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ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF *CITRUS PARADISI* (GRAPEFRUIT SEED) EXTRACTS

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ABSTRACT
The seeds and the seeds extract of *Citrus paradisi* (Rutaceae) have been widely used in traditional medicine to treat a variety of conditions such as ulcers, cataracts, urinary and alimentary tract infections. The juice is taken mainly for its medicinal properties in large area of sub-Saharan Africa. However, very little is known about the cellular actions by which this plant mediates its therapeutic effects. This study investigated the antimicrobial and antioxidant activities of the extracts and isolated compounds from the seeds extract. The structures of the isolated compounds were established using spectroscopy studies and identified as obacunone (**1**), nomilin (**2**), limonin (**3**), nomilinic acid (**4**) and obacunone-17-O-β-D-glucopyranose (**5**). Their antioxidant activity was evaluated using DPPH (2, 2-diphenyl-1-picrylhydrazyl) spectrophotometric assay. None of the isolated compounds showed antimicrobial activities but nomilinic acid showed a weak antioxidant property. It scavenged 13.09 % of the DPPH free radical at the highest concentration of 200 µM tested.
Oil of the seeds of *C. paradisi* investigated using gas chromatographic analysis was found to contain both saturated and unsaturated fatty acids. The major saturated fatty acids present were lauric and stearic acids with percentage composition of 2.61 and 0.07 respectively while the major unsaturated fatty acids present in the seed were linoleic and linolenic acids with percentage compositions of 5.02 and 3.84 respectively.
KEYWORDS: *Citrus paradisi*; antimicrobial; antioxidant activity; DPPH; radical scavenging.

INTRODUCTION

Citrus paradisi (Macfad) family; Rutaceae is an evergreen tree originally of Asiatic South West (Tropical Asia, West India). It has thorny, prickly stems[1]. The grapefruit has a yellow or yellow-red and juicy pulp with a distinctive sour-bitter taste. The fruit has not only been enjoyed for its palatable qualities, but its medicinal values were known to ancient Greeks. The flesh of this fruit is used as a cure in poisoning and also used to refresh the break[2]. The seed extracts of *Citrus paradisi* have been used for the treatment of ulcers, cataracts, urinary and alimentary tract infections[3, 4]. The oil from the peel of grapefruit has been used as insecticide and antifeedant[5].

Dietary polyphenols found in vegetables and fruit could reduce the risk of oxidative stress related diseases such as cancer, inflammation and cardiovascular diseases[6]. It has been demonstrated that the incidence of lung cancer is associated to low intake of vegetabales, fruits and carotenoids[7]. Phytonutruent constituents such as phenols, flavonoids, terpenes and glucosinolates appear to be responsible for the chemoprotection observed in vegetables and fruits. Polyphenols and other reducing agents such as vitamin C and E referred to as antioxidants found in citrus fruits, vegetables and other fruits have been demonstrated to protect the body against oxidative stress related diseases[6, 8, 9].

Grapefruit has high vitamin C content and is therefore valuable to the immune system. It helps protect against colds and flu, has a very positive effect on obesity and also has diuretic properties, helping to remove excess water from the body and is therefore also great for treating cellulite. It has an uplifting effect on the mood and helps with stress and depression. It is used with great success to combat muscle fatigue and stiffness while stimulating the lymphatic system and thereby clearing the body of toxins. It helps to clear congested oily skin and also assist with acne while toning the skin and tissues. Grapefruit is used in hair care to promote hair growth[10].

