content of phenolics and tannins were measured as gallic acid equivalents [18]. One mL juice sample was added with 0.5 mL Folin-Ciocalteu reagent (1 mol/L) and 2.5 mL sodium carbonate solution (20%). Soon after vortexing the reaction mixture, the tubes were placed in the dark for 40 min and the absorbance was recorded at 725 nm with UV-vis spectrophotometer (Cyberlab-UV100, USA) against the reagent blank. Total phenolic content (TPC) of juices were expressed as mg gallic acid equivalents (GAE)/L. Using the same juices and method, the content of tannins were estimated after treatment with polyvinylpolypyrrolidone (PVPP).

2.4. Estimation of total flavonoids

Total content of flavonoids was measured according to the method of Zhishen et al. [19], outlined by Siddhuraju and Becker [20]. Fruit juice was added with 0.3 mL of 5% sodium nitrite and well mixed. After 5 min of incubation, 0.3 mL of 10% aluminum chloride solution was added. Then, after 6 min, 2 mL of 1 mol/L sodium hydroxide was added to the mixture and the volume was made up to 10 mL with water. The absorbance was measured at 510 nm with UV-vis spectrophotometer. Total content of flavonoids were calculated from a rutin (20–100 μg) standard curve and expressed as mg rutin equivalents/L.

2.5. Free radical scavenging activity on DPPH \bullet

The antioxidant activity of juices and standards (BHA, rutin and tannic acid) was measured in terms of hydrogen donating ability using a stable, commercially available organic and nitrogen centered DPPH radical using the method of Brand-Williams et al. [21] with slight modifications. Juice samples were mixed with 3.9 mL methanol containing DPPH \bullet (0.025 g/L) and incubated in the dark for 30 min. The absorbance was measured at 515 nm with UV-vis spectrophotometer. The trolox standards were prepared in the range of 0–2.5 mmol/L. The concentration of DPPH was calculated from a trolox standard curve and expressed as mmol trolox equivalents/L.

2.6. Antioxidant activity by the ABTS \bullet^{+} assay

The ABTS \bullet^{+} radical cation decolorization assay was performed to evaluate the radical scavenging ability of fruit juices using the method of Re et al. [22] with slight modification made by Siddhuraju and Becker [20]. ABTS \bullet^{+} was generated by adding 2.45 mmol/L potassium persulfate to 7 mmol/L ABTS and incubated in the dark at room temperature for 12–16 h. This stock solution of ABTS \bullet^{+} was diluted with ethanol to give an absorbance of 0.70 (± 0.02) at 734 nm, which acted as a positive control. Ten microliters of juice samples was mixed with 1.0 mL diluted ABTS \bullet^{+} solution and incubated at 30°C for 30 min. The absorbance value was measured at 734 nm with UV-vis spectrophotometer. Trolox standard was also prepared (in ethanol: 0–1.5 mmol/L) to get the concentration response curve. The unit of trolox equivalent antioxidant activity (TEA) was defined as

the concentration of Trolox with the equivalent antioxidant activity (expressed as mmol/L) of juice samples. The TEA of BHA, rutin and tannic acid was also measured by ABTS^{•+} method for comparison.

2.7. Ferric reducing antioxidant power assay (FRAP)

FRAP assay can be used to evaluate the electron donating ability of antioxidants according to the method of Pulido et al. [23]. An aliquot of 30 μ L juice samples was mixed with 90 μ L water and 900 μ L FRAP reagent [2.5 mL of 20 mmol/L of TPTZ in 40 mmol/L of HCl, 2.5 mL of 20 mmol/L of ferric chloride, 25 mL of 0.3 mol/L of acetate buffer (pH 3.6)] and incubated at 37 °C for 30 min. After incubation, the absorbance was recorded at 593 nm with UV–vis spectrophotometer. Known ferrous sulfate concentrations ranging from 400 to 2000 μ L/L were used to generate the calibration curve. From the curve, the ferrous ions reduced by the juice sample were calculated using a regression equation. The antioxidant activity was expressed as amount of juices required to reduce 1 mmol of ferrous ions. The antioxidant activity of juice samples was compared with the following standards: BHA, rutin and tannic acid.

2.8. Metal chelating activity

The chelating activities of samples, standards like BHA and α -tocopherol were estimated using the method of Dinis et al. [24]. An aliquot of 0.1 mL juice samples, 0.6 mL distilled water and 0.1 mL ferrous chloride (2 mmol/L) were well mixed and incubated for 30 s. Then, 0.2 mL ferrozine (5 mmol/L) was added to the above mixture and incubated for 10 min at room temperature and the absorbance was recorded at 562 nm with UV–vis spectrophotometer. EDTA (0–2 μ g) was used as standard for the preparation of calibration curve. Metal chelating ability of antioxidant was expressed as mg EDTA/L.

