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Antioxidant activity, type II diabetes related enzymes, cholinesterase and tyrosinase inhibition properties of methanolic extracts of peel, pulp and peel fiber from underutilized fruits of *Citrus hystrix* and *C. maxima*

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Abstract
In the present study, methanolic extract of peel, pulp and peel fiber from underutilized fruits of *Citrus hystrix* and *C. maxima* (red and white variety) were examined phenolic content and antioxidant activity systems. The extracts were also analyzed for α-amylase, α-glucosidase, cholinesterase and tyrosinase inhibition using properties. The major bioactive compounds responsible for all these bioactivities were documented as gallic acid, *p*-coumaric acid, chlorogenic acid, catechin, rutin, caffeic acid, trans cinnamic acid, ferulic acid, quercetin and ellagic acidthrough HPLC analysis. Higher inhibition of 79 and 71% against α-amylase and α-glucosidase respectively was registered by peel and peel fiber extract of both the *C. maxima* varieties.On the other hand, peel and peel fiber extract of *C. hystrix* showed higher inhibition rates of 64% towards both acetylcholinesterase and tyrosinase. The findings of this study explores the potential of underutilized fruits *C. hystrix* and *C. maxima* use of peel, pulp and peel fiber in supplementing human health.

Keywords: *Citrus hystrix*, C. *maxima*, Antioxidant, diabetes, enzymatic inhibitory activity, bioactive components

Introduction

Diet intake changes has attributed for the contribution of nearly 90% diabetes, 80% cardiovascular diseases, 30% cancer and many other lifestyle diseases (WHO/FAO, 2002). Oxidative stress which affects all the biomolecules in the body is found to be the major driving force behind the scenario. Antioxidant which hinders the oxidation and its consequent reactions is the only viable solution to prevent these lifestyle diseases. This can be achieved through intake of diversified diet especially fruits and vegetable which offer a good source of phenolics, the major antioxidant molecules and free radical scavengers. This also acts as an inhibitor of various enzymes involved in the disease progression such as α-amylase, α-glucosidase for hyperglycemia, acetylcholinesterase in Alzheimer's disease and tyrosinase inhibitors in Parkinson disease.

C. hystrix DC (Kaffir lime) and *C. maxima* L (giant citrus/Pummelo) are originates in South East Asia, India and cultivated throughout the tropical and temperate regions. *C. hystrix* is pear-shaped, bumpy, greenish yellow fruit with acidic flavor with very thorny bush and aromatic leaves. The leaves are used for flavouring in Southeast Asian and Thai dishes. It is used in traditional medicine for headache, flu, fever, sore throats, bad breath and indigestion (Hutadilok-Towatana *et al*., 2006) [14]. Many active compounds were isolated from various parts of this plant such as glyceroglycolipids, α-tocopherol, limonoids, furanocoumarins, flavonoids, benzenoid derivative and quinolinone alkaloid (Panthong *et al*., 2013) [30]. Pharmacological activities such as anti-tumor, antimicrobial, anti-inflammation and antioxidant activities (Loh *et al*, 2011) [21] were also reported. *C. maxima* possess a unique feature that, it bears the largest fruit among the citrus genus with 11-14 segments. The pulp appears either as white or pinkish red with spindle-shaped juice sacks that separate easily from one another and sweetish-acidic flavor. It is used in traditional medicine as cardiac stimulant and stomach tonic (Arias and Laca, 2005) [1]. Pummelo composed of water (90.3%), mineral (0.3%), protein (0.5%), fat (0.3%), carbohydrate (8.5%), calcium (37mg), iron (0.2mg), carotene (120µg), vitamin-B1 (0.06mg), vitamin-B2 (0.04mg), vitamin C (105mg) and calories (38Kcal) (Islam, 2007) [16]. These fruits were also recognized for its vitamin C, carotenoids, lycopene, polyphenols, limonoids, pectin and fiber and hence antioxidant activity and antimicrobial activity (Pichaiyongvongdee and Haruenkit, 2009; Breksa *et al*., 2010) [32, 3]. Fruit, leaves and juice of pummelo reported to contain flavanone glycoside naringin, narirutin, hesperidin and neohesperidin (Xu *et al*., 2008) [45]. Fruit extract and juice is used for the treatment of diabetes as it has been shown to significantly lower the blood glucose and cholesterol level (Oyedepo, 2012) [29].

There can be no doubt that nutritional value and pharmacological properties of different parts of indigenous fruits *C. hystrix* and *C. maxima*, but there has been very little information on antioxidant and enzyme inhibition properties of peel, pulp and peel fiber materials. Hence, the present study attempts to investigate the total phenolic content, antioxidant, cholinesterase, tyrosinase, and type II diabetic related enzyme inhibition properties for methanolic extract of peel, pulp and peel fiber of *C. hystrix* and *C. maxima* (Red & White var) fruits.

2. Materials and Methods

2.1 Sample preparation

The fruits of *C. hystrix* & and *C. maxima* (Red and White) were collected from Mayiladuthurai, Nagai district, Tamil Nadu. The fresh fruit samples were separated into peel and pulp. The peel was divided into two parts. One part of the peel and pulp samples was dried at room temperature and another part of the peel was subjected to treatment for preparing the peel fiber (Larrauri, 1999) [20]. The peels were washed in hot water (90° C for 5 min, peel: water 1:2). Afterwards the residue was pressed to reduce excess moisture and dried at 65° C for 24 hour. The dried samples were ground into fine powder using electric blender and stored in a separate container at 4 °C until further analysis.

