

PHENOLIC AND ANTIOXIDANT EVALUATION OF THE AQUEOUS AND ETHANOL EXTRACTS OF UNRIPE *Citrus reticulata* PEELS

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(Received: 16th December, 2018; Accepted: 12th February, 2019)

ABSTRACT

Citrus reticulata is a widely consumed citrus fruit in Nigeria, but its potential as a source of alternative medicine has been poorly explored. The present study, therefore attempts to evaluate the phenolic, tannin, flavonoids and proanthocyanidin contents as well as the free radical scavenging activity of the aqueous and ethanol extracts of the peels of unripe *Citrus reticulata* and was carried out using standard methods. Results from the quantitative phytochemical analysis revealed that the total phenols for both the aqueous and ethanol extracts were 52.50 ± 0.21 and 23.750 ± 0.271 mg GAE/g extract, tannins 29.60 ± 0.25 and 13.006 ± 0.326 mg TAE/g extract, flavonoids 22.96 ± 5.05 and 04.207 ± 5.803 mg QE/g extract and proanthocyanidin 332.75 ± 16.42 and 127.750 ± 11.755 mg AAE/g extract respectively. There was a concentration- dependent increase in the scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, Ferric Reducing Antioxidant Power (FRAP), nitric oxide reducing power. The results suggest that the extracts of unripe peels of *Citrus reticulata* possess appreciable antioxidant activity due to the presence of important phytochemicals, which may be of high nutraceutical and pharmaceutical importance.

Keywords: *Citrus reticulata*, Phenols, Flavonoids, Proanthocyanidin, Antioxidant.

INTRODUCTION

Citrus (*Citrus* spp) is one of the most abundant fruit crops with world production estimated at 115 million tons per year (Manthey, 2004). Citrus is a large family whose dominant members include tangerines (*Citrus reticulata*), sweet oranges (*Citrus sinensis*), lemon (*Citrus limon*), limes (several species) and grape fruits (*Citrus paradisi*). Citrus fruits are notable for their fragrance, partly due to flavonoids and limonoids contained in the rinds (Manthey, 2004). Also, citrus fruits and juices are important sources of bioactive materials including antioxidants such as ascorbic acid, flavonoids and phenolic compounds that are important to human nutrition (Kamran et al., 2009). Citrus fruits are good source of folic acid, vitamin B (thiamine), potassium, phosphorus, calcium, iron, magnesium, sodium and sulphur (Nagy et al., 2007). The endocarp is rich in soluble sugar and contains significant amounts of vitamin C, pectin, fibres, different organic acids and potassium salt which give the fruits its characteristic "citrus flavour" (Ezejiolor et al., 2011). Africa produces 3,741,000 tonnes of varieties of citrus fruits of which Nigeria contributes 3,240,000 tonnes (FAO, 2004). Nigeria produces 3% of fresh citrus in the World.

The production of citrus fruits in Nigeria is significant, with heavy direct consumption due to few and small capacity processing industries to convert the fruit to juice concentrate and canned fruit (FAO, 2004).

The search for new natural compounds with potent biological activities has been increasing with an estimated eighty percent (80%) of the human population in developing countries relying, to some extent, on medicinal plant materials for their primary healthcare. Medicinal plant-based drugs have the added advantage of being readily available, effective, and offering a broad spectrum of activity with a greater emphasis on preventive action. These medicinal plants are usually known to exert their diverse health benefits through the numerous phytochemicals they contain (Jeyachandran et al., 2010; Jothy et al., 2013).

The present study, evaluates the phenolic, tannin, flavonoids and proanthocyanidin content and the antioxidant activities of extracts of unripe *Citrus reticulata* peels with the hope that, the extracts may contain pharmacologically-active compound which may be used as drug.

MATERIALS AND METHODS

Sample Collection: Unripe *Citrus reticulata* fruits were harvested at the University of Benin, Faculty of Arts Citrus Orchard in June, 2018 and was authenticated by Dr. Akinnibosun, H.A., a Botanist at the Department of Plant Biology and Biotechnology, University of Benin and a voucher specimen number UBH_c390 was given.

Preparation of Extract: Unripe *Citrus reticulata* fruit was washed, dried, hand peeled and air dried for two weeks. Peels were ground into a fine powder form by using a grinder. 250g of ground peels powder was soaked in 2.5 litres of distilled water and then periodically stirred for 72 hours. While 500 g of the powder was soaked in 2.5 litres of absolute ethanol solution and then periodically stirred for 72 hours. All extracts were concentrated over a rotary evaporator, freeze-dried and stored in air tight sample bottles.

