Enzyme and Oxidation Inhibitory Activities of Cardenolides from Leaves of *Parquetina nigrescens*

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Abstract: *Parquetina nigrescens* (Asclepiadaceae) is a flowering plant used in traditional medicine as antioxidant and antimicrobial agent. Isolates were obtained and characterized using chromatographic and spectroscopic techniques. The lipid component was characterized using Gas chromatography (GC) and gas – chromatography mass spectrometry (GC-MS). β-sitosterol-β-o-glucoside and cardenolides: strophanthidin, 16-dehydrostrophanthidin, 17α-strophadogenin, convallatoxin, strophanthidol and nigrescigenin were obtained as pure solids while ethyl iso-allocholate, oleyl alcohol, palmitic acid, β-sitosterol, tremulone and 7-methyl-Z-tetradecen-1-ol acetate were the major compounds detected in the oil. The compounds were screened for urease, α-glucosidase and oxidation inhibitory activities. Only, 17α-strophadogenin, nigrescigenin and the lipid component showed significant activity in the three assays when activity was compared with thiourea, 1-deoxynojiromycin hydrochloride (DNJ) and butylatedhydroxyanisole (BHA) respectively. These results underscore the biological relevance of *P. nigrescens* in ethnomedicine.

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1. Introduction

Parquetina nigrescens (Afzel) Bullock is a perennial twine herb, native to Africa and South America. It is a member of the Asclepiadaceae family of flowering plants which consist of 348 genera, with about 2,900 species. Ethnomedicinal application of *P. nigrescence* leaves for treating stomach troubles, parasitic infection, venereal diseases, typhoid, paralysis, epilepsy, convulsions, spasm (Burkill, 1997; Makanjuola *et al*., 2009; Akinyemi and Dada, 2013), malnutrition and as laxative (Owoyele *et al.*, 2008) is common in the tropics. The leaf-sap is used for treating arthritis, rheumatism, dysentery (Odetola *et al.*, 2006) and diabetes (Saba *et al.*, 2010). The root is used as pain killer, genital stimulants/depressants, reptile-repellents, and as an antidote to venom stings and bites while the latex of the plant is used for treating brain tumour, cancer and as antioxidant (Burkill, 1997; Imanga *et al*., 2010; Aderibigbe *et al.*, 2011; Owoyele *et al.*, 2011; Alvarez-Cruz, 2012). Non - ethnomedicinal uses of the stem as arrow poison, making of fishing nets and fishing lines and as ornamental plants (flowers) are well documented (Alvarez-Cruz, 2012). *P. nigrescens* has been reported to contain glycosides and alkaloids however, cardenolides are the major compounds isolated from *P. nigrescens* and are collectively called strophanthins (responsible for the activity in arrow poison). They include strophantidol, strophanthidin-β-D-glucoside, 16-dehydrostrophanthidin, strophanthidin, strophadogenin, convallatoxin, cymarin, nigrescigenin. Others are sterols: β-sitosterol, β-sitosterol-β-o glucoside, campesterol and stigmasterol and the triterpenes, α - and β-amyrine, ursolic acid and flavonoids: apigenin and apigenin-7-O-rutinoside as well as vitamins A and C were isolated from the leaves. Other isolated compounds include 16β-acetoxystrophanthidin, monodigitoxide of 16-dehydrostrophanthidin, rhamnoside of 16-acetoxystrophanthidin and 16-dehydrostrophanthidol (Berthold *et al*., 1965; Datté and Ziegler, 2001). The insecticidal activity of the essential oil of *P. nigrescens* was reported by Osunsami *et a*l., (2016).

Bioactivity-targeted screenings of plants has provided potential therapeutic agents from natural sources. The development of herbal medicine has to some extent depended on the scientific findings (Lam, *et a*l., 2008). Many ethnomedicinal claims on *P. nigrescens* exist in literature but this present work was carried out to determine the bioactivity of compounds isolated from *P. nigrescence* leaves as enzyme inhibitors and antioxidant. Enzyme Inhibition and antioxidant (free radical scavenging) activities were

investigated based on ethnomedicinal claims and previous scientific findings. Inhibitor of enzymatic reactions in biological system has played great role in ameliorating many disease conditions. The bacteria *Helicobacter pylori* induces gastrointestinal diseases, particularly peptic ulcer, gastric cancer and gastritis. It is persistently habituated by urease enzyme therefore urease deficiency effectively risks the bacteria existence (Andrade-Cetto, *et al.,* 2008). Also inhibition of α-glucosidase delays the breaking down of carbohydrates in the small intestine and diminishes hyperglycemia. Urease inhibitory activity of the isolates was examined against Jack Bean urease activity using Berthelot reaction while α-glucosidase inhibitory property was determined by measuring the yellow-colored paranitrophenol released from p-nitrophenyl glucopyranoside (pNPG) at 405 nm. In addition to this, oxidation inhibitory activity was determined since literature has it that many degenerative diseases such as cancer, ageing, Parkinson's disease to mention a few is caused by oxidation reactions. This is due to overproduction of reactive oxygen species arising either from mitochondrial electron transport chain or excessive stimulation of NAD (P)H oxidase (Onocha *et al.*, 2016; Oloyede *et al*, 2016). The antioxidant activity of the isolates was determined using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) method and butylated hydroxylanisole was used as standard. There is no report of the enzyme and oxidation inhibitory activities of the pure compounds in literature. The pure compounds used for these assays were isolated and characterized by carrying out gradient elution column, preparative and analytical thin layer Chromatography. Compounds were characterized by Nuclear Magnetic Resonance (NMR) and Electron Impact (EI/EI-HR) mass spectrometry. GC and GC-MS was used to characterize the lipid component. Research of this nature is therefore relevant in this dispensation as it gives credence to use of herbs and isolates from the plant could be a source of compounds used in disease management.