2.2 Sample extraction

The peel, pulp and peel fiber of *C. hystrix* and *C. maxima* were subjected to extraction. The samples were extracted with 80% methanol (1:5 w/v) for 48 h at room temperature. The extract were filtered, air dried and stored at 4 °C for further analysis.

2.3 Determination of total phenolics and tannin contents

The total phenolics and tannins were measured as gallic acid equivalents (Makkar *et al*., 2007) [24]. One milliliter of the sample extract was transferred to a test tube and 0.5 ml of Folin-Ciocalteu reagent and 2.5 ml of sodium carbonate solution (20% w/v) were added. After an incubation period of 40 min in dark, the absorbance was recorded at 725 nm with UV-visible spectrophotometer (Cyberlab-UV100, USA) against the reagent blank. Using the same extracts and method, the tannins were estimated after treatment with polyvinylpolypyrrolidone (PVPP). One hundred mg of PVPP in 1.0 mL of distilled water is mixed with 0.1 mL of sample extracts. The content was vortexed and kept at 4 °C for 4 h. Then the sample was centrifuged (3000 × g for 10 min at room temperature) and the supernatant was collected. This supernatant has only simple phenolics other than tannins (the tannins would have been precipitated along with the PVPP). The phenol content of the supernatant was measured, as mentioned above and expressed as the content of non-tannin phenolics. From the above results, the tannin content of the sample was calculated as follows:

Tannin (%) = Total phenolics (%) – Non-tannin phenolics (%)

2.4 Estimation of total flavonoids

Total flavonoid content was measured according to the method of Zhishen *et al*. (1999) [46], described by Sasipriya and Siddhuraju (2012) [36]. One mL of sample extract was added to 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 M 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–visible spectrophotometer. Total flavonoids was determined using a rutin (20–100 µg) standard curve and expressed as mg rutin equivalents/g extract.

2.4.1 Extraction and hydrolysis

The phenolic acid and flavonoids in investigated samples were extracted using the procedure described by Siddhuraju and Becker (2003) [38]. The methanolic extracts of the peel, pulp and peel fiber samples (10 mg) were dispersed in 2 ml of 62.5% aqueous methanol containing 200 mg /L of 2(3)-*tert*-butyl-4-hydroxy-anisoloe (BHA). The mixture was ultrasonicated for 5 min and 1 ml of 6M HCL was added. The extraction solution thus obtained consisted of 1.2 M HCl in 50% aqueous methanol (v/v). The sample was bubbled with nitrogen for 40-60 s and hydrolysis was carried out in a shaking water bath at 90 °C for 2 h. After hydrolysis, the extract was allowed to cool, filtered, and made up to 5 mL with methanol. The extract was sonicated for 5 min and filtered through a 0.45 µm membrane filter prior to injection in HPLC.

2.4.2 Identification and quantification of phenolics

Chromatographic separation of phenolics was done using Shimadzu LC-6AD pumps, SPD-20A prominence UV/VIS detector and a LUNA C_{18} (4.6×250 mm i.d., 5 µm) column. Gradient elution was employed for phenolic acid and flavonoids separation. A mobile phase consisting of 3% aqueous acetic acid (Solution A), and a mixture of 3% acetic acid, 25% acetonitrile and 72% water (Solution B) was used. Gradient elution, using a flow rate of 1ml/min, was conducted as follows: 0-40 min, 100% A to 30% A; 40-55 min, 30% A, 70% B to 10% A, 90% B (Kubola and Siriamornpun, 2008) [18]. Operating conditions were as follows: column temperature, 40 °C; injection volume, 20µl and UV-diode array detection at 280nm.

2.5 Free radical scavenging activity on 2, 2-diphenyl-1-picrylhydrazyl (DPPH$^\cdot$)

The antioxidant activity of extracts and standards (BHA, rutin and tannic acid) was measured in terms of electron transferring ability using a stable, commercially available DPPH radical by the method of Brand-Williams *et al*. (1995) [2] outlined by Sasipriya and Siddhuraju (2012) [36]. Sample extracts prepared in methanol (0.1 mL) were mixed with 3.9 mL of methanol containing DPPH$^\cdot$ (0.025 g/L) and incubated in dark for 30 min. The absorbance was measured at 515 nm.The concentration of DPPH was calculated from trolox standard curve (0-2.5 mM) and expressed as mmol trolox equivalents/g extract.

2.6 Antioxidant activity by the ABTS$^{\cdot+}$ assay

The ABTS$^{\cdot+}$ radical cation decolorization assay was performed by the method of Re *et al*. (1999) with slight modification made by Sasipriya and Siddhuraju (2012) [36]. ABTS radical cation (ABTS$^{\cdot+}$) was generated by adding 2.45 mM potassium persulfate to 7 mM ABTS and incubated in dark at room temperature for 12–16 h. This stock solution of ABTS$^{\cdot+}$ was diluted with ethanol to give an absorbance of 0.70 (± 0.02) at 734 nm, which act as a positive control. Ten microliters of crude extract was mixed with 1.0 mL of diluted ABTS$^{\cdot+}$ solution and incubated at30 °C for 30 min. The absorbance value was measured at 734 nm with UV–visible spectrophotometer. Trolox standard was also prepared (0–1.5 mM) to get the concentration response curve. The unit of trolox equivalent antioxidant activity (TEA) was defined as the concentration of Trolox having the equivalent antioxidant