2.9. Superoxide anion radical scavenging assay

The superoxide anion radical ($O_2^{\bullet-}$) scavenging capacity of standards (BHA, catechin, trolox and rutin) and juice samples were determined using the method of Martinez et al. [25] for the determination of superoxide dismutase with some modifications made by Dasgupta and De [26] in the riboflavin–light–nitroblue tetrazolium system. Each 3 mL reaction mixture consisting of 50 mmol/L phosphate buffer (pH 7.8), 13 mmol/L methionine, 2 μ mol/L riboflavin, 100 μ mol/L EDTA, 75 μ mol/L NBT and 1 mL juices/standard was kept for 10 min under illumination with 20 W fluorescent lamps. The production of blue formazan was monitored and recorded at 560 nm with UV–vis spectrophotometer. The degree of superoxide anion radical scavenging activity (SRSA) was calculated as follows:

$$SRSA = (A_c - A_s)/A_c \times 100$$

where A_c is the absorbance of the control; A_s is the absorbance of juice samples. The scavenging activity was compared with positive standards BHA, rutin and trolox (150 μ g).

2.10. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging ability of juices and standard (catechin) was measured according to the method of Klein et al. [27]. Juice samples and standards were mixed with 1 mL iron-EDTA solution (0.13% ferrous ammonium sulfate in 0.26% EDTA), 0.5 mL of 0.018% EDTA and 1 mL DMSO solution (0.85% in 0.1 mol/L phosphate buffered saline pH 7.4). The reaction was terminated by the addition of 1 mL ice cold trichloroacetic acid (17.5, *m/V*). Then, 3 mL Nash reagent (7.5 g ammonium acetate, 0.3 mL glacial acetic acid, 0.2 mL acetyl acetone and distilled water – 100 mL) was added to the above mixture and incubated at room temperature for 15 min and the absorbance values were recorded at 412 nm with UV–vis spectrophotometer. Sample control was also run with phosphate buffer instead of ascorbic acid. The percentage of hydroxyl radical scavenging activity (HRSA) was calculated using the following formula:

$$HRSA/\% = 1 - (\text{Difference in absorbance of sample} / \text{Difference in absorbance in blank}) \times 100$$

The activity was compared with the positive standard catechin (250 μ g).

2.11. β -Carotene/linoleic acid bleaching activity

The antioxidant activity of juice samples and standards (BHA, rutin and trolox) was analyzed according to the method of Taga et al. [28] with slight modifications. Two mg of β -carotene were dissolved in 1 mL chloroform containing 40 mg linoleic acid and 400 mg Tween 40. The chloroform was removed by rotary vacuum evaporator at 45 °C for 4 min and 100 mL distilled water was added slowly to the semisolid residue with vigorous agitation to form an emulsion. Five mL of the emulsion was added to a tube containing standards (50 μ g) and juice samples (50 μ L), and the absorbance was measured at 470 nm with UV–vis spectrophotometer, immediately, against a blank consisting of the emulsion without β -carotene. The tube was placed in a water bath at 50 °C and the absorbance measurements were conducted at 120 min. All determinations were carried out in triplicates. The antioxidant activity (AA) of the juices was evaluated in terms of bleaching of β -carotene using the following formula:

$$AA = [1 - (A_0 - A_t)/(A'_0 - A'_t)] \times 100$$

where A_0 and A'_0 are the absorbance at 0 min of the incubation for test sample and control, respectively, and A_t and A'_t are the absorbances measured in the test sample and control, respectively, after incubation for 120 min.

2.12. Antihemolytic assay

Protective effects of standards (BHA, tannic acid and quercetin), juice samples against haemolysis was done as described by Valente et al. [29]. Blood (10 mL) was collected from a healthy human volunteer by venipuncture in a

citrate-coated tube. It was centrifuged immediately at 1500 r/min for 10 min at 4 °C and washed three times with phosphate buffered saline (PBS) (0.02 mol/L, pH 7.4) to get red blood cells (RBCs). It is re-suspended to 2% using the same buffer. To study the protective effects of the extracts/standards against AAPH-induced haemolysis, erythrocyte suspension was pre-incubated with the extracts/standards (50 µg standards and 100 µL juice samples) at 37 °C for 30 min, followed by incubation with and without AAPH (dissolved in PBS; final concentration 50 mmol/L). This reaction mixture was shaken gently during incubation for 4 h at 37 °C. In all experiments, a negative control (erythrocytes in PBS), as well as extract controls (erythrocytes in PBS with extract) were used for comparison. The extent of haemolysis was determined spectrophotometrically at 540 nm by the procedure of Ko et al. [30]. Briefly, the reaction mixture was centrifuged at 4000 r/min for 10 min to separate the erythrocytes at the end of incubation and the data was calculated for antihaemolytic activity.

$$\text{Antihaemolytic activity}/\% = (A_c - A_s)/A_c \times 100$$

where A_c is the absorbance of the control; A_s is the absorbance of juice samples.

