# Characterization of the antioxidant properties of phenolic extracts from some citrus peels

**G. Oboh · A. O. Ademosun**

**Abstract** This study sought to determine the distribution of free and bound phenolics in some Nigerian citrus peels [orange (*Citrus sinensis*), grapefruit (*Citrus paradisii*) and shaddock (*Citrus maxima*)] and characterize the antioxidant properties. The free phenolics were extracted with 80% acetone, while the bound phenolics were extracted from the alkaline and acid hydrolyzed residue with ethyl acetate. Free phenolic extracts had significantly higher ($P<0.05$) DPPH* scavenging ability than the bound phenolic extracts, except in orange peels where the bound phenolic extracts had significantly higher ($P<0.05$) DPPH* scavenging ability. Bound phenolics from orange peels had the highest ABTS* scavenging ability (6.09 mmol./TEAC g) and ferric reducing antioxidant properties (FRAP) (71.99 mg/GAE 100 g), while bound phenolics from shaddock peels had the least ABTS* scavenging ability (1.35 mmol./TEAC g) and FRAP (2.58 mg/GAE 100 g) . Bound phenolics from grapefruit peels had the highest OH* scavenging ability ($EC_{50}=3.8$ mg/ml), while bound phenolics from shaddock peels had the least ($EC_{50}=16.1$ mg/ml). The phenolics chelated $Fe^{2+}$ and inhibited malondialdehyde production in rat's pancreas in a dose-dependent manner. The additive and/or synergistic action of the free and bound phenolics could have contributed to the observed medicinal properties of the peels; therefore, the high antioxidant properties of the free and bound phenolic extracts from orange peels could be harness in the formulation of nutraceuticals and food preservatives.

## Introduction

Reactive oxygen species (ROS) are very unstable, and react rapidly with other substances including DNA, membrane lipids and proteins. Health disorders such as diabetes mellitus, hypertension, cancer, neurodegenerative, gastric ulcers, reperfusion, arthritis and infammatory diseases are linked to the unchecked activities of ROS (Halliwell 1989; Vajragupta et al. 2000). Antioxidants are well known to scavenge free radicals and ROS, and interrupting the radical chain reaction of lipid peroxidation, thereby preventing oxidative damage (Halliwell 1999). A practical approach to the management of the deleterious effect of ROS is through eating foods that are rich in antioxidants (Oboh and Rocha 2007a)

In the last decades of the 20th century, world production of citrus fruit has experienced continuous growth. Annual citrus production was totaled at an estimated 105 million tons in the period 2000–2004. Citrus fruits have a small edible portion and large amounts of waste materials such as peels and seeds and the peels are used in folk medicine for the management of degenerative diseases, such as diabetes and hypertension, though there is very limited information on the mode of action of these peels in the management of diabetes and hypertension. Belitz and Grosch (1999) reported that the content of total phenols was higher in peels of citrus fruits than in peeled citrus fruits. Citrus fruits' peels contain significant amount of phenolic compounds, especially phenolic acids and flavonoids (Sawalha et al. 2009).

Reactive oxygen species attack and damage body cells to get the missing electron they need, but antioxidants protect the body by contributing an electron of their own,

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and in so doing, they neutralize free radicals and help prevent cumulative damage to body cells and tissues. Much of the total antioxidant activity of fruits and vegetables is related to their phenolic content, not only to their vitamin C content, also, a correlation exists between the polyphenol content and antioxidant activities (Ramakrishnan et al. 2010). Research suggests that many flavonoids are more potent antioxidants than vitamins C and E (Oboh and Rocha 2007a). Natural polyphenols exert their beneficial health effects by their antioxidant activity, these compounds are capable of removing free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce α-tocopherol radicals, and inhibit oxidases (Amic et al. 2003; Alia et al. 2003).