activity expressed as mmol/ mg of extracts. The TEA of BHA, rutin and tannic acid was also measured 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*. (2000) [33]. An aliquot of 30 µL sample was mixed with 90 µL of water and 900 µL of FRAP reagent (2.5 mL of 20 mmol/L of TPTZ in 40 mM 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 values were recorded at 593 nm. Ferrous sulphate (400 - 2000 µmol) was used for calibration curve. The antioxidant activity was expressed as amount of extract required to reduce 1 mmol of ferrous ions. The antioxidant activity of samples was compared with the following standards: BHA, rutin and tannic acid.

2.8 Metal chelating activity
Iron chelating activity of samples, standards (BHA and α-tocopherol) was estimated by the method of Dinis *et al*. (1994) [6]. An aliquot of 0.1 mL sample, 0.6 mL of distilled water and 0.1 mL of ferrous chloride (2 mmol/L) were well mixed and incubated for 30 s. Then, 0.2 mL of 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. EDTA (0-2 µg) was used as standard for the preparation of calibration curve. Metal chelating ability of antioxidant was expressed as mg EDTA/g extract.

2.9 In vitro antidiabetic activity
Peel, pulp and peel fiber extract of *C. hystrix* and *C. maxima* (Red and White) fruits were assessed for *in vitro* antidiabetic activity by the α-amylase and α-glucosidase inhibition activity. *2.11.1 α-amylase inhibition activity*
The sample extracts (250 µg) were mixed with 100 µL of 0.02 M sodium phosphate buffer (pH 6.9) and 100 µL of 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 of dinitrosalicylic acid reagent (1 g 3,5-dinitrosalicylic acid in 20 mL of 2 N NaOH + 50 mL distilled water + 30 g Rochelle salt. The contents were dissolved and 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 (Worthington, 1993) [43]. α-amylase inhibition activity (%) was calculated

$$\% \text{ inhibition} = (Abs_{ref} - Abs_{sam})/Abs_{ref} \times 100\%$$

Where Abs_{ref} – Abs_{sam} were defined as absorbance of the control and the sample, respectively.

2.9.1 α-glucosidase inhibition activity
The sample extracts (250 µg) were mixed with 100 µL of 0.1 M 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 of *p*-nitrophenyl-α-D-glucopyranoside (5 mM) 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 (Worthington, 1993) [43].

$$\% \text{ inhibition} = (Abs_{ref} - Abs_{sam})/Abs_{ref} \times 100\%$$

Where Abs_{ref} – Abs_{sam} were defined as absorbance of the control and the sample, respectively.

2.10 Tyrosinase inhibitory activity
Tyrosinase inhibitory activity was determined according to the modified method of Chang *et al*. (2007) [4]. A 20 µl volume of tyrosinase solution (1000 U/ml in 50 mM phosphate buffer, pH 6.8) was mixed with 100 µl of the extract (dissolved in DMSO) and 1.9 ml of 50 mM phosphate buffer (pH 6.8). The reaction mixture was incubated at 25 °C for 5 min. Then, 880 µl of L-3,4-dihydroxyphenylalanine (L-DOPA) was added. The increase in absorbance at 475 nm was measured. Kojic acid was used as a positive control. The tyrosinase inhibitory activity was calculated as
Tyrosinase inhibitory activity (%) = [(A0 - A1)/A0] × 100
Where, A0 - absorbance of control (DMSO) and A1 - absorbance of sample.

2.11 Determination of anticholinesterase activity
Acetylcholinesterase inhibitory activity was measured according to Ellman *et al*. (1961) [8] with slight modification. 300 µl of 100 mM sodium phosphate buffer (pH 8.0), 10 µl of sample solution and 40 µl of acetylcholinesterase (AChE) (5.32×10^{-3}U) solution were mixed and incubated for 15 min at 25° C, and then 20 µl of 5,5′-dithio-bis(2-nitrobenzoic) acid (DTNB) (0.5 mM) was added. The reaction was then initiated by the addition of acetylthiocholine iodide (0.71mM). The hydrolysis of this substrate was monitored at 412 nm. Percentage of inhibition of AChE was determined using the formula: (E-S)/E × 100, where E is the activity of enzyme without test sample, and S is the activity of enzyme with test sample. Eserine was used as a reference compound.

2.12 Statistical analysis
Values are expressed as mean of triplicate determinations ± standard deviation. The data were subjected to one way analysis of variance and the significant difference between means was determined by Duncan's multiple range 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 Total phenolics content (TPC) and tannins
TPC of pulp, peel and peel fiber of *C. hystrix* and *C. maxima* is shown in Table 1. TPC was found to be in the order of peel (60-116 mg GAE/g) > peel fiber (66-80 mg GAE/g) > pulp (39-53 mg GAE/g). Similar trend was observed for tannins on *C. hystrix* alone; whereas both the *C. maxima* varieties registered in the order of peel fiber > peel > pulp. TPC and tannin content vary considerably with respect to different parts of the fruit and also the citrus variety. Generally, extracts that contain a higher polyphenols also exhibit high antioxidant