MATERIALS AND METHODS

General

Adsorption chromatography was performed with Accelerated Gradient Chromatography (AGC) on silica gel, particle size 0.040-0.063 µm (Mereck, 230-400 mesh ASTM) in ascending mode using AGC workstation from Baeckstron Saparo AB, Lindigo, Sweden. The NMR (^{1}H and ^{13}C-NMR) spectra were recorded at 200, 600 (^{1}H) and 50, 150 (^{13}C) MHz on Varian NMR (Gemini 200MHz) and Bruker NMR 600 MHz respectively. Chemical shifts were reported in ppm (δ). TLC were performed using silica gel F_{254} (Merck) pre-coated plates and spots were detected by visualizing under UV lamp at 254nm and 366nm spraying with vanillin-sulphuric acid solution (1 %), ferric chloride and solution of DPPH in methanol (0.1 %). UV Versa-max® microplate reader was employed for the DPPH spectrophotometric assay. All solvents used were General Purpose Reagents (GPR) and were redistilled before use. L-ascorbic acid (Sigma), DPPH 2, 2-dipheny-1-picrylhydrazyl (Sigma) and absolute methanol (Fluka).

Plant Materials

The grapefruit seeds were collected on campus premises of Obafemi Awolowo University (OAU), Ile-Ife, Nigeria in February 2002. The grapefruits were authenticated by Mr. T.K. Odewo, a taxonomist in the Forest Reserve Institute of Nigeria (FRIN) and voucher specimen was deposited at FRIN Herbarium, Ibadan, Nigeria with voucher number FHI 107722.

Extraction and Isolation of Compounds

The air-dried and powdered seeds (1.5 kg) of *Citrus paradisi* was extracted at room temperature for 24 h with 50 % aqueous EtOH. The extract was concentrated, suspended in H_2O and sequentially partitioned with EtOAc and *n*-BuOH. The EtOAc portion was subjected to Accelerated Gradient Chromatographic (AGC) separation on silica using a gradient of *n*-hexane (200 ml), a doubling gradient of EtOAc - *n*-hexane, EtOAc, doubling gradient of EtOAc - MeOH and finally MeOH to give eight fractions (Fr CPEA-CPEH). Fr

CPEE was pure and designated as compound **1** (20 mg). Repeated column chromatography of Fr CPEG (1.45 g) on silica *n*-hexane (100 ml), a doubling gradient of EtOAc - *n*-hexane and EtOAc afforded compound **2** (249 mg) and fraction CPEGa (488 mg) which was further chromatographed on silica n-hexane (100 ml), a doubling gradient of EtOAc - *n*-hexane and EtOAc afforded a compound designated as compound **3** (74 mg). Fr CPEH (626 mg) was subjected to repeated column chromatography on silica *n*-hexane (100 ml), a doubling gradient of EtOAc - *n*-hexane, EtOAc, doubling gradient of EtOAc - MeOH and MeOH which afforded compound **4** (40 mg).

The *n*-BuOH portion (12.0 g) was subjected to repeated Accelerated Gradient Chromatographic separation on silica using a gradient of n-hexane (100 ml), a doubling gradient of EtOAc – *n*-hexane, EtOAc, doubling gradient of EtOAc - MeOH, MeOH and sephadex LH-20 (Toluene - EtOH; 1:1, 1:3) gave an unclean fraction CPBC (133 mg). This was further purified on a reverse phase RP-18 lobar column on a doubling gradient of MeOH - H_2O which afforded compound **5** (23 mg).

The structures of compounds **1-5** were deduced by comparison of their spectral data (^{1}H and ^{13}CNMR) with those of literature.

Spectroscopic Analysis of Compounds 1-5

Obacunone 1 is a white crystalline solid. ^{1}H NMR (200 MHz, acetone-d_6) δ ppm: 5.85 (1H, d, J = 12 Hz, H-1), 6.80 (1H, d, J = 12 Hz, H-2), 3.65 (1H, s, H-15), 5.5 (1H, s, H-17), α-furans; 7.63 (1H, m, H-21), 7.58 (1H, m, H-23) and β-furan; 6.5 (1H, m, H-22), 5C-Methyls at δ1.5, 1.8, 1.4, 1.8, 2.0 (each 3H,s).