Phenolics in many plant foods are in both soluble free and bound forms. Bound phenolics, mainly in the form of β-glycosides, could survive the human stomach and small intestine digestion and reach the colon intact, where they are released to exhibit their bioactivity with health benefits (Sun et al. 2002; Chu et al. 2002; Oboh and Rocha 2007b). Most earlier reports on the phenol constituents and antioxidant properties of citrus peels are mainly determined on the soluble free phenolics (Oboh and Ademosun 2006; Sawalha et al. 2009), which is on the basis of the solvent-soluble extraction, this gives an underestimation of the phenolic and antioxidant properties of the peels, since the bound phenolic content and activities were not accounted for, therefore this studies sought to determine the antioxidant properties of free soluble and bound phenolics of orange peels as a potential material for the formulation of nutraceuticals and food preservatives.

## Materials and methods

### Sample collection and preparation

Orange (*Citrus sinensis*), grapefruit (*Citrus paradisii*) and shaddock (*Citrus maxima*) peels were collected from the Akure main market during the month of November. The peels were sun dried to a moisture content of 12% and ground to fine powder using Warring Commercial heavy Duty Blender (Model 37BL18; 24ØCB6). The water used was glass distilled, while the chemicals were of analytical grade.

### Extraction of free soluble phenolics

The extraction of free soluble phenolics was carried out according to the method reported by Chu et al. (2002). 10 g of the ground peels was extracted with 80% acetone (1: 5 w/v) and filtered (filter paper Whatman no. 2) under vacuum. The

filtrate was then evaporated using a rotary evaporator under vacuum at 45 °C until about 90% of the filtrate had been evaporated. The phenolic extracts were frozen at -40 °C, while the residues were kept for the extractions of bound phenolics.

### Extraction of bound phenolics

The residue from free soluble extraction above was flushed with nitrogen and hydrolyzed with about 20 ml of 4 M NaOH solution at room temperature for 1 hr with shaking. Then, the pH of the mixture adjusted to pH 2 with concentrated HCl and the bound phytochemicals were extracted with ethylacetate and repeated five times. The ethyl acetate fractions were then evaporated at 45 °C (Chu et al. 2002).

### Determination of total phenol content

The total phenol content was determined according to the method of Singleton et al. (1999). Briefly, appropriate dilutions of the extracts were oxidized with 2.5 ml 10% Folin-Ciocalteau's reagent (v/v) and neutralized by 2.0 ml of 7.5% sodium carbonate. The reaction mixture was incubated for 40 min at 45 °C and the absorbance was measured at 765 nm in the spectrophotometer. The total phenol content was subsequently calculated as gallic acid equivalent.

### Determination of total flavonoid content

The total flavonoid content of both extracts were determined using a slightly modified method reported by Meda et al. (2005), briefly 0.5 ml of appropriately diluted sample was mixed with 0.5 ml methanol, 50 µl 10% AlCl₃, 50 µl 1 M Potassium acetate and 1.4 ml water, and allowed to incubate at room temperature for 30 min. The absorbance of the reaction mixture was subsequently measured at 415 nm; the total flavonoid content was subsequently calculated. The non-flavonoid polyphenols were taken as the difference between the total phenol and total flavonoid content.

### DPPH free radical scavenging ability

The free radical scavenging ability of the extracts against DPPH (1,1-diphenyl–2 picrylhydrazyl) free radical was evaluated as described by Gyamfi et al. (1999). Briefly, appropriate dilution of the extracts (1 ml) was mixed with 1 ml, 0.4 mM methanolic solution containing DPPH radicals, the mixture was left in the dark for 30 min and the absorbance was taken at 516 nm. The DPPH free radical scavenging ability was subsequently calculated.

ABTS* scavenging ability

The ABTS* scavenging ability of both extracts were determined according to the method described by Re et al. (1999). The ABTS* was generated by reacting an (7 mmol/l) ABTS aqueous solution with $K_2S_2O_8$ (2.45 mmol/l, final concentration) in the dark for 16 h and adjusting the Abs734 nm to 0.700 with ethanol. 0.2 ml of appropriate dilution of the extract was added to 2.0 ml ABTS* solution and the absorbance were measured at 734 nm after 15 mins. The trolox equivalent antioxidant capacity was subsequently calculated.