2.13. *In vitro* anti-diabetic activity

Juice samples of *C. hystrix* and *C. maxima* (Red and White) fruits were assessed for *in vitro* anti-diabetic activity by the α -amylase and α -glucosidase inhibition.

2.13.1.1. α -Amylase inhibition activity

The juice sample (100 µL) was mixed with 100 µL of 0.02 mol/L sodium phosphate buffer (pH 6.9) and 100 µL α -amylase solution (4.5 units/mL/min) and pre-incubated at 25 °C for 10 min. Then, 100 µL of 1% starch solution was added and incubated at 25 °C for 30 min and the reaction was stopped by the addition of 1.0 mL dinitrosalicylic acid reagent (1 g 3,5-dinitrosalicylic acid in a solution containing 20 mL of 2 mol/L NaOH, 50 mL distilled water and 30 g Rochelle salt. The contents were dissolved and the volume was made up to 100 mL with distilled water). The test tubes were then incubated in a boiling water bath for 5 min and then cooled to room temperature. The reaction mixture was then diluted 10-fold times with distilled water and the absorbance was measured at 540 nm. The readings were compared with the control (the sample was replaced by buffer) and α -amylase inhibition activity (%) was calculated [31].

2.13.1.2. α -Glucosidase inhibition activity

The juice samples (100 µL) were mixed with 100 µL of 0.1 mol/L phosphate buffer (pH 6.9) and 100 µL α -glucosidase solution (1 unit/mL/min) and incubated at 25 °C for 5 min. After the pre-incubation, 100 µL *p*-nitrophenyl- α -*D*-glucopyranoside (5 mmol/L) solution was added and the reaction mixture was incubated at 25 °C for 10 min. After the incubation, the absorbance was recorded at 405 nm and α -glucosidase inhibition (%) was calculated [31].

2.14. Tyrosinase inhibitory activity

Tyrosinase inhibitory activity was determined according to the modified method of Chang et al. [32]. Twenty µL tyrosinase (1000 U/mL in 50 mmol/L phosphate buffer, pH 6.8) was mixed with 100 µL juice samples and 1.9 mL of 50 mmol/L phosphate buffer (pH 6.8). The reaction mixture was incubated at 25 °C for 5 min. Then, 880 µL *L*-DOPA as a substrate in the same buffer was added to start the reaction. The increase in absorbance at 475 nm was monitored with the spectrophotometer. Kojic acid was used as a positive control. The tyrosinase inhibitory activity was calculated as:

$$\text{Tyrosinase inhibitory activity}/\% = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance at 475 nm with DMSO instead of the tested sample and A_1 is the absorbance at 475 nm with the tested sample.

2.15. Determination of anticholinesterase activity

Acetylcholinesterase inhibitory activity was measured using spectrophotometric method of Ellman et al. [33] with slightly modification. 300 µL of 100 mmol/L sodium phosphate buffer (pH 8.0), 10 µL juice sample and 40 µL AChE (5.32×10^{-3} U) solution were mixed and incubated for 15 min at 25 °C, and then 20 µL 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) (0.5 mmol/L) was added. The reaction was then initiated by the addition of acetylthiocholine iodide (0.71 mmol/L). The hydrolysis of this substrate was monitored spectrophotometrically at 412 nm. Percentage of inhibition of AChE enzymes was determined by comparison of reaction rates of samples relative to blank using the formula of $(E - S)/E \times 100$, where E is the activity of enzyme without the test sample and S is the activity of enzyme with the test sample. Eserine was used as a reference compound.

2.16. β -Glucuronidase inhibition assay

The effect of the juice samples on activity of β -glucuronidase was determined according to Kawasaki et al. [34] with slight modification. The reaction mixture contained 1.5 mL of 0.1 mol/L acetate buffer (pH 5.0), 90 µL *p*-nitrophenyl- β -*D*-glucuronide (0.4 mmol/L), 30 units of enzyme, and 50 µL juice sample. After incubation for 30 min at 37 °C the reaction mixture was interrupted by the addition of 200 µL sodium carbonate (0.2 mol/L). The increase of absorbance with the release of *p*-nitrophenol from the *p*-nitrophenyl- β -*D*-glucuronide was measured at 403 nm by means of a spectrophotometer. Saccharic acid 1,4-lactone was used as a standard inhibitory agent for positive control. The β -glucuronidase inhibitory activity was calculated as:

$$\beta\text{-glucuronidase inhibitory activity}/\% = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of the control at 403 nm and A_1 is the absorbance of the juice sample at 403 nm.

Table 1
Total content of phenolics, tannins and flavonoids of fresh juice of *C. hystrix* and *C. maxima* (Red & White).