2. Materials And Methods

2.1 Chemicals and Reagents

Hexane, chloroform, dichloromethane, ethyl acetate, methanol, hydrochloric acid, ammonia solution, conc. tetraoxosulphate (VI) acid, conc. hydrochloric acid, ammonia solution, sodium potassium tartarate, potassium chloride, glacial acetic acid (BDH chemicals), were used. Those that are general purpose chemicals were distilled prior to use. 2, 2 - diphenyl-1-picrylhydrazyl (DPPH), and butylatedhydroxylanisole (BHA) (Sigma Chemical Co, St Louis, MO), Silica gel (230–400 mesh) and Sephadex LH-20 (Merck, Germany).

2.2 Plant Material

Parquetina nigrescence fresh leaves (3.45 kg) were collected in July, 2014 from the Botanical garden, University of Ibadan, Nigeria premises and duly identified. The leaves were air dried and ground into fine powder.

2.3 Isolation Procedure

The dried and powdered *P. nigrescence* sample (1.86 kg) was extracted with methanol (3.0 L) for 72 h. The extract was concentrated under reduced pressure at 37 ^{0}C using a rotary evaporator to give the crude methanol extract (367 g). The crude methanol extract was partitioned in hexane and ethylacetate to give the non polar and moderately polar fractions respectively. The ethylacetate soluble fractions were adsorbed in silica gel and subjected to gradient elution column chromatography using silica gel 230 – 400 mesh size and *n*-hexane, ethylacetate (EtOAc), and methanol MeOH as eluent in order of increasing polarity. Sub fractions were obtained. Thin Layer Chromatography was performed on the fractions collected from the column chromatography in other to determine purity, ascertain the number of components so as to pool together fractions which contain the same component based on their R_f values. Fractionation gave 121 fractions and was pulled into 12 based on the similarity of their TLC profile. Fractions 1-10 obtained from 100% hexane was β-sitosterol and was confirmed by its melting point only. Colorless crystal (PN1, 24 mg) was obtained from fractions 11-27 (hexane-ethyl: acetate 98:2) and recrystallized and washed with ethanol. Factions 28-51 gave compound PN2 (18 mg) which was obtained from 85% hexane and 15% ethylacetate (EtOAc) and recrystallized with DCM to give white crystalline substance. PN3 (15 mg) was obtained using Sephadex LH-20 (30 g in 150ml of solvent system 50% Hex+ 100% methanol) to purify fractions 52-87 obtained from hexane-ethyl acetate 70:30. PN4 (12 mg) was obtained from the ethylacetate fractions 32-64 and purified by preparative TLC using Hex:EtOAc 2:1). Solid obtained was recrystallised with DCM (R_f 0.5 in hexane: ethylacetate 2:1). PN5 (16 mg) was precipitated from fractions 65-97 (R_f 0.3, methanol: acetone 7:3). Purification in a micro column with silica gel and solvent system Hex: EtOAc 1: 2 afforded PN6 (8 mg) (R_f 0.6). Methanol was used to remove the yellow oil (PN7) from the fraction. It was analyzed using Gas chromatography and Gas Chromatography-Mass spectrometry (GC and GC-MS). PN8 (6 mg) was obtained from fractions 134-161, washed with methanol and recystallized with DCM (R_f 0.8, Hex: EtOAc 7:3).

2.4 General experimental procedures

Soxhlet extractor was used to obtain methanol fraction. Buchi Rotary Evaporator fitted with Vacuum

pump V-700 and B-490 heating bath was used to concentrate samples. All weighing was achieved by the use of Analytical balance KERN ALS 220-4 KERN & SOHN, Germany. Silica gel (230–400 mesh) and Sephadex LH-20 were used for column chromatography (CC) depending on the polarity of the fractions while TLC aluminium sheets (precoated) Silica gel 60 F_{254} (20 cm × 20 cm, 0.2 mm thick; Merck, Germany) for analytical thin layer chromatography and Alugram Sil G/UV_{254} for preparative chromatography (Macherey-Nagel MN, Germany). Visualization of the TLC plates was carried out under UV light at 254 and 366 nm and by spraying with ceric sulfate reagent solution with heating and vanillin sulphuric acid. Melting points of all crystalline solids were determined by Barnstead Electrothermal melting point apparatus, 9100. Ultra violet (UV) spectra were run in methanol on Spectro UV-VIS Double beam PC scanning spectrometer, UVD - 2960 (190-400 nm) while Infra red (IR) spectra were recorded on Fourier Transform-IR PerkinElmer Spectrum Two (LABOMED, INC)FT-IR-8900 IR spectrometer (4000-400 cm^{-1}). Proton Nuclear Magnetic Resonance (NMR), ^1H NMR spectra were recorded on AVANCE AV-500 spectrometer operating at 500 MHz for ^1H. The chemical shift values (δ) are reported in ppm. The EIMS, HREIMS were recorded on JEOL MS 600H-1 with a data system showing mass to charge (*m/z*). Gas chromatography (GC) was performed on a Shimadzu gas chromatograph (GC-17A) (FAME-MOI, column oven temperature set at 70-300 °C, injection port: 220-400 °C and detector temperature 240–450 °C, control mode is split, column pressure (kPa):71, column flow rate 8.19 mL/min, linear velocity (cm/s), 50.01, column length (m): 60, column diameter (mm): 0.53, flame-ionization detector). The percentage compositions were obtained from electronic integration measurements using flame ionization detector (FID), set at 250ºC. Triple quadrupole Acquisition Method using electron impact (EI) as ion source at 70eV was used for the GC-MS with the following parameters, injection volume: 2µL, oven equilibrium time: 0.5 min, maximum temperature 325 °C. Oven was programmed to 50 °C for 3 min, then 6°C /min to180°C for 20 min and then 6°C /min to 290°C for 40 min with a Run time of 103 min. Split mode was used and column type was ZEBRON –ZB-5 column with dimensions 360°C: 30 m x 250 µm and 0.25 µm. Initial temperature was 50 °C, pressure 9.78 psi, flow 1.2 mL/min with average velocity 39.92 min, hold on time 1.25 min and Flow programme 1.2 mL/min for 0 min. Mass Hunter Workstation Software Qualitative analysis version 13.04.00 Agilent Technologies In.2011 was used and individual constituents of the oil were identified on the basis of their retention indices compared with data previously reported in literature (Oloyede *et al*., 2017).