^{13}C NMR (50 MHz, acetone-d_6) δ ppm; 157.8(C-1), 122.4(C-2), 166.7(C-3), 86.4(C-4), 56.9(C-5), 39.8(C-6), 208.0(C-7), 53.0(C-8), 49.0(C-9), 43.4(C-10), 19.4(C-11), 32.5(C-12), 37.6(C-13), 65.5(C-14), 53.4(C-15), 166.7(C-16), 78.0(C-17), 20.6(C-18), 16.5(C-19), 121.0(C-20), 143.4(C-23), 110.0(C-22), 141.7(C-21), 16.2(C-28), 26.5(C-29), 31.5(C-30). The spectral data agreed very well with literature[11].

Nomilin 2 is a white solid, m.p. 166^0 C IR (Nujol mull): C=O, 1721 cm^{-1}, C-O, ester 1292 cm^{-1}, C-O-C ether 1114 and 1155 cm^{-1}. ^{1}H NMR (200 MHz, acetone-d_6) δ ppm: 3.81(1H, s, H-15), 4.95(1H, d, J = 12 Hz, H-1), 5.5(1H, s, H-17), α-furans; 7.61(1H, m, H-23), 7.58(1H, m, H-21), and β-furan; 6.5(1H, m, H-22) 5C-methyls at δ 1.12, 1.30, 1.40, 1.50, 1.53 (each 3H, s), an acetyl methyl at δ 2.85(3H, s).

^{13}C NMR (50 MHz, acetone-d_6) δ ppm: 70.8(C-1), 35.5(C-2), 169.3(C-3), 84.5(C-4), 51.2(C-5), 38.9(C-6), 207.0(C-7), 53.0(C-8), 44.5(C-9), 44.3(C-10), 17.2(C-11), 32.4(C-12), 37.6(C-13), 65.6(C-14), 53.5(C-15), 166.9(C-16), 78.1(C-17), 20.9(C-18), 17.3(C-19), 120.3(C20), 143.3(C-21), 109.8(C-22), 141.4(C-23), 16.6(C-28), 23.5(C-29), 33.6(C-30), acetate carbonyl 169.3 and acetate methyl 20.9. The spectra data were in agreement with literature[12].

Limonin 3 is a white solid, m.p. 298 ^{0}C and EI-MS m/z of 471. ^{1}H NMR (600 MHz, acetone-d_6) δ ppm: 4.29(1H,s,H-1), 2.81(2H, d, J = 2.87 Hz, H-2), 2.60(1H, dd, J = 13 Hz, H-5), 3.17(2H, dd, J = 15 Hz, H-6), 2.42(2H, dd, J = 3,15 Hz, H-6), 4.10(1H, s, H-15), 5.5(1H, s, H-17), 5.0(2H, dd, J = 13 Hz, H-19), 4.64(2H, dd, J = 13 Hz, H-19, germinal coupling), α-furans; 7.62(1H, m, H-23), 7.58(1H, m, H-21), and β-furan; 6.5(1H, m, H-22).

^{13}C NMR (150 MHz, acetone-d_6) δ ppm: 79.6(C-1), 36.0(C-32), 169.6(C-3), 80.1(C-4), 59.7(C-5), 36.7(C-6), 207.7(C-7), 51.5(C-8), 48.0(C-9), 46.3(C-10), 18.6(C-11), 30.5(C-12), 38.5(C-13), 66.8(C-14), 54.4(C-15), 167.2(C-16), 78.1(C-17), 20.1(C-18), 65.4(C-19), 121.2(C-20), 143.6(C-21), 110.5(C-22), 142.0(C-23), 17.1(C-28), 21.3(C-29), 29.8(C-30). The spectra data were in agreement with literature[11].

Nomilinic acid 4 is a light yellowish non-crystalline solid, m.p. 108-110 ^{0}C. ^{1}H NMR (600 MHz, MeOD) δ ppm: 6.47(1H, s, H-1), 3.2(2H, br., H-2), 2.1(1H, q, H-5), 2.9(2H, t, H-6), 2.28(1H, d, J = 12, H-9), 2,48(2H, m, H-11), 1.87(2H, m, H-12), 3.68(1H, s, H-15), 5.52(1H, s, H-17), α-furans; 7.55(1H, m, H-23), 7.5(1H, m, H-21), β-furan; 6.45(1H, m, H-22), 5C-methyls at δ 1.12, 1.32, 1.37, 1.35, 1.18(each 3H, s), an acetate methyl 2.25(3H, s).