Samples	Phenolics ^a	Tannins ^a	Flavonoids ^b
<i>C. hystrix</i>	836.90 ± 7.43 ^b	507.61 ± 5.15 ^b	224.88 ± 1.38 ^c
<i>C. maxima</i> (Red)	769.05 ± 10.91 ^c	333.33 ± 8.43 ^c	245.77 ± 2.34 ^b
<i>C. maxima</i> (White)	909.52 ± 8.25 ^a	523.21 ± 12.87 ^a	262.22 ± 2.34 ^a

Values are mean of triplicate determinations ± standard deviations. Mean values followed by different superscript letters in the same column are significantly ($P < 0.05$) different. Similarly hereinafter.

^a mg gallic acid equivalents/L juice.

^b mg rutin equivalents/L juice.

2.17. Statistical analysis

Values are expressed as mean of triplicate determinations ± standard deviation. The data was subjected to one way analysis of variance (ANOVA) and the significant difference between means was determined by Duncan's multiple test ($P < 0.05$) using Statistical Package for the Social Sciences Version 13.0. (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Juice yield, total phenolics and tannins contents

Yield (%) of citrus juice is shown in Table 1. The data showed a considerable variation in juice content from different citrus fruits. *C. hystrix* fruit achieved the highest juice yield (54.15%) among other citrus fruits. The lowest juice yield (19.58%) was registered from *C. maxima* (Red) followed by (18.29%) *C. maxima* (White). Barros et al. [35] investigated the juice yield (%) of different varieties of citrus (35.5%–47.3%) from Brazil and found a lower yield (%) than that of *C. hystrix* and a higher yield than that of the *C. maxima*. Plant phenolics are a major class of bioactive constituents. It possesses the ideal chemistry for free radical scavenging activity due to the presence of high reactivity as hydrogen or electron donors and metal chelating activity. So the regular consumption of phenolics rich fruits and vegetables along with foods will inhibit carcinogenesis and mutagenesis in humans [36]. Total phenol and tannin contents of the fresh juices from *C. hystrix* and *C. maxima*

fruits are expressed in terms of mg GAE per liter, and shown in Table 1. In 1 L juice, 769.05–909.52 mg GAE of phenols and 333.33–523.21 mg GAE of tannins were detected. Among the juices, *C. maxima* (White) had the highest contents of phenolics and tannin, followed by *C. hystrix* and *C. maxima* (Red) juices. Kelebek et al. [37] found that the total content of phenolic compounds was 317.36 mg/L in orange juice. Rekha et al. [38] reported the total content of phenolics in several juices obtained from ripe and unripe fruits of *C. limon*, *C. reticulata*, *C. sinensis* and *C. aurantium* ranged from 532 to 960 µg GAE/mL. According to our results, three juices from citrus fruits contained significant amount of total phenolics that may increase antioxidant intake in human diet.

3.2. Flavonoids

The flavonoids are a prominent group of secondary metabolites in citrus fruits that may possess biological activity and have beneficial effects on human health as antimicrobial, anti-inflammatory, anti-diabetic, anti-cholesterolemic, antioxidant and anti-cancer agents [16,39,40]. Flavonoids are reported to possess strong free radical scavenging activities based on their ability to act as hydrogen or electron donors and chelate transition metals [41]. Total flavonoid contents of juices were expressed as mg rutin equivalents/L. The results, shown in Table 1, varied from 224.88 to 262.22 rutin equivalents/L. Among the juices, *C. maxima* (White) contained the highest amount of flavonoid content, followed by *C. hystrix* and *C. maxima* (Red) juices. These results indicated that a higher flavonoid content was associated with a higher total phenolic content of the juice. Our results showed a higher flavonoid contents than those in the edible portions of eight other varieties of citrus fruits (8.41–21.6 mg RE/g db), juice sac (47–78 mg RE/100 g FW) and segment (84.5–136 mg RE/100 g FW) of citrus fruit of four species but showed lower values than those in segment membrane (278–713 mg RE/100 g FW) of the same species [5].

3.3. DPPH• and ABTS•+ scavenging activity

Free radical scavenging potential of juices from *C. hystrix* and both varieties of *C. maxima* fruit is initially screened using

Table 2
DPPH•, ABTS•+, FRAP and metal chelating activity of fresh juice of *C. hystrix* and *C. maxima* (Red & White).

Samples	DPPH (mmol TE/L) ^a	ABTS (mmol TE/L) ^a	FRAP (mmol Fe(II)/L) ^b	Metal chelating (mg EDTA/L) ^c
<i>C. hystrix</i>	10,903.28 ± 36.5 ^d	33,830.69 ± 1469.01 ^d	30,504.40 ± 148.58 ^d	7.73 ± 0.08 ^d
<i>C. maxima</i> (Red)	11,380.78 ± 43.12 ^d	34,659.62 ± 822.42 ^d	28,339.28 ± 179.63 ^d	7.02 ± 0.04 ^c
<i>C. maxima</i> (White)	11,058.39 ± 27.37 ^d	31,343.90 ± 1712.02 ^d	26,650.01 ± 109.03 ^d	7.98 ± 0.13 ^c
Standards				
BHA	814,172.7 ± 187 ^b	655,137.01 ^b ± 61,415.86	350,760.45 ^b ± 72,476.70	10.49 ^b ± 0.06
RUT	748,175.2 ± 598 ^c	433,569.06 ^c ± 23,178.34	174,032.83 ^c ± 26,869.47	–
TAN	848,540.1 ± 547 ^a	751,735.57 ^a ± 62,890.85	562,955.03 ^a ± 42,130.92	–
α-TOC	–	–	–	12.67 ^a ± 0.26 ^a

BHA – butylated hydroxyl anisole; RUT – rutin; TAN – tannic acid; α-TOC – α-tocopherol.