activity. *C. hystrix* peel possesses higher phenolic compounds compared to pulp and peel fiber. The higher amount of phenolic compounds accumulates in the outer parts of the fruits and vegetables than the inner portions. Both varieties of *C. maxima* peel fiber possess higher TPC than peel and pulp. The major constituent of plant cell wall, dietary fiber is regarded as abundant sources of phenolic acids. Overall, the total polyphenols content in the peels was significantly higher than in the edible parts of fruits. Ghasemi *et al*. (2009) [12] observed higher values for TPC of peel and pulp extract of 13 citrus varieties (104.2-223.2 mg GAE/g extract) than the present study. On the other hand TPC of peel fiber in the present study is found to be higher than the previously reported citrus byproduct fibers (Fernandez-Lopez *et al*., 2009) [11]; but lower than orange fiber (Crizel *et al*., 2013). These variations might be due to the variation in the citrus variety, environmental factors of growing site, solvent used, temperature and extraction process (Martinez *et al*., 2012) [25].

3.2 Flavonoids

Flavonoid content of peel, pulp and peel fiber of *C. hystrix* (120.44-178.01 mg RE/g extract), *C. maxima* (Red) (123.66-169.21 mg RE/g extract) and *C. maxima* (White) (128.01-146.33 mg RE/g extract) were estimated and results are presented in Table 1. All the peel extracts exhibited higher flavonoid content than the pulp and peel fiber. Similar flavonoid content was noticed by Rehman (2006) [35] on kaffir lime peel and pummel peel. Siahpoosh and Javedani (2012) [37] and Wang *et al*. (2011) [21] also reported total flavonoid content of peel extract of *C. deliciosa* (4.55 mg RE/g extract) and peel and pulp extract of *C. sulcata* (22.67-60.92 mg RE/100 g extract). Citrus fruits rich in flavonoids are of great importance in the maintenance of health due to their key role in antiallergic, anticancer, anti-inflammatory activities, lowering blood pressure, plasma lipids neuroprotective activities and antioxidant activity (Hwang *et al*., 2012) [15].

Table 1: Total phenolics, tannins and flavonoids of methanolic extracts of C. hystrix and C. maxima (Red &White) peel, pulp and peel fiber.

Samples	Phenolics[a]	Tannins[a]	Flavonoids[b]
C. hystrix			
Peel	116.43[a]±0.71	78.47[a]±1.21	178.01[a]±2.13
Pulp	49.05[f]±1.09	30.01[f]±1.22	120.44[h]±1.31
Peel fiber	80.00[b]±1.43	50.47[bc]±1.90	175.55[a]± 150
C. maxima (Red)			
Peel	60.24[d]±2.94	34.28[e]±1.98	169.21[b]±1.70
Pulp	39.52[e]±2.89	20.90[g]±4.00	123.66[g]±1.52
Peel fiber	77.38[b]±1.09	53.52[b]±0.91	157.66[c] ±2.08
C. maxima (White)			
Peel	62.24[d]±2.64	38.52[d]±1.93	146.33[d]±1.02
Pulp	53.09[g]±2.29	28.42[f]±3.19	128.01[f]±2.21
Peel fiber	66. 19[c]±1.80	47.09[c]±2.12	140.44[e]±1.88

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.

[a] mg gallic acid equivalents/g extract.

[b] mg rutin equivalents/ g extract.

3.3 Identification of phenolic compounds in Citrus peel, pulp and peel fiber extracts

The phenolic compounds identified in methanolic extracts of peel, pulp and peel fiber from indigenous fruits of *C. hystrix* and *C. maxima* (Red and White) are shown in Fig 1 and Table 2. Gallic, *p*-coumaric, chlorogenic, caffeic, trans cinnamic, ferulic acid and quercetin were identified in the peel extract of *C. hystrix* (Fig 1A and Table 2). *P*-coumaric, rutin, caffeic, ferulic acid and ellagic acid were found in *C. maxima* (Red) peel extracts (Fig 1B and Table 2). *P*-coumaric, rutin, caffeic and trans cinnamic acid were identified in *C. maxima* (White) peel (Fig 1C and Table 2).

Table 2: Quantitative determination of phenolic acids and flavonoid of methanolic extracts of *C. hystrix* and *C. maxima* (Red & White) peel, pulp and peel fiber.

S. No	Compounds (mg/100g)	*C. hystrix*			*C. maxima* (Red)			*C. maxima* (White)		
		Peel	Pulp	Peel fiber	Peel	Pulp	Peel fiber	Peel	Pulp	Peel fiber
1	Gallic acid	16.12±0.2	ND	6.17±0.3	ND	ND	ND	ND	5.66±0.3	ND
2	*p*-coumaric acid	83.87±0.4	387.07±1.0	4.28±0.7	260.86±1.0	30.19±0.6	8.16±0.4	351.51±0.5	76.12±0.6	7.02±0.3
3	Chlorogenic acid	56.11±0.7	ND	1.69±0.2	ND	3.66±0.2	12.63±0.9	ND	23.24±0.1	ND
4	Catechin	ND	ND	3.94±0.2	ND	0.63±0.4	ND	ND	12.56±0.3	ND
5	Rutin	ND	ND	ND	2.92±0.1	ND	ND	1.92±0.3	ND	59.90±0.6
6	Caffeic acid	3.32±0.6	ND	4.45±0.4	4.53±0.5	3.75±0.6	ND	3.08±0.4	5.58±0.3	ND
7	Trans cinnamic acid	11.91±0.2	ND	ND	ND	5.65±0.2	ND	6.35±1.0	0.69±0.7	ND
8	Ferulic acid	258.34±0.4	8.37±0.3	5.29±0.1	61.54±0.4	ND	17.03±0.1	ND	1.36±0.2	53.51±1.0
9	Quercetin	432.99±0.3	ND	5.19±0.2	ND	ND	2.81±0.8	ND	ND	37.23±0.7
10	Ellagic acid	ND	26.70±1.0	ND	54.24± 0.1	ND	ND	ND	3.131±0.3	ND

