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COMPARISON OF PHYTOCHEMICALS, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF PEELS AND JUICES OF CITRUS SPECIES

***[1]Jesumirhewe, C., [2]Omeke, N. M. and [2]Ogunlowo, O. P.**

[1]Department of Pharmaceutical Microbiology, College of Pharmacy, Igbinedion University Okada, Edo state, Nigeria.
[2]Department of Molecular Sciences, Macquarie University, Australia

*Corresponding Author's Email: ebarunosen2002@yahoo.co.uk

ABSTRACT

Antimicrobial resistance and decreasing efficiency of antimicrobial drugs have resulted in the search for antimicrobial agents as an important strategy for the establishment of alternative therapies in handling difficult infections. This study was carried out to compare the phytochemical components, antioxidant properties and to investigate the antimicrobial activities of juice and peels of some selected *Citrus* spp (*Citrus aurantifolia*, *Citrus sinensis* and *Citrus limon*) against *Escherichia coli* isolates. The undiluted juice and ethanol extracts of the peels were obtained from various *Citrus* spp. The antimicrobial activities of the resulting crude extracts were screened using agar well diffusion assay methods. Gentamicin was used as a standard in the antimicrobial activity studies. The free radical-scavenging activities of the samples were determined using the stable 2, 2-diphenyl-2-picrylhydrazil (DDPH). The Ferric reducing antioxidant power (FRAP) assay and the β-carotene-linoleate bleaching assay were used to determine the total antioxidant activity in the extracts. The qualitative presence of terpenoids, flavonoids, saponins, alkaloids, tannins and cardiac glycosides were also analysed. The extracts of both the juice and the peels of *Citrus aurantifolia* had the highest antimicrobial activity compared to *Citrus limon* and *Citrus sinensis.* Gentamicin also had better inhibitory effects on the isolates used. The crude extracts of the juice and peels of the fruits exhibited significant antioxidant activity and free radical scavenging effects. The phytochemical tests revealed the presence of various active medicinal constituents analysed. Our study showed good promising evidence for the antimicrobial effects of *Citrus aurantifolia* juice and peels.

Keywords: Medicinal plants, Antioxidant activity, Antibacterial activity, *Citrus spp*

INTRODUCTION

The emergence of antibiotic resistant microorganism has reversed the advances of previous years of discovery of antibiotics (Mahida and Mohan, 2006). Antimicrobial resistance and the decreasing efficiency of antimicrobial drugs have resulted in the search for antimicrobial agents as an important strategy for the establishment of alternative therapies in handling difficult infections. Medicinal plants have been described as any plant which contains substances that can be used for therapeutic purpose or which are precursors of chemo-pharmaceutical semi-synthetic drugs (Salih and Abass, 2003). They are one of the best sources to obtain natural occurring antimicrobials and antioxidants as their phytochemicals are more specific (Yusuf, 1991). Medicinal plants have been used in traditional medicine for various human diseases and microbial infections including diarrheal diseases which remain a major cause of morbidity and mortality throughout the world.

The term citrus fruit includes different types of fruits and products, oranges (*citrus sinensis*) been the major fruit in the group and accounting for about 70% of *Citrus spp* (Omidaro and Umekwe, 2013). Citrus fruits are commonly consumed because they contain a high amount of vitamins (Vitamin C), carbohydrate, proteins, amino acids, minerals and antioxidant compounds such as flavonoids. Citrus fruits and juices are also important sources of bioactive compounds such as phenolic compounds and pectins that are important to human nutrition (Kumar *et al*., 2013). Citrus fruits are known to have shown potent antibacterial and antifungal potentials (Mathur *et al.*, 2011). Previous studies indicate that Citrus fruit juice exhibit significant antibacterial effect which is associated with its mineral contents and biologically active constituents (Bansode and Chavan 2012).