^a mmol of trolox equivalents/L juice.

^b mmol of ferrous equivalents/L juice.

^c mg of EDTA equivalents/L juice.

nitrogen-centered, organic radicals, DPPH• and ABTS•⁺, since both of them have different absorption spectra and reaction kinetics with antioxidant molecules. DPPH• and ABTS•⁺ scavenging potential of the juices are presented in Table 2. DPPH assay is based on the principle that a hydrogen donor is an antioxidant. It measures the activity of an antioxidant to directly scavenge DPPH• by spectrophotometrically determining its absorbance at 515 nm. The DPPH• is stable organic nitrogen centered free radical with a dark purple color which becomes colorless when it reacts with antioxidants to form non-radicals [42]. The results also expressed as a trolox equivalent. The juices were able to reduce the DPPH• to the yellow-color diphenylpicrylhydrazine (10,903.28–11,380.78 mmol TE/L). Among the juices *C. maxima* (Red) had the highest DPPH• scavenging ability, followed by *C. maxima* (White) and *C. hystrix*. The antioxidant standards BHA and rutin showed tremendously higher scavenging activity than that of the juices. Rekha et al. [38] showed DPPH• scavenging activity of fresh juice of four ripe and unripe citrus fruits. Zvaigzne et al. [43] reported the highest radical scavenging activity of freshly squeezed orange and reconstituted grapefruit juices.

ABTS•⁺, a cation free radical soluble in both water and organic media, is produced by reacting ABTS solution with potassium persulfate. In the absence of antioxidants, ABTS•⁺ is rather stable, but it reacts energetically with an H atom donor and is converted into a non-color form of ABTS. ABTS•⁺ scavenging activity was expressed as mmol trolox equivalents (TE)/L juice. Among the juice samples *C. maxima* (Red) (34,659.62 mmol TE/L juice) exhibited greatest scavenging activity, followed by *C. hystrix* (33,830.69 mmol TE/L juice) and *C. maxima* (White) (31,343.90 mmol TE/L juice). The higher trolox equivalents antioxidant activity value indicated that the mechanism of action of the citrus fruits was as a hydrogen donor and it could terminate the oxidation process by converting free radicals to the stable forms [44]. The DPPH• and ABTS•⁺ scavenging activities of citrus fruits were due to some of phytoconstituents found in the juices that may donate electrons and react with free radicals to terminate radical chain reactions and, therefore, were able to boost the natural antioxidant defense mechanism.

3.4. FRAP assay

FRAP assay is a commonly used method for measuring the antioxidant potential of dietary antioxidants [23] because it is simple, robust and inexpensive. The antioxidant activity of fruit extracts is determined by the ability of the antioxidants in these juices to reduce ferric to ferrous iron in FRAP reagent. The reducing properties are generally associated with the presence of reductones, which have the capacity to donate an electron to free radicals and convert them into more stable forms. Furthermore, reductones can react with certain precursors of peroxide, thus reducing the oxidized intermediates generated from lipid peroxidation reaction [45]. Juices from three citrus species were examined for their antioxidant potential by using FRAP assay (Table 2). The antioxidant potentials were of

varying levels in all the juices (26,650.01–30,504.40 mmol Fe(II)/L juice). The highest antioxidant potential was found in *C. hystrix* juice (30,504.40 mmol/L Fe(II)/g extract), followed by *C. maxima* (Red) (28,339.28 mmol Fe(II)/L juice) and *C. maxima* (White) (26,650.01 mmol Fe(II)/L juice). Toh et al. [46] reported the FRAP value of peel and pulp of red and white variety of pomelo fruit (0.51–1.01 mmol Fe(II)/100 g FW) and showed lower values than those in our reports.

3.5. Metal chelating activity

Iron can stimulate the lipid peroxidation by Haber–Weiss and Fenton reactions, resulting in the generation of hydroxyl radicals. Ferrous ions are also commonly found in food systems and considered as prooxidants. Ferrozine form complexes with the ferrous ion, generating a violet color [47]. The formation of this color can be interrupted by metal chelating agents. The chelating activity was measured against ferrous ions and reported as EDTA equivalents. The chelating activity of juices from three different citrus fruits were 7.02–7.73 mg EDTA/g extract (Table 2). Iron chelating ability may be involved in antioxidant activity and affect other functions that contribute to antioxidant activity [48]. So the chelating ability of the fruit juices on ferrous ion may influence other scavenging activities of free radicals which protect the organisms against oxidative damage.

