ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF CITRUS PARADISI (GRAPEFRUIT SEED) EXTRACTS
Faley, F J*1, Ogundaini AO2, Olugbade AT3
1Faculty of Science, Ekiti State University, Ado-Ekiti, Ekiti State, Nigeria
2Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria
3Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria
*Email: fjalleye2002@yahoo.com

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 the 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.

INTRODUCTION
Citrus paradisi (Macfayd) family; Rutaceae is an evergreen tree originally of Asiatic South West (Tropical Asia, West India). It has thorny, prickly stems. 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. The seed extracts of Citrus paradisi have been used for the treatment of ulcers, cataracts, urinary and alimentary tract infections. The oil from the peel of grapefruit has been used as insecticide and antifeedant.

Dietary polyphenols found in vegetables and fruit could reduce the risk of oxidative stress related diseases such as cancer, inflammation and cardiovascular diseases. It has been demonstrated that the incidence of lung cancer is associated to low intake of vegetabales, fruits and carotenoids. Phytoalexin 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. 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.

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 Sarapo AB, Lindigo, Sweden. The NMR (1H and 13C-NMR) spectra were recorded at 200, 600 (1H) and 50, 150 (13C) 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 F254 (Merck) precoated 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 Vesa-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-diphenyl-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. Ode, 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 H2O 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 - H2O which afforded compound 5 (23 mg).

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

Spectroscopic Synthesis of Compounds 1-5

**Obacunone 1** is a white crystalline solid. 1H NMR (200 MHz, acetone-d6) δ ppm: 8.54 (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), SC-Methyls at δ1.5, 1.4, 1.8, 2.0 (each 3H, s).

13C NMR (50 MHz, acetone-d6) 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), 143.3(C-23), 110.0(C-22), 141.7(C-21), 62.8(C-28), 26.5(C-29), 31.5(C-30). The spectral data agreed very well with literature.

**Nomilin 2** is a white solid, m.p. 166°C IR (Nujol mull): C=O, 1721 cm-1, C-O, ester 1292 cm-1, C-O-C ester 1114 and 1155 cm-1. 1H NMR (200 MHz, acetone-d6) 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) SC-methyls at δ1.2, 1.30, 1.40, 1.50, 1.53 (each 3H, s), an acetyl methyl at δ 2.85(3H, s),

13C NMR (50 MHz, acetone-d6) 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(C-20), 143.3(C-23), 109.8(C-22), 141.4(C-21), 66.8(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.

**Limonin 3** is a white solid, m.p. 298°C and El-MS m/z of 471.1H NMR (600 MHz, acetone-d6) δ ppm: 4.29(1H, 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).
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 °C for 30 minutes to allow for diffusion and then incubated at 37 °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 cm²), 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 cm²). The spread agar was allowed to set on the plates and thereafter incubated at 37 °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 F254, 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:

\[
\text{AA} \% = 100 - \left\{ \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}}{\text{Abs}_{\text{control}}} \right\} \times 100
\]

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 (r² ≥ 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.
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 (EC50 = 13.18 ± 0.63 μM) used as standard antioxidant agent.

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.

REFERENCES