2.5 Determination of Antioxidant (DPPH Free Radical Scavenging) Activity

Free radical scavenging activity was measured using 2,2 -diphenyl-1-picryl-hydrazyl (DPPH) method as described by Oloyede et al., 2016 and 2017. Different concentration of each sample (62.5 µg - 500 µg) was prepared and five microlitres of each of the sample was mixed with 95 µl of 0.3 mM DPPH solution in ethanol. The mixture was dispersed in 96 well plates and incubated at 37° C for 30 min. The absorbance at 517 nm was measured by micro titre plate reader (Spectramax plus 384 Molecular Device, USA) and percent radical scavenging activity was determined. Butylatedhydroxyanisole (BHA) was used as standard. Inhibition concentration at 50% (IC_{50}) was calculated (Gulcin *et al*., 2005; Oloyede *et al*., 2016).

2.6 Urease assay inhibition

The determination of urease activity was done by measuring ammonia production from sodium nitroprusside. Isolated compounds were prepared in different concentrations, ranging from 25-500 µg. 5 µL (1 mM concentration) of each prepared solution was mixed with 25 µL Jack bean Urease enzyme solution. The mixture was incubated at 30 ^oC. Aliquots were taken after 15 minutes and transferred immediately to assay mixtures containing 100 mM urea in 55 µL of buffer and incubated again for 30 min in 96 well plates. 45 µL each of phenol reagent and 70 µL of alkali reagent (0.5% w/v NaOH and 0.1 % NaOCl) were added to each well. Increase in absorbance at 630 nm was measured after 50 min, using a microplate reader (Spectramax plus 384 Molecular Device, USA). All reactions were performed in triplicate in a final volume of 200 µL. The results (change in absorbance per min) were processed by using SoftMax Pro software (Molecular Device, USA). All the assays were performed at pH 8.2 (0.01 M $K_2HPO_4.3H_2O$, 1 mM EDTA and 0.01 M $LiCl_2$). Thiourea was used as the standard inhibitor of urease. The percent inhibition was computed according to: % Inhibition = Test activity of control - test activity of sample x 100/Test activity of control. Median inhibitory concentrations were determined by plotting percent inhibition vs the logarithms of final toxicant concentrations. The concentration giving 50% inhibition (ICs_0) was derived from least squares linear regression (Jung, *et al.,* 1995; Ferheen, *et al*., 2009; Mahemia *et al*., 2015).

***2.7* α-Glucosidase Inhibitory Assay**

α-Glucosidase activity of the pure compounds was determined according to the method described below. The substrate solution, p-nitrophenyl glucopyranoside (pNPG) was prepared in 20 mM phosphate buffer (pH 6.9). 100 µL of α-glucosidase obtained from *Saccharomyces cerevisiae* (1.0 U/mL) was

pre-incubated with 50 µL of the different concentrations of the isolates for 10 min. 50 µL of 3.0 mM substrate (pNPG) dissolved in 20 mM phosphate buffer (pH 6.9) was added to the reaction mixture. The reaction mixture was incubated at 37°C for 20 min and stopped by adding 2 mL of 0.1 M Na_2CO_3. α - glucosidase activity was determined by measuring the yellow-colored paranitrophenol released from pNPG at 405 nm. 1-Deoxynojiromycin hydrochloride (DNJ) at various concentrations was used as standard. The results were expressed as percentage of the blank control. Concentrations of extracts resulting in 50% inhibition of enzyme activity (IC_{50}) were calculated (Kim, *et al.*, 2005 and Kazeem, *et al*., 2013).

2.8 Statistical Analysis

Measurements were done in triplicate and results are expressed in terms of mean ± standard deviation (Values of $P \leq 0.05$ were considered to be significant) and IC_{50} values were calculated using GraphPad Prism 5 version 5.01 (Graph pad software, Inc., La Jolla, CA, USA.) software. Experimental data were analyzed statistically by one-way analysis of variance, using the SPSS software package (version 12.0; SPSS, Inc., Chicago, Illinois, USA).