3.6. Superoxide anion radical scavenging activity

Superoxide radical is the most common free radical generated *in vivo*. It plays an important role in the formation of more reactive oxygen species such as hydroxyl radical and hydrogen peroxide, which result in oxidative damage in biomolecules, such as DNA, lipids and proteins. In this assay all the juices exhibited superoxide anion scavenging activity and the results are presented in Table 3. All the juices showed lower activity (18.70%–20.50%) than that of the standards (49.87%–76.72%). The superoxide radical scavenging activities of the fruits were in the following order: *C. maxima* (Red) > *C. hystrix* > *C. maxima* (White). The superoxide anion radical scavenging activity of the citrus fruits may be due to the presence of phenolic compounds [49]. Our results further support the view that strong scavenging properties of citrus fruits extracts on superoxide anion possibly render them as promising antioxidants. The results suggested that all the three types of citrus fruits are capable for scavenging free radicals, so it is able to prevent the initiation of free radical-mediated chain reactions by stabilizing reactive species before they can participate in deleterious reactions.

3.7. Hydroxyl radical scavenging activity

Hydroxyl radicals are high reactive-oxygen species capable to attack most biological substrates, e.g. carbohydrates, DNA, polyunsaturated fatty acids, and proteins. The prevention of such deleterious reactions is highly significant in terms of both human health and the shelf-life of foodstuffs, cosmetics and pharmaceuticals. The hydroxyl radical scavenging activity of juice from *C.*

Table 3
Superoxide and hydroxyl radical scavenging activity of fresh juice of *C. hystrix* and *C. maxima* (Red & White).

Samples	SRSA	HRSA	β -Carotene bleaching inhibition	Antihemolytic activity %
<i>C. hystrix</i>	19.89 \pm 0.27 ^a	42.91 \pm 0.90 ^b	62.18 \pm 1.87 ^a	92.69 \pm 0.73 ^a
<i>C. maxima</i> (Red)	20.50 \pm 0.66 ^a	46.43 \pm 1.19 ^a	59.24 \pm 1.30 ^b	93.60 \pm 1.09 ^a
<i>C. maxima</i> (White)	18.70 \pm 0.33 ^b	48.09 \pm 0.51 ^a	56.99 \pm 0.90 ^b	92.03 \pm 0.96 ^a
Standards				
BHA	69.29 \pm 2.71	–	67.70 \pm 6.07	91.06 \pm 2.41
Rutin	76.72 \pm 2.82	–	37.48 \pm 9.29	–
Trolox	49.87 \pm 1.31	–	49.57 \pm 1.96	96.07 \pm 0.77
Catechin	–	82.46 \pm 2.29	–	–

SRSA- superoxide radical scavenging activity; HRSA – hydroxyl radical scavenging activity; BHA – butylated hydroxyl anisole.

hystrix (42.91%), *C. maxima* (Red) (46.43%) and *C. maxima* (White) (48.09%) are shown in Table 3. The hydroxyl radical scavenging activities of the fruits were in the following order: *C. maxima* (White) > *C. maxima* (Red) > *C. hystrix*. The results suggested that the extracts display a scavenging effect on hydroxyl radicals that could help to protect proteins, DNA, polyunsaturated fatty acids in membranes, and almost any biological molecules from free radical attacking. The potential scavenging ability of flavonoid compounds might be due to the active hydrogen-donating ability of extracts [50]. Therefore, we concluded that the activities revealed in this study may be due to the presence of flavonoids as well as other polyphenolic compounds in the fruit extracts.

3.8. β -Carotene linoleic acid emulsion assay

In the β -carotene linoleate system, β -carotene undergoes rapid discoloration in the absence of antioxidants. This test measures the potential of samples for inhibiting conjugated diene hydroperoxides formation via linoleic acid oxidation. The inhibition effect of fruit juices is presented Table 3. From this study, oxidation of β -carotene was effectively inhibited by all the juices which showed the moderate inhibition (56.99%–62.18%). However, all the juices showed lower antioxidant activity compared with the reference compound, BHA, rutin and trolox. The results indicated that the presence of phenolic compounds in fruit juices largely prevented the bleaching of β -carotene which indicates a good capacity for reduction of the radicals generated by the oxidation of linoleic acid. Karoui and Marzouk [51] and Tounsi et al. [52] reported the inhibiting of β -carotene bleaching by the juice sample of bitter orange (15.92%, 18.27%), lemon (22.67%) and found lower values than our results.