Determination of Total Phenolic Content

The total phenolic content was determined using the Folin-Ciocalteu method as described by Cicco et al. (2009). Concentrations, ranging from 0.2-1 mg/ml of gallic acid or extracts, were prepared in methanol. Then, 4.5 ml of distilled water was added to 0.5 ml of the extract and mixed with 0.5 ml of a ten-fold diluted Folin-Ciocalteu reagent. Five milliliters of 7% sodium carbonate was then added to the tubes and another 2 ml of distilled water was added. The mixture was allowed to stand for 90 min at room temperature; absorbance was then read at 760 nm. All determinations were performed in triplicates with gallic acid utilized as the positive control. The total phenolic content was expressed as Gallic Acid Equivalent (GAE).

Determination of Total Flavonoid Content

The total flavonoid content was determined using the method of Ayoola et al. (2008). The assay is based on the reaction between flavonoids and aluminium chloride to generate a characteristic mixture that has maximum absorption at 420 nm. 2 ml of 2% AlCl₃ in ethanol was added to 2 ml extract. A concentration of 1 mg/ml of the extract dissolved in methanol using serial dilutions was used. Similar concentrations of quercetin, the positive control, were used. The absorbance was

measured at 420 nm after incubation for 60 minutes at room temperature. The standard curve was prepared using quercetin in six different concentrations (0.01, 0.025, 0.050, 0.075, 0.1, 0.15 mg/ml). The total flavonoid content was calculated as mg quercetin equivalent /g of extract.

Determination of Proanthocyanidin Content

The determination of proanthocyanidin was carried out according to the method of Sun et al., (1998). To 0.5 ml of 1.0 mg/ml of each extract was added 1 ml of 4% methanol solution and 0.75 ml of concentrated hydrochloric acid. The mixture was left undisturbed for 15 minutes and the absorbance was read at 500 nm. Ascorbic acid was used as standard.

Determination of Total Tannin Content

The level of tannin in the plants was determined using the method of Van-Burden and Robinson (1981). Determination is based on reaction with phosphotungstomolybdate in solution of Na₂CO₃. Concentrations ranging from 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.12, 0.15 mg/ml of tannic acid or extracts were prepared in methanol to a total volume of 1 ml, then 6.5 ml of distilled water, followed by 0.5 ml of Folin-Denis reagent and 1 ml of 7.5% Na₂CO₃ were added. The absorbance was read at 700 nm. The total tannin content was expressed as Tannic Acid Equivalent (TAE) using the standard calibration curve.

Estimation of Diphenyl-2-Picryl-Hydrazyl (DPPH) Radical Scavenging Activity

The free radical scavenging capacity of the aqueous and ethanol extracts against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was determined by a slightly modified method of Brand-Williams et al. (1995). Briefly, 0.5 ml of 0.3 mM DPPH solution in methanol was added to 2 ml of various concentrations (0.2 - 1.0 mg/ml) of the extracts. The reaction tubes were shaken and incubated for 15 min at room temperature in the dark. Absorbance was read at 517 nm and all tests were performed in triplicate. Ascorbic acid was used as standard control, with similar concentrations as the test samples prepared. A blank containing 0.5 ml of 0.3 mM DPPH and 2 ml methanol was prepared and treated as the test samples. The radical scavenging activity was

calculated using the following formula: DPPH radical scavenging activity (%) = $[(A_0 - A_1) / (A_0)] \times 100$, where A_0 was the absorbance of DPPH radical + methanol; A_1 was the absorbance of DPPH radical + sample extract or standard.

Ferric Reducing Antioxidant Power (FRAP) Assay
The Ferric Reducing Antioxidant Power (FRAP) assay was carried out using a modified method of Benzie and Strain (1996). To 1.5 ml of freshly prepared FRAP solution (25 ml of 300 mM acetate buffer pH 3.6, 2.5 ml of 10 mM 2,4,6-tripyridyls-triazine (TPTZ) in 40 mM HCl, and 2.5 ml of 20 mM ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) solution) was added to 1 ml of the extracts at concentrations of 0.1 - 1.0 mg/ml. The reaction mixtures were incubated at 37 °C for 30 min and the increase in absorbance at 593 nm was measured. FeSO_4 was used for the calibration curve and ascorbic acid served as the positive control. FRAP values (expressed as mg Fe (II)/g of the extract) for the extracts were then extrapolated from the standard curve.