^{13}C NMR (150 MHz, MeOD) δ ppm: 77.0(C-1), 60.3(C-2), 171.0(C-3), 73.5(C-4), 52,5(C-5), 38.4(C-6), 210.7(C-7), 52.6(C-8), 44.2(C-9), 46.0(C-10), 18.7(C-11), 31.5(C-12), 36.9(C-13), 65.2(C-14), 52.7(C-15), 168.2(C-16), 78.5(C-17), 19.95(C-18), 15.6(C-19), 120.0(C-20), 141(C-21), 109.5(C-22), 143(C-23), 31.4(C-28), 27.2(C-29), 15.4(C-30), acetate methyl 19.7 and acetate carbonyl 168.5. The spectra data were in agreement with literature[13].

Obacunone-17-O-β-D-glucopyranoside 5 is a colourless solid. ^{1}H NMR (600 MHz, MeOD) δ ppm: 6.47(1H, d, J = 12 Hz, H-1), 5.98 (1H, d, J = 12 Hz, H-2), 3.25 (1H, t, H-5), 2.4 (2H, dd, J = 12, 6 Hz, H-6), 2.63 (1H, t, H-9), 1.85 (2H, q, H-11), 2.13 (2H, q, H-12), 2.83 (1H, s, H-15), 5.5 (1H, s, H-17), 5-C-methyls; (1.47, 1.1, 1.38, 1.47, 0.86, all singlet), α-furans; 7.74(1H, m, H-21), 7.35 (1H, m, H-23), β-furans; 6.64 (1H, m, H-22), sugar; 4.38 (1H, d, J ,= 8.4Hz, H-1'), 3.16 (1H, H-2'), 3.3 (1H, H-3'), 3.3 (1H, H-4′), 3.29 (1H, H-5′), 3.66 (2H, dd, J = 6.6, 2.4 Hz, H-6′).

^{13}C NMR (150 MHz, MeOD) δ ppm: 151 (C-1), 118.5 (C-2), 167.5 (C-3), 84.2 (C-4), 50.4 (C-5), 40.3 (C-6), 212 (C-7), 52.0 (C-8) , 47.0 (C-9), 44.5 (C-10), 17.5 (C-11), 27.1 (C-12), 44.7 (C-13), 69.4 (C-14), 60.0 (C-15), 174.6 (C-16), 78.2 (C-17), 21.8 (C-18), 14.3 (C-19), 125.5 (C-20), 142.4 (C-21), 112.4 (C-22), 140 (C-23), 28.6 (C-28), 24.8 (C-29), 19.2 (C-30), 104 (C-1′), 75.8 (C-2′), 76.8 (C-3′), 70.3 (C-4′), 74.3 (C-5′), 61.4 (C-6′). The spectra data were in agreement with literature[14].

Evaluation of Antimicrobial Activity

Agar diffusion method

Antibacterial activity tests were carried out using agar diffusion (hole-in-plate) method[15].

A stock solution concentration of 20.0 mg/ml was used with reference antibiotic concentration: Streptomycin (l.0mg/ml) and acriflavine (6.0 mg/ml). Negative control was aqueous MeOH (50 %).

The agar diffusion (hole-in-plate) method was used, with 18 h broth cultures of the following organisms: *Staphylococcus aureus*, NCTC 6571; *Escherichia coli* NCTC 10418, *Bacillus subtilis* NCTC 8236 and *Pseudomonas aeruginosa* ATCC 10145. A 0.5 ml of the appropriate culture was inoculated into 50 ml of molten and cooled nutrient agar (Oxoid). This was mixed, poured into the petri dish (14 cm, diameter) and allowed to set. Eight equidistant holes were bored in the agar using a sterile glass cork borer (9.0 mm, diameter) and the plates were incubated at 37^0 C for 30 minutes. A 100 µl of the stock solution of 20.0 mg/ml of the appropriate sample was each delivered into appropriately labeled hole in each plate

and 100 µl each of streptomycin (reference antibiotic, positive control) and blank solvent (negative control) were delivered into their respective holes. Each sample was tested in duplicate and the plates were incubated at 4 ^{0}C for 30 minutes to allow for diffusion and then incubated at 37 ^{0}C for 18 hours. The diameter of the zones of inhibition was measured using a ruler and recorded (to the nearest mm) for each hole in the plates.