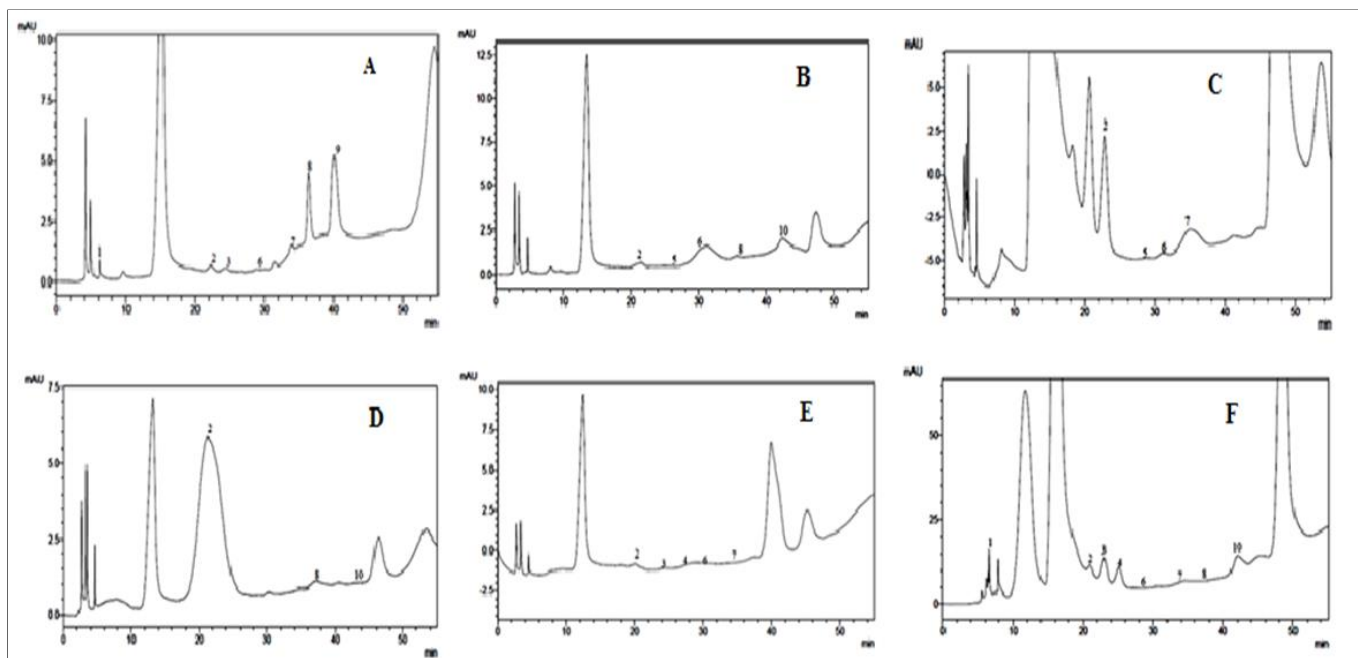
Values are mean of triplicate determinations ± standard deviations. ND- not detected.

In *C. hystrix* peel, the concentration of flavonoids was higher than the phenolic acids. The main flavonoid found in kaffir lime was quercetin (433 mg/100 mL) and the remaining flavonoids were not found in peel. Ferulic acid was the dominant phenolic acid followed by *p*-coumaric, chlorogenic, gallic and trans cinnamic acid, whereas caffeic acid was found at lower concentrations. On the other hand, *C. maxima* (Red and White) peel was characterized by higher concentration of phenolic acids in comparison to flavonoids. *P*-coumaric acid was the dominant phenolic acid, followed by ferulic and ellagic acid in *C. maxima* (Red) peel. Gallic, chlorogenic, trans cinnamic acid were not identified and caffeic acid found in low concentration in the peel of *C. maxima* (Red). The only flavonoid found in *C. maxima* (Red) peel was rutin. The dominant phenolic acid in *C. maxima* (White) peel was *p*-coumaric acid and its concentration was highest in comparison

to red fruit. The concentrations of trans-cinnamic and caffeic acid were low. Similarly, rutin was the only flavonoid found in *C. maxima* (White) peel and its concentrations was lower than in *C. maxima* (Red) peel. Overall, phenolic acids of *C. maxima* (Red and White) peel contained the highest concentration of *p*-coumaric acid than the *C. hystrix* peel. However, *C. hystrix* peel contained highest concentration of flavonoids like quercetin than the other peel. The mentioned values of phenolic acids such as gallic, chlorogenic, trans-cinnamic, *p*-coumaric, ferulic and flavonoids such as catechin and rutin of our reports exhibited higher values than the peel of bitter orange (Karoui and Marzouk, 2013) [17].