Fenton reaction (degradation of deoxyribose)

The method of Halliwell et al. (1987) was used to know the ability of the extract to prevent $Fe^{2+}/H_2O_2$ induced decomposition of deoxyribose. The extract 0–100 μL was added to a reaction mixture containing 120 μL of 20 mm deoxyribose, 400 μL of 0.1 m phosphate buffer, 40 μL of 500 μm of $FeSO_4$, and the volume were made up to 800 μL with distilled water. The reaction mixture was incubated at 37 °C for 30 min and the reaction was then stopped by the addition of 0.5 ml of 28% trichloro acetic acid. This was followed by addition of 0.4 ml of 0.6% thiobarbituric acid solution. The tubes were subsequently incubated in boiling water for 20 min. The absorbance was measured at 532 nm in a spectrophotometer.

Determination of reducing property

The reducing property of the extracts was determined by assessing the ability of the extract to reduce $FeCl_3$ solution as described by Oyaizu (1986). 2.5 ml aliquot was mixed with 2.5 ml 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. and then 2.5 ml 10% trichloroacetic acid was added. This mixture was centrifuged at 650 rpm for 10 min. 5 ml of the supernatant was mixed with an equal volume of water and 1 ml 0.1% ferric chloride. The absorbance was measured at 700 nm, and the ferric reducing antioxidant property was subsequently calculated.

$Fe^{2+}$ chelation assay

The $Fe^{2+}$ chelating ability of both extracts were determined using a modified method of Minotti and Aust (1987). Freshly prepared 500 μM $FeSO_4$ (150 μl) was added to a reaction mixture containing 168 μl 0.1 M Tris–HCl (pH 7.4), 218 μl saline and the extracts (0–25 μl). The reaction mixture was incubated for 5 min, before the addition of 13 μl 0.25% 1, 10-phenanthroline (w/v). The absorbance was

subsequently measured at 510 nm in a spectrophotometer. The Fe (II) chelating ability was subsequently calculated.

Lipid peroxidation assay

*Preparation of tissue homogenates*

The rats were decapitated under mild diethyl ether anaesthesia and the pancreas was rapidly isolated and placed on ice and weighed. This tissue was subsequently homogenized in cold saline (1/10 w/v) with about 10-up-and –down strokes at approximately 1,200 rev/min in a Teflon glass homogenizer. The homogenate was centrifuged for 10 min at 3000xg to yield a pellet that was discarded, and a low-speed supernatant (S1) was kept for lipid peroxidation assay (Belle et al. 2004).

*Lipid peroxidation and thiobarbibutric acid reactions*

The lipid peroxidation assay was carried out using the modified method of Ohkawa et al. (1979), briefly 100 μl S1 fraction was mixed with a reaction mixture containing 30 μl of 0.1 M pH 7.4 Tris–HCl buffer, extract (0–100 μl) and 30 μl of 250 μM freshly prepared $FeSO_4$. The volume was made up to 300 μl by water before incubation at 37 °C for 1 h. The colour reaction was developed by adding 300 μl 8.1% SDS (Sodium doudecyl sulphate) to the reaction mixture containing S1, this was subsequently followed by the addition of 600 μl of acetic acid/HCl (pH 3.4) mixture and 600 μl 0.8% TBA (Thiobarbituric acid). This mixture was incubated at 100 °C for 1 h. TBARS (Thiobarbituric acid reactive species) produced were measured at 532 nm and the absorbance was compared with that of standard curve using MDA (Malondialdehyde).

Data analysis

The results of the three replicates were pooled and expressed as mean±standard error (S.E.). Student $t$-test, one-way analysis of variance (ANOVA) and the least significance difference (LSD) were carried out (Zar 1984). Significance was accepted at $p \leq 0.05$. $EC_{50}$ was determined using linear regression analysis.