In recent years, citrus processing waste (CPW) has been used for the production of single cell protein (Kalara *et al*., 1989), bio-hydrogen (VenkataMohan *et al*., 2009), bio-ethanol (Boluda-Aguilar *et al*., 2010) and multiple enzymes (Puri *et al*., 2008; Puri *et al*., 2011). Functional compounds from CPW such as flavonoids and their further processing can be of great interest to the food and pharmaceutical industry as they retard oxidation of low-density protein, thereby reducing the risk of heart disease (Peluso *et al*., 2006). Flavonoids from citrus peel are comprised primarily of flavanone glycosides (naringin) (Mamma *et al*., 2008), polymethoxylated flavones aglycons (tangeritin) and flavone glycosides (rutin) (Sawalha *et al*., 2009). Thus as a

result of recovery and enzymatic conversion of naringin, the abundant major flavonoid in citrus fruits, citrus peels holds promises for byproduct utilization from citrus production. Naringin can be converted to naringenin, a free radical scavenger compound that reduces oxidative damage to DNA (Gao *et al*., 2006), and L-rhamnose. Rhamnose from naringin hydrolysis can be used as a precursor for the industrial production of aromatic compounds and flavors, as well as a chiral compound for chemical synthesis and as an inducer of recombinant protein expression in *Escherichia coli* (Zverlov *et al*., 2000).

Citrus peel which represents almost one half of the fruit mass contains the highest concentrations of flavonoids (Anagnostopoulou *et al*., 2006). The peels are also rich in nutrients and contain many phytochemicals, they can be efficiently used as drugs or food supplements (Kumar *et al*., 2011). They contain volatile essential oils which are said to be effective in inhibiting microbial growth and disinfecting wounds, among its other medicinal capabilities (Najimu *et al*., 2013). This study was carried out to compare the phytochemical components, antioxidant properties and investigate the antimicrobial activities of *Citrus* fruit juice and peels (*Citrus aurantifolia*, *Citrus sinensis* and *Citrus limon*) against *Escherichia coli* isolated from patients with gastroenteritis in Benin City, Nigeria.

MATERIALS AND METHODS

Plant materials

Fresh fruits of *Citrus aurantifolia*, *Citrus sinensis* and *Citrus limon* were obtained from vegetable market, airport road, Benin City, Nigeria.

Preparation of extracts

The fresh fruits were thoroughly washed under running water and then with sterile distilled water just before they were neatly peeled. The peels were air-dried at room temperature (34ºC) for five days and 100g of peels for each *Citrus sp* was pulverized using a sterilized grinder before extraction. Dried and powdered samples of the peels were extracted separately using a soxhlet extractor for 5hours (Lin *et al.*, 1999). Ethanol 95% was used for the extraction. The extracts were afterwards filtered and concentrated to dryness using a steam bath at 37ºC. Percentage yield of crude extracts obtained was calculated as

$$\text{The \% yield} = \frac{\text{weight of crude extract} \times 100}{\text{weight of dried sample}}$$

Each extract were then stored in a freezer (4ºC) until further use. Juice was extracted from the different fruits using a juice extractor. The different juices obtained were lyophilized, and the concentrates obtained were preserved at 4°C in airtight containers until subsequent use (Oikeh *et al*., 2015). Concentrated juice samples and ethanol extracts of the peels were obtained.

Phytochemical study

The qualitative presence of terpenoids, flavonoids, saponins, alkaloids, tannins and cardiac glycosides were analysed. Phytochemical screening was carried out on both the concentrated juice samples and ethanol extracts of the peels using established protocols as described by Trease and Evans (1989), Sofowora (1993) and Harborne (1998).

Determination of Antioxidant activity

The Ferric reducing antioxidant power (FRAP) assay and the β-carotene-linoleate bleaching assay were used to determine the total antioxidant activity in the concentrated juice samples and ethanolic extracts. The FRAP assay was carried out according to the method of Benzie and Strain (1996) with a little modification in terms of time lapse. The FRAP reagent was freshly prepared containing 1020µL of 300mM sodium acetate pH 3.6, 100µL of 10mM TPTZ solution and 100µL of 20mM ferric chloride. The FRAP reagent was mixed with 10µL aliquots of each extract and the mixture incubated at 37ºC for 15, 30 and 60mins. After this time, the absorbance was read at 593nm. Results were expressed as mol Fe^{+2} per gram of fresh weight (µmol $Fe^{+2}g^{-1}$FW).