Nitric Oxide Scavenging Assay

The method of Garrat (1964) was followed to determine the nitric oxide radical scavenging activity of the extracts. Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions which is measured by Griess reaction. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite. To 0.5 ml of varying concentration of extract, 2 ml of (10 mM) sodium nitroprusside, 0.5 ml of phosphate buffer saline (pH 7.4) was added and incubated at 25 °C for 2½ hours. To 0.5 ml of this reaction mixture, 1 ml of (0.33%) sulfanilic acid was added and allowed to stand at room temperature for 5 minutes. Then 1 ml of (0.1 %) naphthylene diamine chloride was added and incubated at room temperature for 30 minutes. Absorbance was read at 540 nm. Percentage (%) inhibition was calculated as:

Percentage inhibition of nitric oxide = $(\text{Abs control} - \text{Abs sample}) \times 100 / \text{Abs control}$.

Reducing Power

This method is based on the principle of increase in the absorbance of the reaction mixtures.

Increase in the absorbance indicates an increase in the antioxidant activity. In this method, antioxidant compound forms a colour complex with potassium ferricyanide, trichloroacetic acid and ferric chloride, which is measured at 700 nm. Increase in absorbance of the reaction mixture indicates the reducing power of the samples (Jayaprakash et al., 2001). In the method described by Oyaizu (1986), 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of $\text{K}_3\text{Fe}(\text{CN})_6$ (1% w/v) are added to different concentrations of the extract (0.02-0.2 mg/ml). The resulting mixture is incubated at 50 °C for 20 min, followed by the addition of 2.5 ml of trichloroacetic acid (10% w/v). The mixture was then mixed with distilled water (2.5 ml) and 0.5 ml of FeCl_3 (0.1%, w/v). The absorbance is then measured at 700 nm against blank sample. The reducing power increases with absorbance values.

Statistical Analysis

All analyses were carried out in triplicate and the results were expressed as mean \pm SEM. The data were subjected to one-way analysis of variance (ANOVA) where applicable p values of < 0.05 were regarded as significant.

RESULTS

The total phenol, flavonoid, proanthocyanidin and tannin content of extracts of unripe *C. reticulata* peels are shown in table 1. Aqueous extract had a higher total phenol, flavonoid, proanthocyanidin and tannin content than the ethanol extract. The results of the DPPH radical scavenging activities of extracts of unripe *C. reticulata* peels are shown in figure 1. The results show that the ethanol extract was a better inhibitor of the DPPH radical than the aqueous extract. The Ferric Reducing Antioxidant Potential (FRAP) results are presented in figure 2. The results revealed that the aqueous extract had a significantly higher ability to reduce Fe^{3+} to Fe^{2+} than the ethanol extract. The nitric oxide scavenging activities of the extracts of unripe *C. reticulata* peels are shown in figure 3. The results show that the ethanol extract was a better scavenger of nitric oxide than the aqueous extract. Figure 4 depicts the reducing power of unripe *C. reticulata* peels. The results show that the aqueous extract had a higher reducing power than the ethanol extract. However, it could be inferred

from the results that increasing the concentration of the extracts may ultimately raise the potency to scavenge free radicals; hence an increase in the antioxidant properties.

TABLE 1: Total Phenol, Flavonoid, Proanthocyanidin and Tannin Content of Extracts of Unripe *Citrus reticulata* Peels.

	Total Phenolic Content (mg GAE/g extract)	Total Flavonoid Content (mg QE/g extract)	Proanthocyanidin Content (mg AAE/g extract)	Total Tannin Content (mg TAE/g extract)
Aqueous Extract	52.50±0.21 ^a	22.96±0.50 ^a	332.75±16.42 ^a	29.60±0.25 ^a
Ethanol Extract	23.75±0.27 ^b	04.21±0.80 ^b	127.75±11.76 ^b	13.01±0.33 ^b

GAE: Gallic acid equivalent; QE: Quercetin equivalent; AAE: Ascorbic acid equivalent; TAE: Tannic acid equivalent. Values are expressed as mean± SEM, n = 3/group. Different lowercase letters represent significant difference between means at p<0.05.