3. Result Analysis

Compound PN1 (24 mg): colourless crystal. M.pt: 361 - 362 ^{0}C. R_f 0.6 (Silica gel F_{254}, methanol: ethylacetate 3:1). UV (methanol) λ_{max} nm (logε): 218 (0.029), 245 (0.116), 308 (0.256). IR (KBr) λ_{max} cm^{-1}: 3682 (O-H stretch), 2975.12 (C-H stretch), 2851.04 (C-H stretch), 1586 (C=C stretch), 1473.16 (C-H bend), 1075.76 (C-O), 934.7, 778.5. ^{1}H NMR (500 MHz, MeOD) δ ppm: 0.96 (m, 3H, CH_3), 0.98 (d, 3H, 2x CH_3), 1.01 (s, 3H, CH_3), 1.08 (s, 3H, CH_3), 1.19 (s, 3H, CH_3), 1.29 (m, 2H, 3xCH_2 alicyclic), 1.46-1.48 (dd, 1H, 3xCH, cyclohexane), 1.49 (t, 1H, CH, cyclopentane), 1.53 (t, 2H, 2xCH_2, cyclopentane), 1.62 (t, 2H, CH_2, (-CH_2-OH)), 1.63-1.84 (m, 1H, 3 x CH alicyclic), 1. 63- 2.11 (m, 2H, 6x CH_2 cyclohexane), 3.41 (m, 1H, 3xCH, sugar), 4.57 (s, 1H, 2xOH-alcohol), 4.65 (s, 1H, OH-alcohol), 4.84 (s, 1H, OH-alcohol), 5.01 (m, 1H, -O-CH-), 5.03 (m, 1H, -O-CH-), 5.09 (m, 1H, -O-CH-O-sugar), 5.51 (s, 1H, =CH- cyclohexane).

EI-HRMS *m/z*: 576.567 Molecular weight (calc) for $C_{35}H_{60}O_6$:576.859 g/mol. PN1 is β-sitosterol-β-o-glucoside (2S,3R,4S,5S,6R)-2-[[(3S,8R,9S,10S,13R,14S,17R)-17-[(2R,5R)-5-ethyl-6-methylheptan-2-yl]-10,13-dimethyl -2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclo penta [a]phenanthren-3-yl]oxy]-6-(hydroxymethyl) oxane-3,4,5-triol). Soluble in methanol, water, acetone, slightly soluble in chloroform.

Compound PN2 (18 mg): colourless crystals; M.pt: 240-241^{0}C. R_f 0.4 (Silica gel F_{254}, hexane: ethylacetate 3:1). UV (methanol) λ_{max} nm (logε): 211 (0.109), 239 (0.225), 281(0.305), 300 (0.112), IR (KBr) λ_{max} cm^{-1}: 3554.23 (O-H stretch), 2925.1 (C-H stretch), 2654.33, 1737.6, 1642.5 (C=O), 1454.54 (C-H bend), 1102.51, 1067.41 (C-O). ^{1}H NMR (400 MHz, CD_3Cl, δ ppm): 1.02 (s, 3H, CH_3), 1.49 (dd, 1H, CH, cyclohexane), 1.61 (dd, 1H, CH, cyclohexane), 1.62 and 1.93 (t, 2H, 2xCH_2, cyclopentane), 1.54 - 2.09 (m, 14x 2H, CH_2 cyclohexane), 2.32 (t, 1H, CH, cyclopentane), 3.15 (m, 1H, CH, cyclohexane), 4.68 (s, 1H, 2xOH-alcohol, cyclohexane), 4.80 (s, 1H, 1xOH-alcohol, cyclopentane), 4.90 (s, 2H, furanone), 5.93 (s, 1H, =CH-, furanone), 9.54 (s, 1H, CHO). EI-HRMS *m/z*. = 404.219 Molecular weight (calc) for $C_{23}H_{32}O_6$: 404.497 g/mol. PN2 is strophanthidin, (3,5,14-trihydroxy-13-methyl-17-(5-oxo-2,5-dihydrofuran-3-yl) hexadecahydro-1*H*-cyclopenta [α]phenanthrene-10-carbaldehyde). Soluble in chloroform, ethylacetate, slightly soluble methanol, acetone.

Compound PN3 (15 mg): colourless crystals; M.pt: 241-242^{0}C. R_f 0.7 (Silica gel F_{254}, hexane-ethyl acetate 7:3). UV (methanol) λ_{max} nm (logε): 200 (0.029), 229 (0.135), 286 (0.105), 311 (0.252), IR (KBr) λ_{max} cm^{-1}: 3654.24 (O-H stretch), 2913.10 (C-H stretch), 2654.87, 1712.14, 1631.87 (C=O), 1454.54 (C-H bend), 1006.56, 1091.45 (C-O). ^{1}H NMR (400 MHz, CD_3Cl, δ ppm): 1.09 (s, 3H, CH_3), 1.43 (dd, 1H, 2xCH, cyclohexane), 1.45-1.51 (dd, 2H, CH_2, cyclohexane), 1.23-1.71 (m, 2H, 4xCH_2 cyclohexane), 1.59 and 1.88 (d, 2H, CH_2, cyclopentene), 1.87 (m, 2H, 2xCH_2 cyclohexane) 2.13 (t, 1H, CH, cyclopentene), 3.15 (m, 1H, CH, cyclohexane (CH-OH)), 4.68 (s, 1H, OH-alcohol, cyclohexane), 4.70 (s, 1H, OH-alcohol), 4.85 (s, 1H, OH-alcohol), 4.90 (s, 2H, CH_2 furanone), 5.94 (s, 1H, =CH-, furanone), 9.56 (s, 1H, CHO). EI-HRMS *m/z*. = 402.203 Molecular weight (calc) for $C_{23}H_{30}O_6$: 402.480 g/mol. PN3 is 16-Dehydrostrophanthidin (3S,5S,10S,13R,14S)-3,5,14-trihydroxy-13-methyl-17-(5-oxo-2H-furan-3-yl)-2,3,4,6,7,8,9,11,12,15-decahydr o-1H-cyclopenta [α]phenanthrene-10-carbaldehyde. Soluble in chloroform, ethylacetate, slightly soluble methanol, acetone.