Determination of antioxidant activity using β-Carotene-linoleate bleaching method was done according to the method by Velioglu *et al.* (1998). One mililitre of β-carotene solution (0.2 mg/ml chloroform) was pipetted into a round-bottom flask (50ml) containing 0.02 ml of linoleic acid and 0.2 ml of 100 % Tween 20. The mixture was then evaporated at 40°C for 10 mins by means of a rotary evaporator to remove chloroform. After evaporation, the mixture was immediately diluted with 100 ml of distilled water. The distilled water was added slowly to the mixture with vigorous agitation to form an emulsion. A 5ml aliquot of the emulsion was transferred into different test tubes containing 0.2ml of samples in 80% methanol at 1 mg/ml. The tubes were then gently mixed and placed at 45°C in a water bath for 2 hours. Absorbance of the samples was measured at 470 nm using a spectrophotometer at initial time (t=0) against a blank, consisting of an emulsion without β-carotene. Standards BHT at the same concentration with samples were used as comparison. An amount of 0.2 ml of 80% methanol in 5 ml of the above emulsion was used as the control. The measurement was carried out at 30mins intervals for 90mins. All determinations were performed in duplicates. The antioxidant activity (AA) was calculated according to the following equation:

$$AA = [1 - (Ao - At) / (A^{o}o - A^{o}t)] \times 100$$

where, Ao and $A^{o}o$ are the absorbance values measured at initial time of the incubation for samples and control respectively, while At and $A^{o}t$ are the absorbance values measured in the samples or standard and control at T = 90 min.

The Determination of DPPH radical scavenging activity was also used in estimation of the antioxidant activity in both the concentrated juice samples and ethanolic extracts of the sample peels. The free radical scavenging activity of the *Citrus spp* and the ascorbic acid (standard) were determined according to the 2, 2-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay as described by Lee *et al.* (2003). Two hundreds

microliters of sample extract (1-8mg/ml in 80% (v/v) methanol) or ascorbic acid (standard) (0.005-1.28 mg/ml) was mixed with 1 ml of 0.05 mM DPPH in 80% methanol. The mixture was shaken vigorously and left to stand for 30 minutes at room temperature in a dark room. The absorbance was read using a UV-Vis spectrophotometer at 517 nm with 200 µl of 80% methanol and 1 ml DPPH served as a blank. The scavenging effect on the DPPH radical is calculated using the following equation:

DPPH scavenging effect (%) = $[(A_0 – A_1)/A_0) \times 100]$, where A_0 is the absorbance of the control reaction, and A_1 is the absorbance of presence of all of the extract samples and standard.

EC50 (the 50% inhibitory concentration value) is defined as the total antioxidant necessary to decrease the initial DPPH radical concentration by 50%. Triplicate measurements were carried out, and their scavenging effect was calculated based on the percentage of DPPH scavenged.

Antimicrobial assay

Test microorganism

Eleven *Escherichia coli* isolates were collected from the medical microbiology laboratory of University of Benin Teaching Hospital, Benin City, Nigeria. The isolates were obtained from patient's stools diagnosed for gastroenteritis.

Antimicrobial susceptibility assay

The antimicrobial activities of the resulting crude extracts were screened using agar well diffusion assay methods (Perez *et al*., 1990). Ditches were made on each culture plate of the organisms with the aid of a sterile cork borer {6mm}. The juice and peel extracts were reconstituted to 100mg/ml. Water (negative control) and 50µg/ml gentamicin (positive control). Exactly 0.2ml of the extract, negative and positive control were dispensed into the wells and labelled accordingly. The plates were left on the bench for 1 hour at room temperature to enhance the diffusion of the extract into the agar and later incubated at 37^0C for 24hrs. The zones of inhibition were measured and recorded.

Results and Discussion

This study revealed important medicinal phytochemicals such as terpenoids, flavonoids, tannins, alkaloids, saponins and cardiac glycosides considered as active medicinal chemical constituents in the citrus samples. Table 1 shows *Citrus aurantifolia, Citrus sinensis* and *Citrus limon* were rich in the phytochemicals investigated.