## Results and discussion

The results of the total phenol and flavonoid distribution in the citrus peels are presented in Table 1. The free phenolic content of the citrus peels [6.5 (shaddock)—13.1 mg/g (grapefruit)] was significantly ($P<0.05$) higher than the bound phenolic content [0.7 (grapefruit)—6.8 mg/g (orange)]. However, grapefruit peels had the highest free phenolic content

**Table 1** Phenolic distribution in some citrus peels (mg/g)

| Sample | Free | Bound |
|---|---|---|
| Total phenol | | |
|   Orange peels | $10.5 \pm 0.19^a$ | $6.8 \pm 0.11^b$ |
|   Grapefruit peels | $13.1 \pm 0.21^a$ | $0.72 \pm 0.12^b$ |
|   Shaddock peels | $6.5 \pm 0.83^a$ | $3.4 \pm 0.31^b$ |
| Total flavonoid | | |
|   Orange peels | $1.3 \pm 0.12^a$ | $0.21 \pm 0.01^b$ |
|   Grapefruit peels | $0.93 \pm 0.13^a$ | $0.10 \pm 0.02^b$ |
|   Shaddock peels | $0.31 \pm 0.03^a$ | $0.40 \pm 0.09^a$ |

Values represent means of triplicate ($n=3$). Values with the same alphabet along the same row (for the same parameter) are not significantly different ($P>0.05$)

(13.1 mg/g), while orange peels had the highest bound phenolic content (6.8 mg/g). The total flavonoid distribution of the citrus peels is also presented in Table 1, free flavonoid content ranged from 0.3 mg/g (shaddock) to 1.3 mg/g (orange), while bound flavonoid ranged from 0.1 mg/g (grapefruit) to 0.4 mg/g (shaddock); however, the free flavonoid content of the citrus peels were significantly higher ($P<0.05$) than the bound flavonoid content except in shaddock peels where there was no significant difference ($P>0.05$) between the free flavonoid (0.3 mg/g) and bound flavonoid content (0.4 mg/g).

The phenolic distribution in the citrus peels as shown in Table 1 agrees with the phenolic distribution in many plant foods such as fruits (Chu et al. 2002), vegetables (Sun et al. 2002), peppers (Oboh and Rocha 2007a, b), legume seeds (Oboh and Rocha 2008) and mushrooms (Oboh and Shodehinde 2009), because they have more free phenolic than the bound phenolic content. However, the free phenolic content of the citrus peels was significantly higher ($P<0.05$) than the free phenolic content of red pepper, potato, lettuce, cucumber, carrot, onion, spinach, cranberry, broccoli, mushrooms and legumes (Sun et al. 2002; Chu et al. 2002; Oboh and Rocha 2007a, b, 2008; Oboh and Shodehinde 2009); but lower than that of green and sour teas (Oboh and Rocha 2008). Likewise, the bound phenolics content of the citrus peels was higher than that of broccoli, cucumber, onion and red pepper (Sun et al. 2002; Chu et al. 2002; Oboh and Rocha 2007a, b).

Phenolics are capable of scavenging free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce $\alpha$-tocopherol radicals, and inhibit oxidases (Alia et al. 2003; Amic et al. 2003). Their potent antioxidant activity is due to the redox properties of their hydroxyl groups (Materska and Perucka 2005; Rice-Evans et al. 1996; Rice-Evans et al. 1997). Phenolics are present in plant in both free and bound forms; bound phenolics mainly in the form of $\beta$-glycosides, may survive human stomach and small intestine digestion and reach the colon intact,

where they are released and exert their bioactivity (Sosulski et al. 1982); while free phenolics are more readily absorbed and thus, exert beneficial bioactivities in early digestion; however, the significance of bound phytochemicals to human health is not clear (Chu et al. 2002; Sun et al. 2002).