P-coumaric, ferulic and ellagic acid were identified in the pulp extract of *C. hystrix* (Fig 1D and Table 2). *P*-coumaric, chlorogenic, catechin, caffeic and trans cinnamic acid were found in *C. maxima* (Red) pulp extracts (Fig 1E and Table 2). Gallic, *P*-coumaric, chlorogenic, catechin, caffeic, trans cinnamic, ferulic and ellagic acid were identified in *C. maxima* (White) pulp (Fig 1F and Table 2). The major phenolic acids found in *C. hystrix* pulp were *p*-coumaric acid followed by ellagic and ferulic acid. The remaining phenolic acids were not identified in these pulp extracts. No flavonoids were identified in the pulp. Both red and white varieties of *C. maxima* pulp contained higher concentrations of *p*-coumaric acid in the pulp extracts. On the other hand, *C. maxima* (Red) pulp contained low concentrations of trans cinnamic, caffeic and chlorogenic acid. Xi *et al*. (2014) found ferulic acid was the predominant phenolic acids followed by caffeic and *p*-coumaric acid in 14 genotypes of Chinese wild mandarin pulp. The only flavonoid found in *C. maxima* (Red) pulp was catechin at a low concentration (0.63 mg/100 g). Chlorogenic acid was the second most abundant phenolic acid while caffeic, ellagic, ferulic and trans-cinnamic acid were present at low concentrations in the *C. maxima* (White) pulp. Similarly, the single flavonoid identified in the White pulp was catechin.

Gallic acid, *p*-coumaric acid, chlorogenic acid, catechin, caffeic acid, ferulic acid and quercetin were identified in the fiber extract of *C. hystrix* (Fig 1G and Table 2). *P*-coumaric, chlorogenic, ferulic acid and quercetin were found in *C. maxima* (Red) fiber extracts (Fig 1H and Table 2). *P*-coumaric, rutin, ferulic acid and quercetin were identified in *C. maxima* (White) fiber (Fig 1I and Table 2). The order of phenolic acids in *C. hystrix* fiber from more abundance to lowest was gallic, ferulic, caffeic, *p*-coumaric and chlorogenic acid. Flavonoids found in lower concentrations in kaffir lime were quercetin and catechin. The major phenolic acids identified in *C. maxima* (Red) were ferulic followed by chlorogenic and *p*-coumaric acid. The only flavonoid found in this fiber was quercetin. The phenolic acids found in *C. maxima* (White) were ferulic acid and *p*-coumaric acid. The major flavonoids identified in this fiber were rutin followed by quercetin. According to Fernandes *et al*. (2010) [10] rutin has been associated with markedly decreased hepatic and cardiac levels of triglycerides. Erlund (2004) [9] reported that quercetin exhibits antioxidative, anticarcinogenic, anti-inflammatory, anti-aggregation and vasodilating effects. Based on these suggestions, our results suggest that the presence of rutin in citrus peel fiber could have beneficial effects on human health. Larrauri *et al*. (1996) [19] identified the polyphenolics such as caffeic, ferulic acid, myricetin, naringin and hesperidin in the orange and lime peel fiber. In addition lime peel fiber contained ellagic acid, quercetin and kaempferol. The mentioned values of *p*-coumaric acid, quercetin and rutin were higher than the peel fiber of tomato fruits (Navarro-Gonzalez *et al*., 2011) [28].



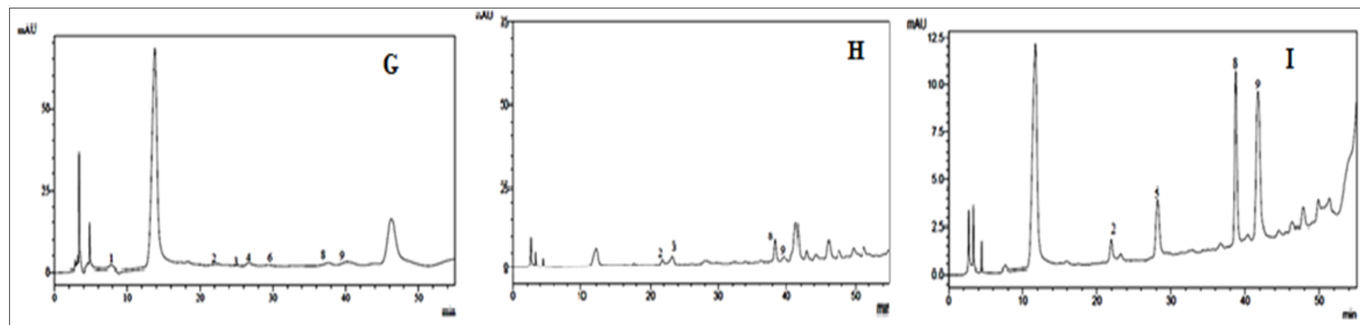


Fig 1: HPLC chromatograms of methanolic extract of peel, pulp and peel fiber from C. hystrix and C. maxima (Red & White) fruits recorded at 280 nm. 1A- C. hystrix peel; 1B- C. maxima (Red) peel; 1C- C. maxima (White) peel; 1D- C. hystrix pulp; 1E- C. maxima (Red) pulp; 1F- C. maxima (White) pulp; 1G- C. hystrix peel fiber; 1H- C. maxima (Red) peel fiber; 1I- C. maxima (White) peel fiber.