Table 1: Phytochemical results of citrus extracts

	PEELS			JUICE		
	Citrus sinensis	*Citrus aurantifolia*	*Citrus limon*	*Citrus sinensis*	*Citrus aurantifolia*	*Citrus limon*
Tannins	+	-	+	-	-	+
Flavonoids	+	+	+	+	+	+
Saponins	+	-	+	-	-	-
Cardiac glycosides	+	+	+	-	+	-
Alkaloids	+	+	-	+	+	+
Terpenoids	+	-	+	-	+	+

Key

+ Present ; - absent

These results agree in part with findings of Kumar *et al*. (2011) who reported the presence of alkaloids, terpenoids and flavonoids in *Citrus aurantifolia* and *Citrus limon* juice. This study shows absence of saponins both in *Citrus aurantifolia* and *Citrus limon* juices and cardiac glycosides was only absent in *Citrus limon* juice. Alkaloids and flavonoids were detected in the *Citrus sinensis* juice. Variation in phytochemicals detected could be as a result of differences in fruit species and plants geographical location. The phytochemical screening of *Citrus sinensis* peel extracts showed the presence of all the tested phytochemicals while absent in *Citrus limon* and *Citrus aurantifolia* peels. This correlates with an earlier report that citrus peels are rich in nutrients and contain many phytochemicals hence, they can be efficiently used as drugs or food supplements (Kumar *et al*., 2011). Phytochemical constituents in the plant samples are known to be biologically active and they have different activities such as antioxidant, antimicrobial, antifungal, and anticancer activities (Hossain and Nagooru, 2011). Results from this study confirm that citrus fruits are rich sources of phytochemicals (Okwu *et al*., 2008).

Fig 1.0 below shows the measurements of the reductive ability of the extracts investigated from the Fe^{+3} - Fe^{+2} transformations using the method of Okwu and Morah (2007).The crude extracts exhibited significant antioxidant activity both in the juice and peel extracts. An earlier report Divya *et al*., (2016) had observed a direct correlation between antioxidant activity and reducing power of certain citrus plant extracts. The reducing power of the different extracts of fruit components increased with increasing concentration in all solvent hence an increase in antioxidant property. The FRAP assay show that the juice had more antioxidant property than the peel extracts. This result correlates with a previous report (Divya *et al*., 2016). It may be suggested that the reducing power of the juices contribute significantly towards the observed antioxidant effect.

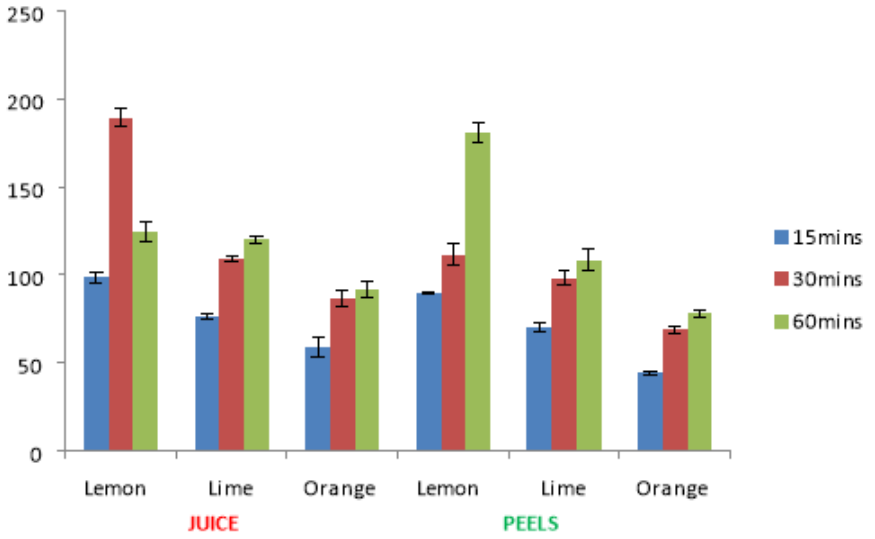

Fig 1: FRAP Activity result

The results of antioxidant activities observed for *C. limon*, *Citrus aurantifolia* and *Citrus sinensis* are 98.56± 2.89, 76.32 ± 1.92, 58.74 ± 5.5 µmol/L Fe(II)/g of the extract for juice respectively. This concentration was observed to increase with time, but this was generally lower in the peels with 89.34± 0.60, 70.22 ± 2.96, 44.05 ± 0.97 µmol/L Fe(II)/g of the extract for *C. limon*, *Citrus aurantifolia* and *Citrus sinensis*. The FRAP results show *Citrus limon* and juice to have a good reductive power and hence may have significant antioxidant activity.