Compound PN4 (12 mg): colourless crystals; M.pt: 256 -257^{0}C. R_f 0.5 (Silica gel F_{254}, hexane: ethylacetate 2:1). UV (methanol) λ_{max} nm (logε): 212 (0.119), 236 (0.125), 285(0.305), 300 (0.212), IR (KBr) λ_{max} cm^{-1}: 3606.12 (O-H stretch), 2825.63 (C-H stretch), 2654.98, 1606.26 (C=O), 1519.00(C=C stretch), 1454.54 (C-H bend), 1067.41 (C-O). ^{1}H NMR (400 MHz, CD_3Cl, δ ppm): 1.02 (s, 3H, CH_3), 1.49 (dd, 1H, CH, cyclohexane), 1.61 (dd, 1H, CH, cyclohexane), 1.63 and 1.93 (t, 2H, CH_2,

cyclopentane), 1.54 - 2.09 (m, 14x 2H, CH_2 cyclohexane), 2.30 (t, 1H, CH, cyclopentane), 3.15 (m, 1H, CH, cyclohexane), 3.21 (t, 1H, CH, cyclopentane), 4.69 (s, 1H, 2xOH-alcohol, cyclohexane), 4.80 (s, 1H, 2xOH-alcohol, cyclopentane), 4.91 (s, 2H, furanone), 5.95 (s, 1H, =CH-, furanone), 9.54 (s, 1H, CHO). EI-HRMS *m/z*. = 420.496 Molecular weight (calc) for $C_{23}H_{32}O_7$: 420.214 g/mol. PN3 is 17α-strophadogenin (3,5,14,16-tetrahydroxy-13-methyl-17-(5-oxo-2,5-dihydrofuran-3-yl)hexadecahydro-1*H*-cyclopenta [α] phenanthrene-10-carbaldehyde). Soluble in chloroform, dichloromethane, slightly soluble in acetone, methanol and insoluble in water.

Compound PN5 (16 mg): colourless crystals; M.pt: 235-236 ^{0}C. R_f 0.3 (Silica gel F_{254}, methanol: acetone 2:1). UV (methanol) λ_{max} nm (logε): 200 (0.189), 215 (0.247), 230 (0.252), 285(0.305), 305 (0.112), IR (KBr) λ_{max} cm^{-1}: 3598.88 (O-H stretch), 2921.70 (C-H stretch), 2717.09, 1692.68 (C=O), 1586.09 (C=C stretch), 1462.73 (C-H bend), 1060.14 (C-O). ^{1}H NMR (400 MHz, MeOD, δ ppm): 1.06 (s, 3H, CH_3), 1.16 (s, 3H, CH_3), 1.49 (dd, 1H, CH, cyclohexane), 1.62 (dd, 1H, CH, cyclohexane), 1.63 (t, 2H, CH_2, cyclopentane), 1.76 (t, 2H, CH_2, cyclopentane), 1.56- 2.10 (m, 14x 2H, CH_2 cyclohexane), 2.18 (t, 1H, CH, cyclopentane), 2.75 (m, 1H, CH, cyclohexane), 3.41 (m, 1H, tetrahydropyran), 3.49 (m, 1H, tetrahydropyran), 3.76 (m, 1H, tetrahydropyran), 4.59 (s, 1H, 2xOH-alcohol), 4.84 (s, 1H, 3xOH-alcohol), 4.93 (s, 2H, furanone), 5.03 (m, 1H, -O-CH-O-tetrahydropyran), 3.83 (m, 1H, tetrahydropyran), 5.91 (s, 1H, =CH-, furanone), 9.49 (s, 1H, CHO). EI-HRMS *m/z* = 550.274. Molecular weight (calc) for $C_{29}H_{42}O_{10}$: 550.645 g/mol, PN5 is convallatoxin, (3S,5S,8R,9S,10S,13R,14S,17R)-5,14-dihydroxy-13-methyl-17-(5-oxo-2H-furan-3-yl)-3-[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxy-2,3,4,6,7,8,9,11,12,15,16,17-dodecahydro-1H-cyclopenta [a]phenanthrene-10-carbaldehyde. Soluble in acetone, methanol, slightly soluble in water, ethyl acetate and chloroform; insoluble in petroleum ether.

Compound PN6 (8 mg): colourless crystals; M.pt: 139-140^{0}C. R_f 0.6 (Silica gel F_{254}, hexane-ethyl acetate 1:2). UV (methanol) λ_{max} nm (logε): 201 (0.009), 228 (0.113), 285 (0.125), 301 (0.162), IR (KBr) λ_{max} cm^{-1}: 3600.24 (O-H stretch), 2905.70 (C-H stretch), 2617.34, 1734.53, 1654.21 (C=O), 1431.75 (C-H bend), 1056.29 (C-O). ^{1}H NMR (400 MHz, CD_3Cl, δ ppm): 1.01 (s, 3H, CH_3), 1.40 (dd, 1H, 2xCH, cyclohexane), 1.48 (dd, 1H, 2xCH, cyclohexane), (1.23 - 1.85 (m, 2H, 6xCH_2 cyclohexane), 1.35 and 1.60 (t, 2H, CH_2, cyclopentane), 1.45 and 1.73 (t, 2H, CH_2, cyclopentane), 2.13 (t, 1H, CH, cyclopentane), 3.34 and 3.55 (m, 2H, CH_2, methylene (α-O)),3.15 (m, 1H, CH, cyclohexane (CH-OH)), 4.66 (s, 1H, 2xOH-alcohol), 4.75 (s, 1H, OH-alcohol), 4.80 (s, 1H, OH-alcohol), 4.90 (s, 2H, CH_2 furanone), 5.94 (s, 1H, =CH-, furanone), EI-HRMS *m/z*. = 406.236 Molecular weight (calc) for $C_{23}H_{34}O_6$: 406.51246 g/mol. PN6 is strophanthidol, 4-(3,5,14-trihydroxy-10-(hydroxylmethyl)-13-methylhexadecahydro-1*H*-cyclopenta [α]phenanthrene-17-yl) furan-2(5*H*)-one. Soluble in chloroform, ethylacetate, slightly soluble methanol, acetone.