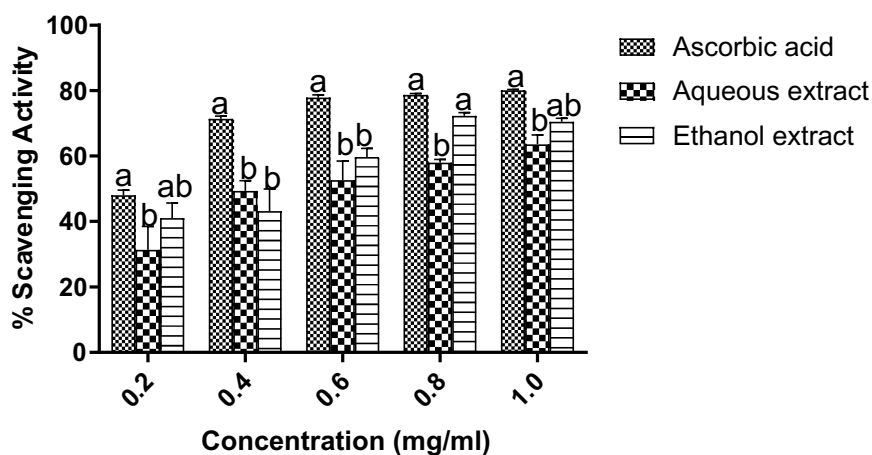


Figure 1: DPPH's radical scavenging activity of extracts of unripe *C. reticulata* peels. Values are expressed as mean± SEM, n = 3/group. Different lowercase letters represent significant difference between means at p < 0.05.

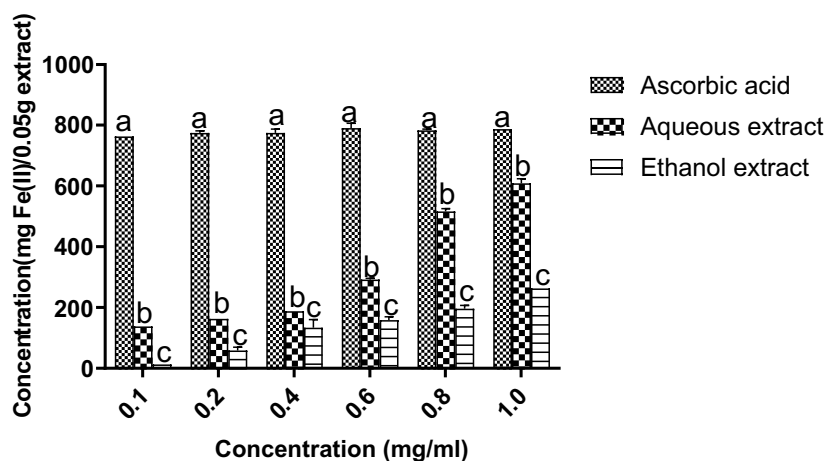


Figure 2: Ferric acid reducing Antioxidant Potential (FRAP) Assessment of Extracts of Unripe *C. reticulata* Peels. Values are expressed as mean± SEM, n = 3/group. Different lowercase letters represent significant difference between means at p<0.05.

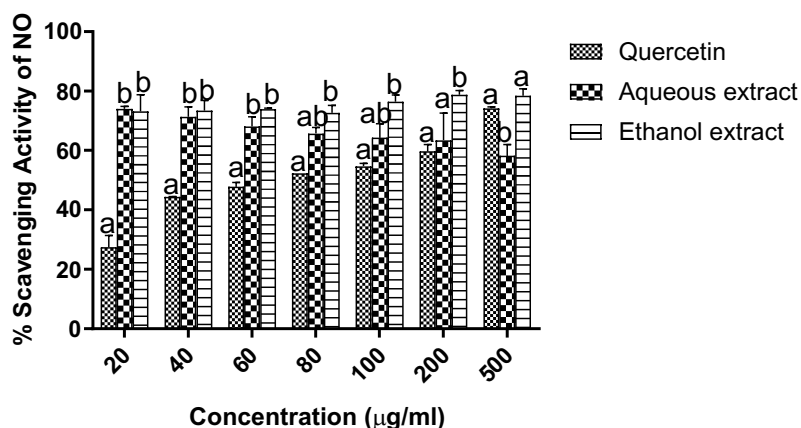


Figure 3: Nitric Oxide Scavenging Activity of Extracts of Unripe *C. Reticulata* Peels. Values are expressed as mean \pm SEM, $n = 3$ /group. Different lowercase letters represent significant difference between means at $p < 0.05$.