Bioautography method

The bioautography test employs the agar overlay technique whereby about 100 µl of stock solution (1.0 mg/ml) of each extract/fraction were loaded as bands (5 mm - 7 mm) onto an appropriately labeled silica gel TLC plate (5 x 10 cm^2), dried and developed in duplicates using appropriate solvent system. Molten agar inoculated with test organisms were spread over the developed thin-layer chromatographic plate (5 x 10 cm^2). The spread agar was allowed to set on the plates and thereafter incubated at 37 ^{0}C for 18 h. After incubation, inhibition zones were made visible by spraying the plates with aqueous solution (2.5 mg/ml) of thiazolyl blue (methyl thiazolyltetrazolium chloride MTT).

Evaluation of Antioxidant Activity

Qualitative Assay

Chromatograms of EtOAc fraction, column fractions and the compounds **1-5** were developed in EtOAc : Hexane (7:3) on silica gel plate F_{254}, dried and sprayed with 0.1 % DPPH in MeOH.

DPPH Quantitative spectrophotometric assay

This was carried out as described by [16] with a slight modification. Sample stocks of 4 (200 µM) and L-ascorbic acid (100 µM) were separately diluted to a final concentration of 200.0, 100.0, 50.0, 25.0, 12.5, 6.25 µM and 50.0, 25.5, 6.25, 3.13, 1.56 µM respectively in MeOH. Twenty micro litres of 0.3 mM DPPH in methanol was added to 50 µL of each concentration of sample tested and allowed to react at room temperature in the dark for thirty minutes. Blank solutions were prepared with sample solution (50 µL) and 20 µL of methanol only while the negative control was 20 µL DPPH solution plus 50 µL methanol. The decrease in absorbance was measured at 515 nm. Absorbance values obtained were converted to percentage antioxidant activity (AA %) using the formula:

$AA\ \% = 100 - \{[Abs_{sample} - Abs_{blank}) \times 100]/Abs_{control}\}$

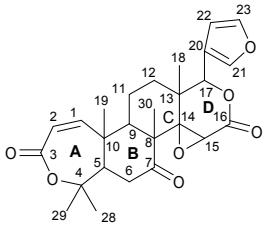
Where Abs_{sample} is the absorbance of the sample, Abs_{blank} is the absorbance of the blank and $Abs_{control}$ is the absorbance of the control). L-ascorbic acid (vitamin C) was used as a positive control.

Effective concentration (EC_{50}) is the concentration of the test sample which will bring about 50 % inhibition of the DPPH free radicals. The value was obtained from the linear regression of plots of mean percentage of the antioxidant activity (AA %) against concentration of the test compounds (µM) from the three replicate assays. A low EC_{50} value is an indication of strong antioxidant activity.

Statistical analysis: The results are expressed as mean ± SEM (Standard error of mean). The EC_{50} values obtained from the regression plots (Stigma Plot® 2001, SPSS Science) showed good coefficient of determination ($r^2 \geq 0.971$).

RESULTS AND DISCUSSION

The ethyl acetate fraction of the ethanolic extract of *Citrus paradisi* seeds afforded compounds **1-4** after purification by repeated column chromatography on silica. The butanolic fraction of the ethanolic extract afforded compound **5** after repeated column chromatography on silica and sephadex LH-20.. These compounds were identified by comparing their spectroscopic data with those reported in literature as Obacunone **1**, nomilin **2**, limonin **3**, nomilinic acid **4** and obacunone-17-O-β-D-glucopyranoside **5.**