The prevention of the chain initiation step by scavenging various reactive species such as free radicals is considered to be an important antioxidant mode of action (Dastmalchi et al. 2007). The DPPH free radical scavenging ability of the free and bound phenolic extracts of the citrus peels as presented in Fig. 1a and their $EC_{50}$ in Table 2, revealed that the free phenolic extracts had significantly higher ($P<0.05$) DPPH* scavenging ability than the bound phenolic extracts. However, free phenolic extracts from orange peels have the highest DPPH* scavenging ability, but lower than that of free phenolics from some leafy spices in Nigeria (Oboh and Rocha 2007a, b); while bound phenolics from shaddock peels had the least DPPH* scavenging ability. Nevertheless, the trend in the results agree with the phenolic distribution in the citrus peels and many earlier research articles, where correlation were reported between phenolic content and antioxidant capacity of some plant foods (Amic et al. 2003; Sun et al. 2002; Chu et al. 2002).

DPPH free radical scavenging ability is frequently used in the determination of free radical scavenging ability; however it has the limitation of colour interference and sample solubility (Dastmalchi et al. 2007). Therefore, the free radical scavenging ability of the free and bound phenolic extracts was further studied using a moderately stable nitrogen-centred radical species -ABTS radical (Re et al. 1999). The ABTS* scavenging ability reported as trolox equivalent antioxidant capacity (TEAC) is presented in Fig. 2a. However, the trend in the ABTS* scavenging ability did not agree with DPPH* scavenging ability earlier discussed (Fig. 1a), the reason for this difference cannot be categorically stated, however it may not be far fetched from the limitations in DPPH* scavenging ability assay method earlier discussed. However, the trend in the ABTS* scavenging ability agrees with the reducing power of the citrus peels' phenolics reported as ascorbic acid equivalent (Fig. 2b) and the OH* scavenging ability of the citrus peels' phenolics [except that grapefruit bound phenolics had the highest OH* scavenging ability (Fig. 1b and Table 2)]. Therefore, bound phenolics from orange peels could be regarded as having the highest antioxidant capacity, while bound phenolics from shaddock peels had the least.

The hydroxyl radical (OH) radical scavenging abilities of the phenolic extracts of the citrus peels are presented in Fig. 1b and their $EC_{50}$ in Table 2; the phenolics scavenged OH* in a dose-dependent manner. However, bound phenolics had significantly higher ($P<0.05$) OH* scavenging ability than the free phenolics, except in shaddock peels. However, bound phenolics from grapefruit