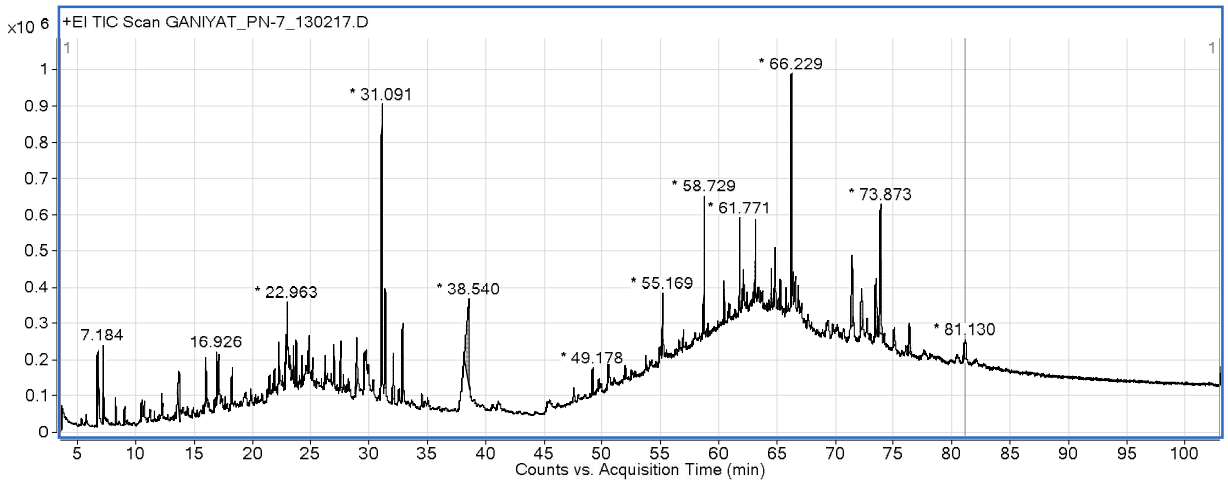


Figure 1: Gas Chromatogram showing Retention time (RT) of compound PN 7 from *P. nigrescence* polar and non polar fractions

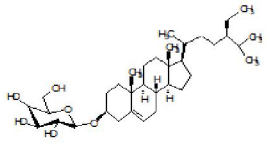
Compound PN7 (28 mg): Yellow oil soluble in methanol and subjected to GC and GC –MS analysis. Figure 1 shows the chromatogram while Table I gives the compounds detected and their relative percentages.

Compound PN8 (6 mg): colourless crystals; M.pt: 273-274^{0}C. R_f 0.8 (Silica gel F_{254}, hexane: ethylacetate 7:3). UV (methanol) λ_{max} nm (logε): 203 (0.009), 241 (0.175), 289 (0.213), 310 (0.118), IR (KBr) λ_{max} cm^{-1}: 3589.09 (O-H stretch), 2973.81 (C-H stretch), 2806.13 (C-H stretch), 2780.11, 1675.84 (C=O), 1586.97 (C=C stretch), 1365.78 (C-H bend), 1014.85 (C-O). ^{1}H NMR (400 MHz, CD_3Cl, δ ppm): 1.02 (s, 3H, CH_3), 1.49 (dd, 1H, CH, cyclohexane), 1.61 (dd, 1H, CH, cyclohexane), 1.63 and 1.93 (t, 2H, CH_2, cyclopentane), 1.54 - 2.09 (m, 14x 2H, CH_2 cyclohexane), 2.30 (t, 1H, CH, cyclopentane), 3.15 (m, 1H, CH, cyclohexane), 3.21 (t, 1H, CH, cyclopentane), 4.10 (s, 1H, OH-alcohol, cyclohexane), 4.69 (s, 1H, 2x OH-alcohol, cyclohexane), 4.80 (s, 1H, OH-alcohol, cyclopentane), 4.91 (s, 2H, furanone), 5.95 (s, 1H, =CH-, furanone), 9.54 (s, 1H, CHO). EI-HRMS *m/z*. = 420.496 Molecular weight (calc) for $C_{23}H_{32}O_7$: 420.214 g/mol. PN8 is Nigrescigenin, (3,5,11,14-tetrahydroxy-13-methyl-17-(5-oxo-2,5-dihydrofuran-3-yl)hexadecahydro-1*H*-cyclopenta [α] phenanthrene -10-carbaldehyde). Soluble in chloroform, dichloromethane, slightly soluble in acetone, methanol and insoluble in water.