3.9. Antihemolytic activity

Erythrocytes are one of the most abundant cells in the human body with potential biological roles. Erythrocytes are considered as major targets of the free radicals owing to the presence of both high concentration of membrane polyunsaturated fatty acids and the oxygen transport by redox active hemoglobin molecules, which are potent promoters of activated oxygen species [53]. So the supply of external antioxidants (via food) is necessary apart from the presence of endogenous antioxidant compounds. Hence these fruits were evaluated for their protection capacity

on RBCs against oxidative damage by AAPH, a peroxy radical generator that attacks the erythrocytes to induce the chain oxidation of lipids and proteins, disturbs the membrane organization and eventually leads to hemolysis. And the results are shown in Table 3. Among the various juices, *C. maxima* (Red) (93.60%) exhibited the highest hemolytic inhibition activity followed by *C. hystrix* (92.69%) and *C. maxima* (White) (92.03%). It is noteworthy that the juice samples showed a higher activity than the positive standard BHA but a lower activity than trolox. Therefore, phenolic compounds of citrus fruit extracts can quench the peroxy radicals formed in the aqueous phase before these radicals attach the biomolecules of the erythrocyte membrane to cause oxidative damage.

3.10. α -Amylase and α -glucosidase potential inhibition activity

Diabetes is characterized by high concentrations of blood sugar which can cause serious complications in the kidneys, eyes and cardiovascular system. The treatment of diabetes therefore mainly focuses on reducing fluctuations in blood sugar and subsequent complications. The α -amylase and α -glucosidase inhibitors are currently used for diabetic treatment as oral hypoglycemic agents. Acarbose is a commercially available enzyme inhibitor for type II diabetes. However, it is reported to cause various side effects such as abdominal distention, flatulence and possibly diarrhea. Searching for safe and effective inhibitors from natural sources are of emerging interest. The inhibitory effects of the juices and acarbose on α -amylase and α -glucosidase are shown in Fig. 1. The potential inhibition of all juices against α -amylase ranged from 75.55% to 79.75%. However, all the extracts showed lower inhibitory activities compared with that of acarbose (82.29%). α -Amylase inhibiting activities of the fruits were in the following order: *C. maxima* (Red) > *C. maxima* (White) > *C. hystrix*.

High levels of α -glucosidase inhibition (70.68%–72.83%) activity observed in all juices of the citrus fruits is comparable to that in acarbose (85.19%). The α -glucosidase inhibition activities of *C. hystrix*, *C. maxima* (Red) and *C. maxima* (White) were 70.68%, 72.83%, and 71.88%, respectively. Therefore, we suggest that inhibition activities against α -amylase and α -glucosidase could be part of the possible mechanisms of *C. hystrix*, *C. maxima* (Red & White) varieties in therapeutic/dietary management of diabetes, by retardation of starch

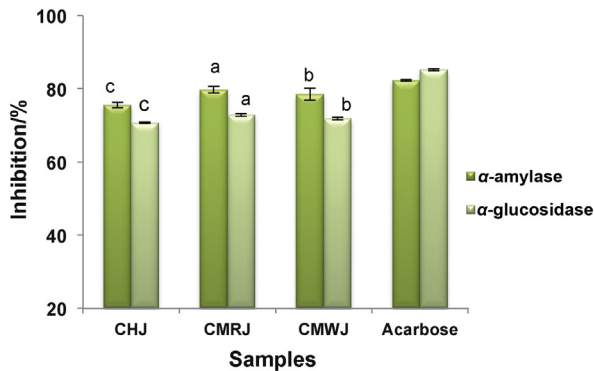


Fig. 1. Inhibitory activity against α -amylase, α -glucosidase, of fresh juice of *C. hystrix* and *C. maxima* (Red & White). CHJ – *C. hystrix* Juice; CMRJ – *C. maxima* Red Juice; CMWJ – *C. maxima* White Juice. Similarly hereinafter.

hydrolysis in the gastrointestinal tract. α -Amylase and α -glucosidase inhibitory potential of citrus fruits like acarbose would delay the degradation of starch and oligosaccharides, which would in turn cause a decrease in the absorption of glucose and consequently inhibit the increase in postprandial blood glucose [54]. This dual inhibitory potentials against the target enzymes might be due to the presence of specific phenolics and flavonoids.

3.11. Acetylcholinesterase inhibitory activity

Acetylcholine breakdown in the brain can be prevented by the inhibition of acetyl cholinesterase activity, which subsequently increases the concentration of acetylcholine. This further result in increased communication between nerve cells that use acetylcholine as a chemical messenger producing a therapeutic effect in patients with Alzheimer's disease, glaucoma, myasthenia gravis, and for the recovery of neuromuscular block in surgery [1]. The interaction of the fruit juices with acetylcholinesterase is shown in Fig. 2. All the juices possess strong anti-cholinesterase activity ranged between 75.71% and 79.74%. Among the juices *C. hystrix* possess the highest activity, followed by *C. maxima* (Red) and (White). All the juices showed lower potent than that of the eserine (86.89%) reference compound. The report by Tundis et al. [55] revealed the inhibition of cholinesterase by essential oil of peel of *C. aurantifolia* and *C. aurantium*. Essential oil from leaf and peel of *C. aurantifolia* is reported to possess