**Fig. 1** Some antioxidant parameters (**a**. DPPH* scavenging ability **b**. OH* scavenging ability **c**. Fe2+ chelating ability **d**. Inhibition of Fe2+ induced lipid peroxidation) of phenolic extracts from citrus peels as a function of concentration of extract($n=3$)
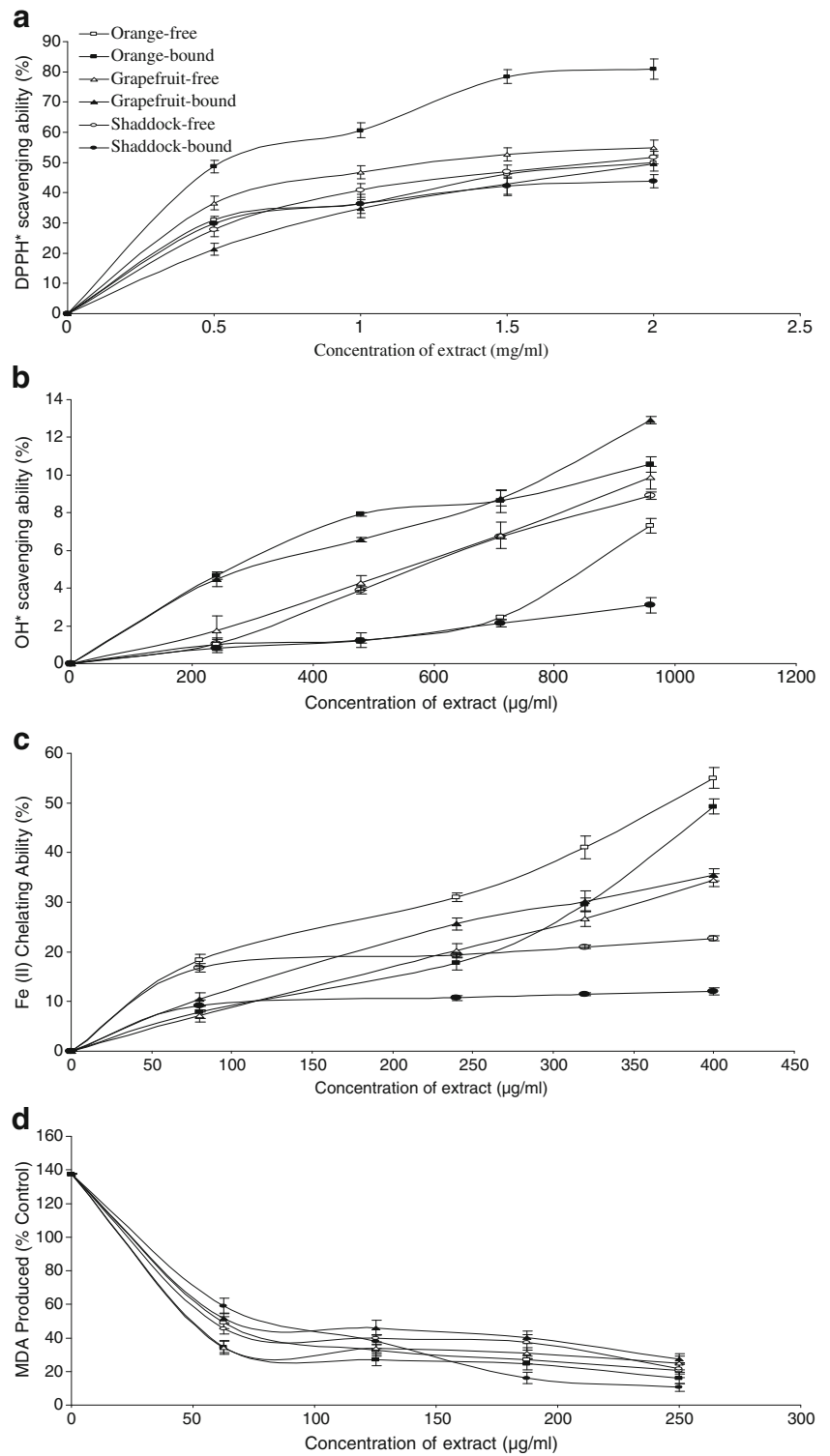


peels had the highest OH* scavenging ability ($EC_{50}=$ 3.8 mg/ml), while bound phenolics from shaddock peels had the least ($EC_{50}=16.1$ mg/ml) (Table 2). The $Fe^{2+}$ chelating ability of the phenolic extracts is presented in Fig. 1c and their $EC_{50}$ in Table 2. The phenolics were able to chelate $Fe^{2+}$ in a dose-dependent manner; however, free

phenolics from orange peels ($EC_{50}=0.31$ mg/ml) had the highest $Fe^{2+}$ chelating ability, while bound phenolics from shaddock peels had the least ($EC_{50}=1.3$ mg/ml) (Table 2). Furthermore, incubation of rat's pancreas in presence of $Fe^{2+}$ caused a significant increase ($P<0.05$) in the malondialdehyde (MDA) content (Fig. 1d); however, the