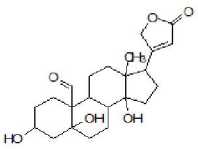
Table 1: Compounds detected in fixed oil (PN 7) obtained from *P. nigrescence*

S/N	RT (min)	KI	Name of compound	M. Formula	(g/mol)	Rel%	Class of compound
1.	6.78	845	Acetonyldimethylcarbinol	C6H12O2	116	0.57	Alcohol
2.	7.18	1453	Ethyl Geranyl acetone	$C_{14}H_{24}O$.	208	1.92	Ketone
3.	13.71	1065	Levulinic acid	C5H8O3	116	2.21	Carboxylic acid derivative
4.	18.23	1272	n-Nonanoic acid	C9H18O2	158	0.99	Carboxylic acid
5.	21.87	1822	7-Methyl-Z-tetradecen-1-ol acetate	C17H32O2	: 268	6.38	Ester
6.	22.25	1458	2,6,10-Trimethyl-9-undecen-1-al	C14H26O	210	0.75	Aldehyde
7.	22.86	2175	cis-Vaccenic acid	C18H34O2	282	0.42	Carboxylic acid
8.	23.52	1506	Isopropyl 9-oxononanoate	C12H22O3	214	0.67	Ester
9.	23.75	2095	Hexanoic acid, tridec-2-ynyl ester	C19H34O2	294	1.17	Ester
10.	24.24	2175	Oleic acid	C18H34O2	282	1.43	Carboxylic acid
11.	27.54	1563	Hexa-hydro-farnesol	C15H32O	228	1.21	Alcohol
12.	31.09	2061	Oleyl Alcohol	C18H36O	268	11.63	Alcohol
13.	31.38	1754	Hexahydrofarnesyl acetone	C18H36O	268	3.84	Ketone
14.	32.07	2165	17-Octadecynoic acid	C18H32O2	280	1.85	Carboxylic acid
15.	32.86	2119	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	C20H40O	296	3.37	Ester
16.	38.54	1968	Palmitic acid	C16H32O2	256	10.57	Carboxylic acid
17.	49.18	2572	Erucic acid	C22H42O2	338	0. 66	Carboxylic acid
18.	58.64	3578	Picrotoxin	C30H34O13	602	0.32	Epoxide derivative
19.	58.73	3942	1-Heptatriacotanol	C37H76O	536	3.05	Alcohol
20.	60.46	3094	Ethyl iso-allocholate	C26H44O5	436	11.79	Ester derivative
21.	71.43	2731	β-Sitosterol	C29H50O	414	7.85	Alcohol
22.	73.48	3090	**Betulin**	C30H50O2	442	4.84	Alcohol derivative
23.	73.87	2696	Tremulone	C29H46O	410	7.83	Ketone derivative
24.	76.36	2987	Lupeol acetate	C32H52O2	468	1.82	Ester derivative
					Total%	87.14%	

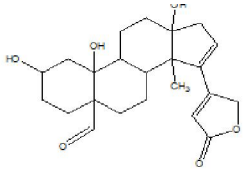
RT-Retention Time (minutes), KI- Kovats retention index, (relative to n-alkane hydrocarbon standards. (Retention Data. NIST Mass Spectrometry Data Center, 2007).



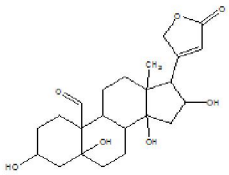
β-sitosterol-β-o glucoside



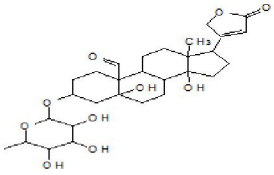
Strophanthidin



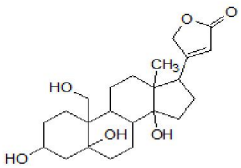
16-Dehydrostrophanthidin



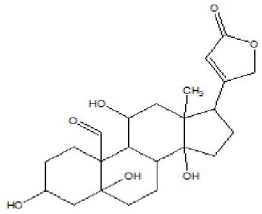
17α-Strophadogenin



Convallatoxin



Strophanthidol



Nigrescigenin

Table 1 shows the twenty four compounds detected in the oil (PN7) obtained from *P. nigrescence*. Ethyl iso-allocholate (11.79%), oleyl alcohol (11.63%), palmitic acid (10.57%), β-sitosterol (7.85%) tremulone (7.83%) and 7-methyl-Z-tetradecen-1-ol acetate (6.38%) were the major constituents. Unlike the fixed oil with varied components, only five identified components was reported in the leaf essential oil of *P. nigrescens* and was dominated by by citral (Owolabi, *et al.*, 2014).

3.1 Enzyme Inhibition and Antioxidant Assays

Isolates from the polar and non-polar fractions of *P. nigrescence* were evaluated for *in-vitro* enzyme inhibition and antioxidant activity. Only, 17α-strophadogenin, strophanthidol, the fixed oil and nigrescigenin showed significant urease inhibitory activity. Nigrescigenin with IC_{50} (μM) of 19.6± 0.06 was the most active when compared with Thiourea. 17α-strophadogenin (2.7 ± 0.10) and Nigrescigenin (2.63 ± 0.07) showed better activity in the α-Glucosidase inhibitory screening when compared to standard DNJ (3.9 ± 0.08). 17α-strophadogenin, the fixed oil and nigrescigenin IC_{50} (μM) of 18.4± 0.19, 37.7± 0.85 and 28.1± 0.21 respectively showed significant free radical scavenging (2,2-diphenyl picryl hydrazyl radical) activity when compared with the standard BHA (44.2 ± 0.09).

Table 2: IC_{50} (μM) values of compounds in the Urease, α-Glucosidase and Free radical inhibitory assays.