The degradation rate of the samples by β-carotene bleaching method is shown in Fig 2.0. The *β*-carotene bleaching estimates the relative ability of antioxidant compounds in plant extracts to scavenge the radical of linoleic acid peroxide that oxidizes *β*-carotene in the emulsion phase. *β*-carotene in the absence of the antioxidant undergoes a rapid decolourization since the free linoleic acid radical attacks the β-carotene, which loses the double bonds and, consequently, its orange colour.

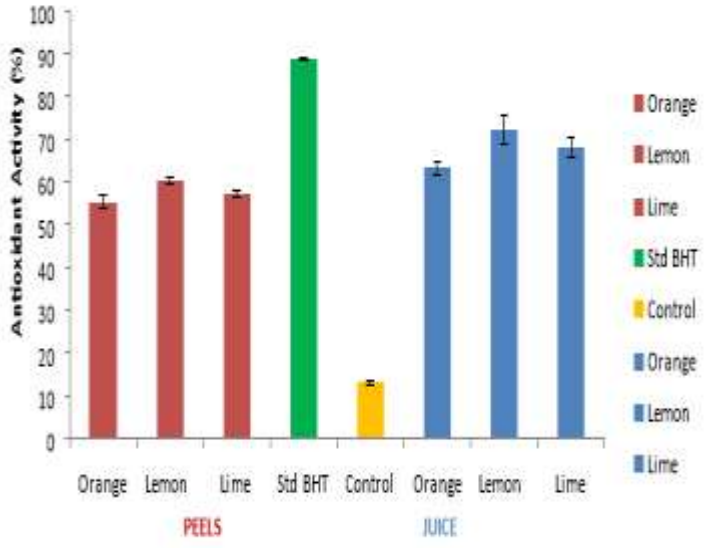

Fig 2: β-carotene bleaching result

The three Citrus fruits were able to inhibit linoleic acid oxidation nevertheless much lower than BHA (88.65±0.22%). As expected, the control (standard BHT) had the highest degradation rate because it contains no antioxidant that can slow down the bleaching of β-carotene. Degradation rate of the peels was slightly higher as compared to the juice. Our study shows that the juice generally exhibited higher antioxidant activities. *Citrus limon* juice exhibited the lowest degradation rate and therefore the highest antioxidant activity of 72.12 ± 3.17 by the β-carotene bleaching method which decreased to 68.22 ±2.36 and 63.30±1.57 in *Citrus aurantifolia* and *Citrus sinensis* respectively. The result is similar in the peels but the antioxidant activities were lower. *Citrus limon, Citrus aurantifolia and Citrus sinensis* had 60.11±0.84, 57.15 ±0.71 and 55.17 ±1.64 respectively.

Fig 3.0 shows mean EC50 values of peels and juice of the citrus fruits. Determination of DPPH radical scavenging activity is a widely used method to evaluate the free radical scavenging activities of various samples (Lee *et al*., 2003). The juices were able to reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine at all concentrations. The juice concentrates had higher DDPH radical scavenging activity compared to the peels extract. The results showed that high flavonoids contents of peels and juice of Citrus fruits may cause high antioxidant activity of this plant. Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities (Aiprakash *et al.*, 2009).