**Table 2** EC50 of citrus peels phenolics on different parameters

| Sample | Free | Bound |
|---|---|---|
| (a) DPPH* Scavenging ability (mg/ml) | | |
| Orange peels | $1.7 \pm 0.31^a$ | $1.0 \pm 0.12^b$ |
| Grapefruit peels | $1.4 \pm 0.14^b$ | $1.8 \pm 0.08^a$ |
| Shaddock peels | $1.6 \pm 0.02^b$ | $1.9 \pm 0.13^a$ |
| (b) OH* Scavenging ability (mg/ml) | | |
| Orange peels | $8.9 \pm 1.11^a$ | $4.0 \pm 0.69^b$ |
| Grapefruit peels | $5.1 \pm 0.32^a$ | $3.8 \pm 0.21^b$ |
| Shaddock peels | $5.6 \pm 0.51^b$ | $16.1 \pm 1.23^a$ |
| (c) $Fe^{2+}$ Chelating ability (mg/ml) | | |
| Orange peels | $0.31 \pm 0.02^a$ | $0.48 \pm 0.11^a$ |
| Grapefruit peels | $0.63 \pm 0.09^a$ | $0.52 \pm 0.18^a$ |
| Shaddock peels | $0.71 \pm 0.21^b$ | $1.3 \pm 0.20^a$ |
| (d) Inhibition of Fe2+ induced lipid peroxidation in pancreas (µg/ml) | | |
| Orange peels | $149.7 \pm 1.21^a$ | $142.8 \pm 1.43^b$ |
| Grapefruit peels | $151.8 \pm 2.32^b$ | $164.0 \pm 1.52^a$ |
| Shaddock peels | $156.1 \pm 2.73^a$ | $142.8 \pm 1.68^b$ |

Values represent means of triplicate ($n=3$). Values with the same alphabet along the same row are not significantly different ($P>0.05$)

phenolic extracts inhibited MDA production in pancreas in a dose-dependent manner, with bound phenolics from orange and shaddock peels having the highest inhibitory ability ($EC_{50}=142.8$ µg/ml), while bound phenolics from grapefruit ($EC_{50}=164.0$ µg/ml) had the least inhibitory effect (Table 2).

The antioxidant capacities of the phenolics from the peels (except bound phenolics from shaddock) were higher

**Fig. 2** Some antioxidant parameters (**a**. ABTS* scavenging ability **b**. Ferric reducing antioxidant property (FRAP)) of phenolic extracts from citrus peels ($n=3$)
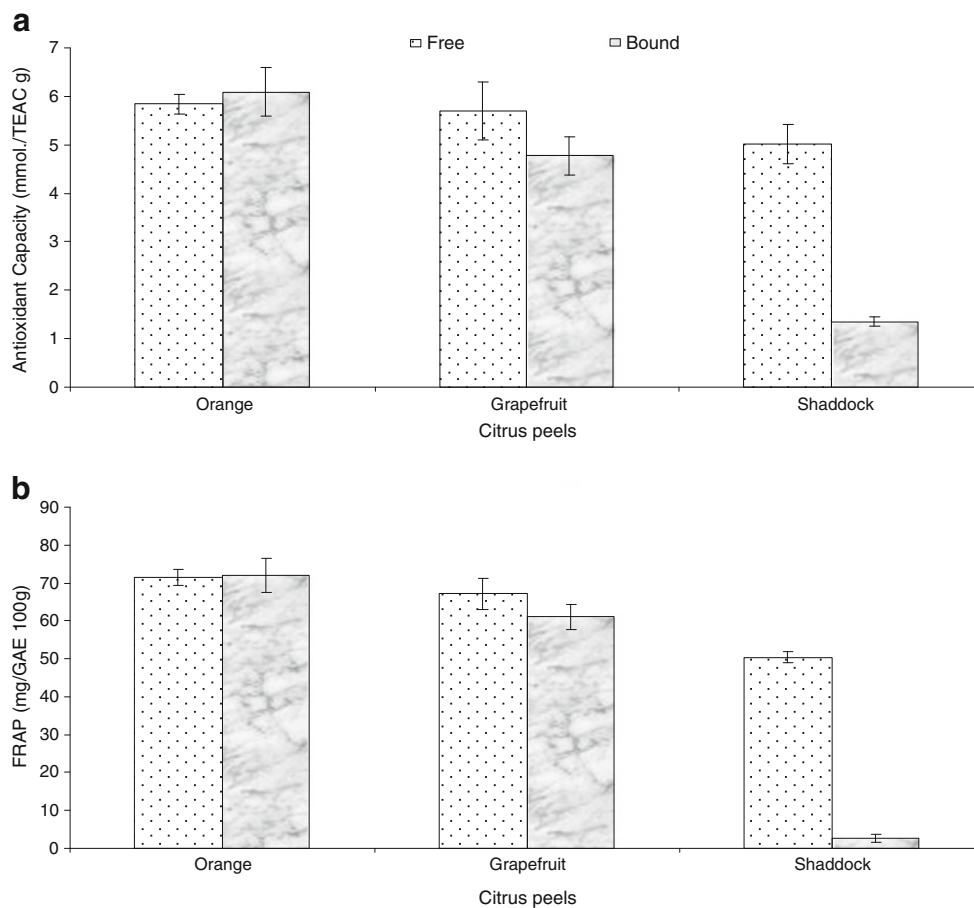
than that of polar and non-polar extracts of some commonly consumed green leafy vegetables in Nigeria (Oboh and Rocha 2008).