S/N	Compound	Urease Inhibition IC_{50} (μM)	Glucosidase Inhibition IC_{50} (μM)	Antioxidant IC_{50} (μM)
1	PN1	> 500	Nil	Nil
2	PN 2	30.9± 0.27	Nil	Nil
3	PN3	35.3± 0.13	16.4± 0.31	> 200
4	PN4	20.4± 0.17	2.7 ± 0.10	18.4± 0.19
5	PN5	> 500	> 500	Nil
6	PN6	42.6± 0.26	65.3 ± 0.11	55.1 ± 0.36
7	PN7	20.7± 0.03	3.7 ± 0.56	37.7± 0.85
8	PN8	19.6± 0.06	2.6 ± 0.07	28.1± 0.21
	BHA	-	-	44.2 ± 0.09
	Thiourea	21.6 ± 0.12	-	-
	DNJ	-	3.9 ± 0.08	-

4. Discussion

Eight compounds, β-sitosterol-β-o-glucoside, strophanthidin, 16-dehydrostrophanthidin, 17α-strophadogenin, convallatoxin, strophanthidol and nigrescigenin were reported from *P. nigrescence* in this present study. These compounds are known compounds from the family of this plant but the chemical constituent of the fixed oil are being reported for the first time. Ethyl iso-allocholate, oleyl alcohol, palmitic acid are the major compounds detected in the oil. These compounds were screened for urease, α-glucosidase and oxidation inhibitory properties. For all the compounds, absorption in the UV at wavelength (nm) 190 - 200 indicates the presence of n-π* of a C=C, 285 nm indicates the presence of decahydronaphthalene and 300 nm suggests that the compound contains hydroxyl moiety while IR analysis result revealed broad peaks in the region of 3372.11 – 3632.97 cm^{-1} indicating a hydrogen bonded O-H_{str} of an alcohol. It was possible to deduce the presence of OH groups which were well resolved in that region. Sharp peaks at 2849.44 - 2921. 67 cm^{-1} revealed the presence of a sp^3 C-H_{str} and peaks at 1692.13 – 1740.53 cm^{-1} confirms the presence of a carbonyl. Generally, peaks at 1719 – 2785 cm^{-1} confirms the presence of aldehyde group in the cardenolides which is due to the presence of C=O and C-H. The proton nuclear magnetic resonance (^{1}H-NMR) of the all the compounds showed signals due to angular momentum at δ (ppm) 0.96-0.98 for PN1 and one hydroxyl-methylene at δ 1.64, olefinic proton at δ 5.51 indicating that PNI is a steroid containing ordinary A and B rings. The glycoside showed anomeric proton signal at 5.09 thus confirming the structure as β-sitosterol-β-o-glucoside. The cardenolides PN2-PN6 and PN8, showed signals for downfield or deshielded peaks at δ 1.01-1.09 corresponding to the methyl group of the octahydro-1*H*-indene. The cardenolides (aglycone) contains OH group at C-16 or C=C bond at 16:17 position. Signals in the range of δ 3. 34 and 3. 55 correspond to the 2H of methylene, which is alpha to oxygen. Tertiary OH occupy positions at C-5 and C-14, secondary one at C-3. The olefinic furanone proton signal was observed around δ 5.94 as a singlet. The signal due to aldehyde proton was observed at about δ 9.54 as a singlet and this was replaced in strophanthidol as a signal due to hydroxyl observed at δ 4.80. Convallatoxin showed anomeric proton signal at δ 5.03 confirming that it is a glycoside.

The cardenolides displayed varied biological activity in the enzyme and oxidation inhibitory assays. Compounds with double bond between C-16 and C-17 are inactive or weakly active. Only, 17α-strophadogenin, strophanthidol, the fixed oil and nigrescigenin showed significant urease inhibitory activity while nigrescigenin was the most active when compared with butylatedhydroxyanisole in the free radical scavenging assay. 17α-strophadogenin, nigrescigenin showed better activity in the α-glucosidase inhibitory screening when compared to standard DNJ.

The antioxidant activity of β-sitosterol-β-o-glucoside in this experiment was beyond detectable limit however it was reported that it showed strong substrate dependence, suggesting involvement of succinate dehydrogenase when stimulatory effects on respiration and reactive oxygen species generation was studied (Panov, et al., 2010). *P. nigrescens* was reported to have cardiotonic and catecholamine-like effects mainly attributed to the presence of cardenolides (Datté and Ziegler, 2001). The presence of **l**ipids *in P. nigrescens* is an indication that the plant have the ability to lower the levels of cholesterol and triglycerides (Oloyede *et al.,* 2017). The presence of these bioactive compounds supports the anti-arrhythmia and cardiotonic effect of the plant (Datté and Ziegler, 2001).

5. Conclusion

Glycosides and cardenolides isolated from *P. nigrescens* include β-sitosterol-β-o-glucoside, strophanthidin, 16-dehydrostrophanthidin, 17α-strophadogenin, convallatoxin, strophanthidol and nigrescigenin. Two of the cardenolides namely, 17α-strophadogenin and nigrescigenin were shown to possess urease, α-glucosidase and oxidation inhibitory properties. Ethyl iso-allocholate, oleyl alcohol, palmitic acid, β-sitosterol, tremulone and 7-methyl-Z-tetradecen-1-ol acetate were the major constituents in the lipid component. It also displayed significant activity in the three assays. The presence of these secondary metabolites justifies the use of *P. nigrescens* plant for treating ailments such as microbial infections and stress related diseases amongst others in ethnomedicine.

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Declaration of Interest

The authors declare that there are no conflicts of interest associated with this work.

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