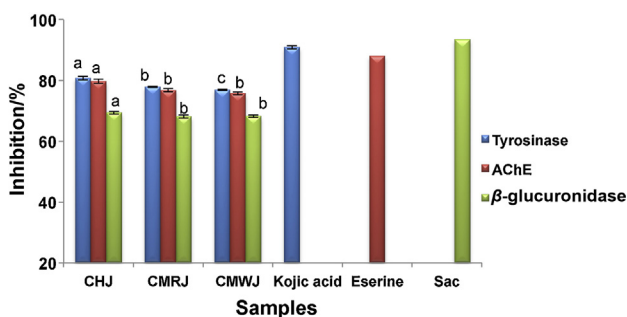


Fig. 2. Inhibitory activity against tyrosinase, acetylcholinesterase, and β -glucuronidase of fresh juice of *C. hystrix* and *C. maxima* (Red & White). AChE – acetylcholinesterase; Sac – saccharic acid 1,4-lactone.

anti-cholinesterase activity due to the presence of active compounds, such as limonene, l-camphor, citronellol, *o*-cymene and 1,8-cineole [56].

3.12. Tyrosinase inhibitory activity

Tyrosinase is a polyphenol oxidase with a dinuclear copper active site and is involved in the formation of mammalian melanin pigments, enzymatic browning of fruits and vegetables. Over-activity of this enzyme leads to hyperpigmentation of the skin. Chemical agents that demonstrated tyrosinase inhibitory activity have been used to suppress melanogenesis and can be clinically useful for the treatment of some dermatological disorders associated with melanin hyperpigmentation. Kojic acid is one of the popular chemicals used in whitening cosmetics but chronic, cytotoxic and mutagenic effects have been proved [57]. To avoid this problem, more attention has been paid to the use of plant-based natural products instead of chemical agents as anti-tyrosinase substances. The tyrosinase inhibition activity of citrus juices are shown in Fig. 2. All the juices revealed significant tyrosinase inhibitory activity ranged between 76.95% and 80.79%. Citrus juices showed lower activity than the reference compound kojic acid (90.87%). Tyrosinase inhibition activity of fruits were in the order of *C. hystrix* > *C. maxima* (Red) > *C. maxima* (White). Previously, tyrosinase inhibitory potential of flavonoids such as naringin, hesperidin and nobiletin from citrus fruits were reported [58–60]. Juices from *C. hystrix* and *C. maxima* (Red & White) fruits can be used as a preventive agent against enzymatic oxidation in food and living systems, especially in the treatment of hyperpigmentation associated with the high production of melanocytes in human cells.

3.13. β -Glucuronidase inhibitory activity

β -Glucuronidase, produced by intestinal bacteria, hydrolyses glucuronide to liberate xenobiotics in the body. The liberated xenobiotics exhibits toxicity in the intestine and decreases excretion rate of xenobiotics via re-absorption. Inhibition of bacterial β -glucuronidase in the intestine will promote excretion of xenobiotics and thus decrease their toxicity. β -Glucuronidase inhibitors decreases the toxicity of xenobiotics [61]. Fresh juices from *C. hystrix* and *C. maxima* (Red & White varieties) are examined for β -glucuronidase inhibition activity using saccharic acid 1,4-lactone as a standard inhibitor for positive control and the results are shown in Fig. 2. All the juices revealed significant β -glucuronidase inhibitory activity ranged between 68.13% and 69.38%. It can be expected that all the juices can reduce the risk factor of liver cancer by inhibiting the hydrolysis to glucuronides of proximate metabolites. Our results showed that citrus fruits can act as natural β -glucuronidase enzyme inhibitors.

4. Conclusions

In the present study, juice samples from indigenous fruits of *C. hystrix*, *C. maxima* (Red) and (White) demonstrated that they not only possesses antioxidant and radical scavenging activities but also exhibits excellent inhibitory potential against

α -amylase, α -glucosidase, acetylcholinesterase, tyrosinase and β -glucuronidase *in vitro*. Consequently, citrus juices that are rich in phenolics and flavonoids are suitable and promising for the development of safe food products, natural additives and cosmetics. Hence, it may be concluded that the antioxidant and inhibitory potentials against α -amylase, α -glucosidase, acetylcholinesterase, tyrosinase and β -glucuronidase action of citrus fruit juices can be used for the future therapeutic medicine due to the presence of potential nutraceuticals. Further studies will be conducted on identification of bioactive constituents, molecular mechanisms involved in antioxidant activity, determination of their efficacy by *in vivo* studies and demonstration of their safety and effectiveness in clinical trials.

Conflict of interest

The authors declare that there are no conflict of interest.

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