3.4 DPPH$^{\cdot}$ and ABTS$^{\cdot+}$ scavenging activity

DPPH and ABTS free radical scavenging potential of the extracts are presented in Table 3. A lower value of IC_{50} indicates a higher antioxidant activity. Among the samples, peel extract of *C. maxima* (Red) (15.69 g extract/g DPPH) had highest DPPH radical scavenging ability followed by peel extract of *C. hystrix* (17.17 g extract/g DPPH) and *C. maxima* (White) (17.18 g extract/g DPPH). Overall, all the peel and peel fiber extracts showed higher DPPH radical scavenging activity than pulp extracts. The antioxidant standards BHA and rutin showed tremendously higher scavenging activity than all the extracts. Similarly the DPPH radical scavenging activity of flavedo extract of *C. limon* fruit was higher than the albedo and pulp extract (Pei-Hsin and Horng-Liang, 2013) [31]. ABTS$^{\cdot+}$, a cationic free radical, scavenging activity was also noticed in the order of peel (97-116 mmol TE/mg extract) > peel fiber (91-114 mmol TE/mg extract) > pulp (68-93 mmol TE/mg extract) in both the *Citrus* spp (Table 3). Among the species, *C. hystrix* exhibited superior scavenging activity followed by *C. maxima* (Red) and (White) varieties. The DPPH and ABTS radical scavenging activities of citrus fruit suggests that, phytoconstituents found in the extracts may act as electron donors and hence terminate radical chain reactions and, boosts natural antioxidant defense mechanism.

Table 3: DPPH$^{\cdot}$, ABTS$^{\cdot+}$, FRAP and metal chelating activity of methanolic extracts of *C. hystrix* and *C. maxima* (Red &White) peel, pulp and peel fiber.

Samples	IC_{50} of DPPH (g extract/g DPPH)^a	ABTS (mmol TE/ mg extract)^b	FRAP (mmol Fe(II)/g extract)^c	Metal chelating (mg EDTA/g extract)^d
C. hystrix				
Peel	17.17^d±0.10	116.21^d±0.95	5439.21^d ± 39.31	2.82^{fg} ± 0.04
Pulp	18.93^{ab}±0.17	72.17^d±0.78	3181.29^d ± 32.70	3.34^d ± 0.03
Peel fiber	17.23^d±0.16	114.75^d±0.18	4901.67^d ± 96.91	3.00^{ef} ± 0.13
C. maxima (Red)				
Peel	15.69^e±0.12	100.46^d±1.56	5148.94^d ± 14.86	2.45^h ± 0.02
Pulp	18.74^b±0.10	93.62^d±2.18	4156.79^d ± 57.25	4.43^c ± 0.03
Peel fiber	17.72^c±0.09	96.83^d±2.18	4197.24^d± 50.13	2.79^{fg} ± 0.10
C. maxima (White)				
Peel	17.18^d±0.20	97.45^d±3.25	4468.47^d ± 21.80	3.07^e ± 0.04
Pulp	19.00^a±0.06	68.54^d±0.82	3252.67^d ± 28.55	2.64^{gh} ± 0.03
Peel fiber	18.87^{ab}±0.13	91.86^d±2.18	4135.38^d ± 47.53	2.97^{ef} ± 0.35
STANDARDS				
BHA	0.15^f ± 0.00	655.14^b±61.42	350760.45^b ± 72476.70	10.49^b ± 0.06
RUTIN	0.18^f ± 0.00	433.57^c±23.18	174032.83^c ± 26869.47	-
TANNIC ACID	-	751.74^a±62.89	562955.03^a ± 42130.92	-
α-Tocopherol	-	-	-	12.67^a ± 0.26

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. BHA- butylated hydroxyl anisole.
[a] g of sample required to decrease 1 g of the initial DPPH concentration by 50%.
[b] mmol of trolox equivalents/ mg extract
[c] mmol of ferrous equivalents/g extract;
[d] mmol of EDTA equivalents/g extract.

3.5 FRAP

FRAP of peel, pulp and peel fiber extracts also reveals the variations in antioxidant power of different fruit tissues (Table 3). The highest reducing power was found in peel of all the three fruits (4468.47-5439.21 mmol Fe(II)/g extract), followed by peel fiber (4135.38-4901.67 mmol Fe(II)/g extract) and pulp (3181.29-4156.79 mmol Fe(II)/g extract). Among the species, *C. hystrix* possess higher reducing power followed by

C. *maxima* (Red) and (White) fruits. Toh *et al*. (2013) [39] also reported FRAP value of pink and white variety of Pomelo peel and pulp extracts.

3.6 Metal chelating activity

The chelating activity of methanolic extract of peel, pulp and peel fiber of citrus fruit were measured against Fe^{2+} and shown in Table 3. The chelating activity was noticed as 2.45-4.43 mg EDTA/g extract. The metal chelating ability of citrus fruits is expected to potentially enhance the neuroprotective properties of the plant particularly in view of the fact that iron overload is involved in the pathogenesis of brain disease such as Alzheimer (Elise and James, 2002) [7]. 3.7 α-Amylase and α-glucosidase potential inhibition activity

The inhibitory effects of the sample extracts and standard acarbose on α-amylase are shown in Table 4. The highest activity is exhibited by peel extracts (75.70-79.28%) followed by peel fiber (68.98-73.83%) and pulp (56.54-67.76%) with the overall activity range of (56.54-79.28%). However, all the extracts showed lower inhibitory activity compared with acarbose (82.29%). α-amylase inhibiting activities of the fruits were in the following order: C. maxima (Red) > C. hystrix > C. maxima (White).