Moreover, the ability of antioxidants to chelate and deactivate transition metals, prevent such metals from participating in the initiation of lipid peroxidation and oxidative stress through metal catalysed reaction (Oboh and Rocha 2007a, b). The $Fe^{2+}$ chelating ability of the phenolics as presented in Fig. 1c; could be attributed to the presence of two or more of the following functional groups: -OH, -SH, -COOH, $PO_3H_2$, C=O, $-NR_2$, -S- and –O- in a favourable structure–function configuration (Lindsay 1996; Yuan et al. 2005; Gülçin 2006). Nevertheless, the $Fe^{2+}$ chelating ability of the free and bound phenolics ($EC_{50}$= 0.31–1.3 mg/ml) is higher than their corresponding OH* scavenging abilities ($EC_{50}$=3.8–16.1 mg/ml) as presented in Table 2; these findings agree with some earlier reports on the OH* and $Fe^{2+}$ chelating abilities of phenolics of some plant foods such as leafy vegetables, peppers and spices that have stronger $Fe^{2+}$ chelating than OH* scavenging abilities (Oboh and Rocha 2007b; 2008). The higher $Fe^{2+}$ chelating ability of these extracts are of immense importance in the protective ability of the extracts against oxidative stress, because it is usually too late to attempt to use OH* scavengers for therapeutic purposes, because of the high reactivity of OH* (Bayır et al. 2006).

Moreover, the incubation of rat's pancreas in the presence of 25 μM $Fe^{2+}$ caused a significant increase in the MDA content (137.65%) of the pancreas (Fig. 1d). The mechanism by which iron causes this deleterious effect is through $Fe^{2+}$ catalyzed hydrogen peroxide ($H_2O_2$) decomposition to produce OH* via Fenton reaction (Bayır et al. 2006; Oboh and Rocha 2007a, b). Nevertheless, the phenolic extracts significantly ($P<0.05$) inhibited MDA production in the pancreas in a dose-dependent manner, bound phenolics from orange and shaddock peels had the highest inhibitory effect on MDA production in the pancreas (in vitro), while bound phenolics from grapefruit peels had the least inhibitory effect (Table 2). The possible mechanism through which the phenolic extracts protect the pancreas could be by $Fe^{2+}$ chelation (Oboh and Rocha 2007a, b) and the scavenging of OH* (Puntel et al. 2005; Oboh and Rocha 2007a, b). Since the phenolic extracts had higher $Fe^{2+}$ chelating ability than OH* scavenging ability, $Fe^{2+}$ chelation could be the domineering mechanism through which the phenolics protect the pancreas membrane from $Fe^{2+}$ induced lipid peroxidation in pancreas.

## Conclusion

The additive and/or synergistic effect of the free and bound phenolics constituent of the orange peels could have contributed to the observed antioxidant and medicinal properties orange peels. Therefore, the high antioxidant properties of the free and bound phenolic extracts from orange peels could be harness in the formulation of nutraceuticals and food preservatives.

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