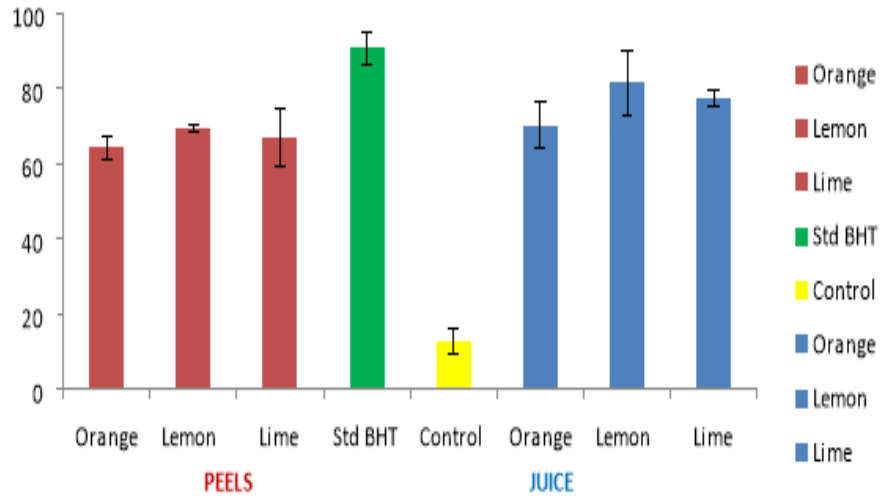

Fig 3: DPPH radical scavenging result

The standard antioxidant ascorbic acid had significantly higher percentage inhibitions of the DDPH radical than the juice concentrates and the peels extract at the concentrations studied. This confirms a previous study that showed the standard antioxidant, ascorbic acid to have a significantly higher percentage inhibition of the DPPH radical than all the juice concentrates at the both concentrations studied (Benzie and Strain, 1996). Previous studies have shown free radicals present in organs of the human body cause oxidative damage to molecules like nucleic acid, proteins resulting in several degenerative diseases (John *et al*., 2017). Phenolic compounds in fruit peels are capable of neutralizing free radicals thereby preventing the onset of degenerative disease (John *et al*., 2017). Table 2.0 below shows the inhibitory activity of the extracts of lime, lemon, and orange on the bacteria at 100mg/ml. Zones of inhibition were seen against all the strains using *Citrus aurantifolia* and *Citrus limon* confirming the antimicrobial activity of both the juice and peels samples. However, *Citrus aurantifolia* extracts (juice and peel) showed higher inhibition on bacteria than the *Citrus limon* and *Citrus sinensis* extracts. For the control, there was no zone of inhibition on any of the bacteria used. The positive control (Gentamicin) had better inhibitory effects on all the isolates used.

Table 2: Results of antimicrobial activity against *E. coli* isolates

	JUICE (mm)			PEELS (mm)			-ve cont (mm)	+ve cont (mm)
	Lime	Lemon	Orange	Lime	Lemon	Orange		
E. coli 1	22	22	23	19	17	15	-	15
E. coli 2	30	19	14	25	19	12	-	13
E. coli 3	28	27	10	22	18	-	-	20
E. coli 4	29	28	-	22	18	14	-	30
E. coli 5	26	28	28	24	16	12	-	30
E. coli 6	30	25	-	23	15	-	-	28
E. coli 7	30	25	22	23	15	14	-	20
E. coli 8	25	24	-	25	18	10	-	22
E. coli 9	26	26	10	22	16	-	-	29
E. coli 10	30	28	27	26	21	12	-	28
E. coli 11	26	24	23	26	22	-	-	28

Key: -ve cont: Negative control, +ve cont: Positive control

CONCLUSIONS

Gastroenteritis remains one of the most prevalent public health problems and this study showed that these extracts possessed various medicinal properties which made them useful in treating gastroenteritis infections. Our study showed good promising evidence for the antimicrobial effects of *Citrus aurantifolia* juice and peels. There are a lot of natural products in our environment which we can explore to combat many emerging and re-emerging infectious diseases. This study has provided additional information on the health benefits of consuming fruits. This study showed that the peels and juices of *C. limon, Citrus aurantifolia* and *Citrus sinensis* can be considered as a good source of natural antioxidants and antimicrobial compounds which can possibly be incorporated into drug formulations. Further studies are required for quantitave analysis of each individual active compound and also *in vivo* studies are needed for better understanding of their mechanism of action as an antioxidant. Studies aimed at isolation of characteristic antimicrobial compounds and *in vivo* studies that may confirm and support the in vitro findings are also recommended.

Conflict of Interest

None declared

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