The α-glucosidase inhibition activity of *C. hystrix* (59.04-68.44%), *C. maxima* (Red) (67.93-70.08%) and *C. maxima* (White) (63.98-71.06%) shows that, all the peel fiber extracts exhibited higher inhibition than the peel and pulp extracts (Table 4). Therefore, we suggest that inhibition of α-amylase and α-glucosidase activities could be part of the possible mechanisms involved in the use of *C. hystrix*, *C. maxima* (Red & White) varieties in therapeutic/dietary management of diabetes, by retardation of starch hydrolysis in the gastrointestinal tract.

3.7 Acetylcholinesterase inhibitory activity

The anticholinesterase activity for all the peel, pulp, peel fiber extracts with the values of 61.61-64.29% by *C. hystrix,* 60.02-62.15% by *C. maxima* (Red), 58.97-60.21%) by *C. maxima* (White) (Table 4). All the extracts showed less potent than the eserine (86.89%) reference compound. Previous reports on anticholinesterase inhibitory activity of citrus essential oil (Loizzo *et al*., 2012; Tundis *et al*., 2012) [12, 40] and our present study results supports the role of citrus constituents in AChE inhibition and thereby reducing nervous system disorders. Some plant extracts with phenolic content and flavonoid inhibited AChE activity *in vitro* (Heo *et al*., 2004) [13] and it could therefore be postulated that the AChE inhibition by the citrus fruits is due to their phenolics.

Table 4: Inhibitory effect of peel, pulp and peel fiber of C. hystrix and C. maxima (Red and White) against α amylase, α glucosidase, tyrosinase and acetylcholinesterase.

Sample	α amylase	α glucosidase	tyrosinase	acetyl cholinesterase
Inhibition (%)				
C. hystrix				
Peel	76.32^{c}±1.18	62.37^{h}±1.01	64.73^{b}±0.74	64.29^{c}±0.40
Pulp	67.76^{e}±2.92	59.04^{i}±0.88	49.44^{f}±0.62	61.61^{f}±0.23
Peel fiber	73.83^{c}±0.91	68.44^{de}±0.36	56.38^{c}±0.37	63.71^{b}±0.12
C. maxima (Red)				
Peel	79.28^{b}±1.43	69.35^{cd}±0.43	55.57^{cd}±0.31	62.15^{e}±0.41
Pulp	62.15^{f}±1.40	67.93^{ef}±0.62	47.13^{h}±0.40	60.02^{g}±0.55
Peel fiber	70.40^{d}±1.18	70.08^{bc}±0.38	55.06^{d}±0.47	60.99^{c}±0.59
C. maxima (White)				
Peel	75.70^{c}±1.87	66.98^{f}±0.75	53.37^{e}±0.62	60.21^{d}±0.47
Pulp	56.54^{g}±0.93	63.44^{g}±0.38	48.33^{g}±0.57	58.97^{h}±0.36
Peel fiber	68.98^{e}±0.71	71.06^{b}±0.25	52.87^{e}±0.40	60.02^{e}±0.47
Standards				
Acarbose	82.29^{a} ±0.23	85.19^{a} ±0.23	-	-
Kojic acid	-	-	90.87^{a} ±0.47	-
Eserine	-	-	-	86.89^{a} ±0.41

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.

3.8 Tyrosinase inhibitory activity

The percentage of tyrosinase inhibition of citrus fruit extracts are shown in Table 4. Among them *C. hystrix* peel (64.73%) exhibited highest inhibition followed by peel extract of *C. maxima* Red (55.57%) and White (53.37%) variety. All the peel (53.37-64.73%) extracts showed higher activity than the peel fiber (52.87-56.38%) and pulp (47.13-49.44%). All the extracts showed less 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). Citrus peel is rich in flavonoids and identified as tyrosinase inhibitors, including nobiletin, naringin and neohesperidin (Lou *et al*., 2012) [23]. Matsuura *et al*. (2006) [26] reported the tyrosinase inhibitory potential of essential oil from citrus fruits. The methanolic extract of *C. grandis* peel showed highest tyrosinase inhibition activity similar to that of Kojic acid (Wu *et al*., 2011) [44]. Therefore, incorporation of such citrus fruit samples in the food materials may enhance the shelf life and also prevent the oxidative browning of food molecules.

4. Conclusions

In the present study, peel, pulp and peel fiber from *C. hystrix*, *C. maxima* (Red & White) fruit extracts, especially methanolic

extracts not only possessed antioxidant and radical scavenging activity but also exhibited inhibitory potential against α-amylase, α-glucosidase, acetylcholinesterase and tyrosinase *in vitro*. These results indicate phenolics and flavonoids were found to mainly responsible for these bioactivities of the extracts of peel, pulp and peel fiber. Based on the results, the citrus fruit peel could be utilized as a cheap renewable bioresource to develop functional food, health-promoting and potential antidiabetic, anticholinesterase, antityrosinase agents. According to these results, we conclude that the consumption of these fruits might display good antioxidant activity and its extracts will probably be used for the development of safe food products, natural additives and cosmetics. 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.

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