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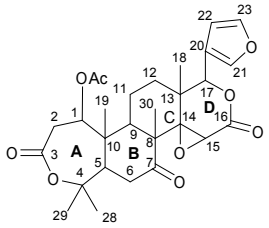

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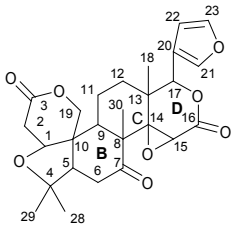

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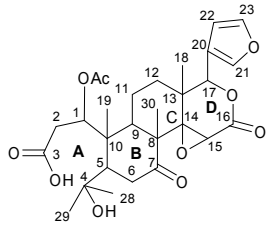

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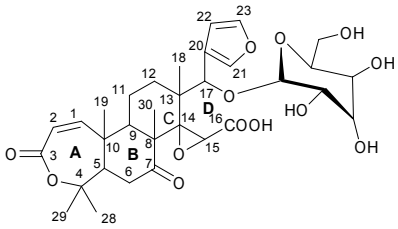

[5]

Compounds **1-5** isolated demonstrated no antimicrobial activity in the hole in plate method or bioautography. This result prompted the gas chromatographic (GC) examination of the seed oil since the whole seed (which contains both the oil and limonoids) was used in the treatment of urinary tract infection[3]. The results of the gas chromatographic analysis of the seed oil of *C. paradisi* revealed the presence of both saturated and unsaturated fatty acids which have been reported in literature to have antibacterial activity[17]. The major saturated fatty acids present were lauric and stearic acids with percentage compositions of 2.61 and 0.07 respectively, while the major unsaturated fatty acids present in the seed oil were linoleic and linolenic acids with percentage compositions of 5.02 and 3.84 respectively. This finding suggests that the observed clinical effectiveness in UTI could be due to the presence of unsaturated fatty acids such as linoleic and linolenic acids since fatty acids had been shown to possess antibacterial properties[17].

Although the hole-in-plate technique and bioautographic methods used in the antibacterial evaluation of the seed extract and the compounds **1-5** isolated did not show any activity, the reported clinical effect may in addition to the effect of unsaturated fatty acid, be due to other effects on the cells, which cannot be detected using these screening methods. The mechanism of action of the compounds may be due, in part, to inhibition of adhesion of infecting bacterial to the uroepithelium. This inhibition would make the organism

more susceptible to host defenses and/or antibiotics than when attached to the epithelium. Infact, in the reported case study showed that a patient with profuse multiply resistant *Ps. aeruginosa* UTI was treatable with a week course of tarivid, to which the organism was initially resistant[3], after chewing and swallowing fresh grapefruit seeds for 2 weeks. Cranberry juice had been similarly reported to be useful in the prevention of the reoccurrence of UTI in women at risk of developing such infections. The main mechanism proposed was that cranberry juice inhibited the adhesion of infecting bacterial such as *E. coli* to the epithelium, which prevents the infection of the mucosal surface of the urinary tract. Two compounds; proanthocyanidin and fructose which in routine screening antibacterial test would not be active, were found to account for the activity of cranberry juice[18]. Further studies are, however, needed to establish whether the compounds isolated from grapefruit seeds in the present study would inhibit adhesion *in vitro* or not. It is instructive to note that alternative test methods may have to be included in initial antimicrobial activity screening protocols to take care of compounds that may be acting as anti-adherents.

Nomilinic acid **4** demonstrated very weak activity. It scavenged 13.09% of the DPPH free radical at the highest concentration of 200 µM tested compared to L-ascorbic acid (EC_{50}= 13.18 ± 0.63 µM) used as standard antioxidant agent. The antioxidant activity of **4** is unique in that it is rare to have non phenolic compounds with antioxidant properties, because most antioxidant compounds are usually phenolic.

CONCLUSION

This study confirmed the earlier report that self made grapefruit seed extract had no antibacterial activity. However, the presence of unsaturated fatty acids as revealed in this study and with antioxidant activity of the nomilinic acid **4** may be responsible for the clinical effectiveness of the seeds and the use of the seed extracts in folk medicine.

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