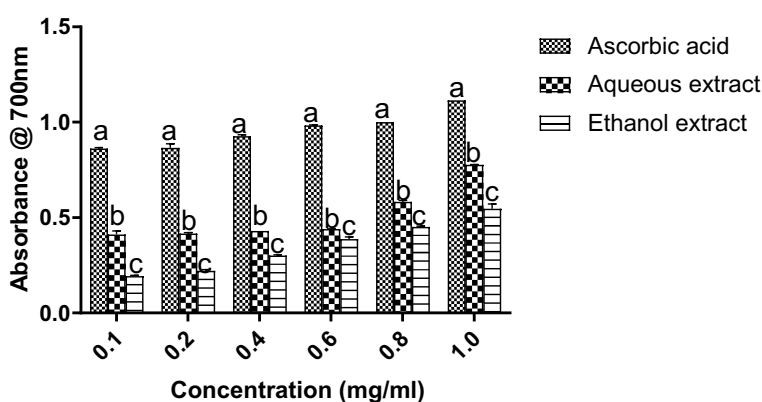


Figure 4: Reducing Power of Extracts of Unripe *C. Reticulata* Peels. Values are expressed as mean \pm SEM, $n = 3$ /group. Different lowercase letters represent significant difference between means at $p < 0.05$.

DISCUSSION

The presence of bioactive metabolites contributes to the medicinal functions of plants (Akinpelu et al., 2018). Phenolics possess diverse biological activities, such as antiulcer, anti-inflammatory, antioxidant, anti-tumour and antidepressant activities (Mamta et al., 2013). Various studies have reported that flavonoids possess potent and appreciable antioxidant, anti-inflammatory and anticancer activities (Adetutu et al., 2015; Oyedapo et al., 2015). The flavonoid content of aqueous and ethanol extracts of unripe *C. reticulata* peels are quite high when compared to methanol extract of ripe *C. reticulata* peels (0.3 mg QE/g of extract powder) as reported by Kamran et al. (2009). Proanthocyanidin is vasodilatory, anti-carcinogenic, anti-allergic, anti-inflammatory, anti-bacterial, cardio-protective, immune-stimulating, antiviral and estrogenic (Yildirim et al., 2015). Tannins are polyphenolic compounds which have been shown to possess antioxidant, anti-inflammatory and

anticarcinogenic effects (Omoregie and Osagie, 2012; Mamta et al., 2013).

Various biological systems naturally generate oxygen free radicals produced from various cellular activities or functions or from exposure to exogenous substances in the environment. Oxygen-centered free radicals play a central role in the pathogenesis of many human diseases resulting in oxidative stress by damaging membrane lipids, proteins and DNA molecules (Steenkamp et al., 2005). Thus, antioxidants may offer resistance against oxidative stress by scavenging free radicals as natural antioxidants which are present in herbs and spices and are responsible for inhibiting or preventing the deleterious consequences of oxidative stress as they have free radical scavenging ability mostly due to the presence of certain bioactive agents contained in them (Adedosu et al., 2013). As a rapid and simple measure of antioxidant activity, the DPPH radical scavenging capacity is based on

the reduction of the stable radical DPPH to yellow colored diphenylpicrylhydrazine in the presence of a hydrogen donor (Omoregie and Oikeh, 2015). The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples (Lee et al., 2003). The values obtained showed that the ethanol extract was relatively comparable with that of the ascorbic acid (standard antioxidant). These plant extracts may thus have potent radical scavenging properties. The aqueous extract was observed to have a significantly higher ($p < 0.05$) FRAP value than the ethanol extract. Both extracts however have significantly lower ($p < 0.05$) FRAP values than the standard antioxidant. Ethanol extract significantly inhibited nitric oxide (NO) production which have health implications and benefits. NO has been implicated in inflammatory and pathogenesis of various human diseases such as cancer and cardiovascular diseases. NO scavenging capacity of extracts may help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to the human health (Olorunnisola et al., 2012). The aqueous extract displayed the highest reducing power. The aqueous extract had higher concentration of phytochemical which are potent antioxidants. It was found that the radical scavenging activities of both extracts increased with increasing concentration. The high proanthocyanidin which possess significant antioxidant potential (Mamta et al., 2013; Yildirim et al., 2015) may cause high antioxidant activity of this plant. Moreover, the unripe *C. reticulata* peels have been shown to possess significant amount of phytochemicals and antioxidant activities which is in line with the report by Tumbas et al. (2010) that ripe *C. reticulata* peels are good source of natural antioxidant. This unripe *C. reticulata* peels by in vitro results appear promising and may be effective as potential source of novel drugs.

CONCLUSION

In conclusion, the unripe *C. reticulata* peels showed that it is a good source of flavonoids, proanthocyanidins, phenols and tannins. The results of our study showed that unripe *C. reticulata* peel have the potential to be applied as a natural constituent of food and medicines as they exhibit a strong antioxidant property.

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