Segun, P, Ismail, FMD, Ogbole, OO, Nahar, L, Evans, AR, Ajaiyeoba, EO and Sarker, SD

Acridone alkaloids from the stem bark of Citrus aurantium display selective cytotoxicity against breast, liver, lung and prostate human carcinoma cells

Acridone alkaloids from the stem bark of *Citrus aurantium* L. (Rutaceae) display selective cytotoxicity against breast, liver, lung and prostate human carcinoma cells

Peter A. Segun[1,2,3], Fyaz M. D. Ismail[2*], Omonike O. Ogbole[1*], Lutfun Nahar[2], Andrew R. Evans[2], Edith O. Ajaiyeoba[1] and Satyajit D. Sarker[2]

[1]Department of Pharmacognosy, Faculty of Pharmacy, University of Ibadan, Ibadan, Nigeria

[2]Medicinal Chemistry and Natural Products Research Group, School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, James Parsons Building, Byrom Street, Liverpool L3 3AF, United Kingdom

[3]Department of Pharmacognosy, Faculty of Pharmacy, Olabisi Onabanjo University, Sagamu Campus, Nigeria

Authors email address

Peter A. Segun: petersegun2020@yahoo.com

Fyaz M. D. Ismail: fyaz.ismail@gmail.com

Omonike O. Ogbole: nikeoa@yahoo.com

Lutfun Nahar: l.nahar@ljmu.ac.uk

Andrew R. Evans: a.r.evans@ljmu.ac.uk

Edith O. Ajaiyeoba: edajaiye@gmail.com

Satyajit D. Sarker: profsarker@gmail.com

*Authors for correspondence

E-mail: nikeoa@yahoo.com , Phone no: +2348056434577

E-mail: fyaz.ismail@gmail.com, Phone no: +447734808544

Abstract

Ethnopharmacological relevance: *Citrus aurantium* L. (Rutaceae) is used, either singly or as a part of a polyherbal preparation, in Nigerian traditional medicine for the management of cancer and inflammatory diseases. Currently, there is a dearth of knowledge demonstrating its anticancer potential.

Aim of the study: This study was carried out to determine the *in vitro* cytotoxicity of the crude extract of the stem bark of *C. aurantium*, identify and isolate the bioactive constituents and to establish the cytotoxicity of such constituents.

Material and Methods The powdered bark of *C. aurantium* was extracted with methanol at room temperature (25-34 °C) and the crude extract was partitioned successively, with *n*-hexane, dichloromethane (DCM) and methanol. Amongst the fractions, the DCM fraction was the most active and compounds were isolated from this fraction using several chromatographic techniques. The structures of the isolated compounds were elucidated by spectroscopic means (mass spectrometry, one-dimensional and two-dimensional nuclear magnetic resonance). The cytotoxicity of the extract, and the isolated compounds were evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) assay against four human cancer cell lines: A549 (lung), HepG2 (liver), MCF7 (breast) and PC3 (prostate). The selectivity of the isolated compounds was assessed using the normal human prostate epithelium cells (PNT2).

Results and Discussion: Of the three plant fractions, the DCM fraction showed significant cytotoxicity, with its highest activity against A549 cells (IC_{50} = 3.88 µg/mL) and the least activity on HepG2 cells (IC_{50} = 5.73 µg/mL). Six acridone alkaloids: citrusinine-I (**1**), citracridone-I (**2**), 5-hydroxynoracronycine (**3**), natsucitrine-I (**4**), glycofolinine (**5**) and citracridone-III (**6**), were isolated from the DCM fraction of *C. aurantium.* The isolated compounds demonstrated potent to moderate cytotoxicity ((IC_{50} = 12.65 – 50.74 µM) against

the cancer cells under investigation. It is noteworthy that the compounds exerted cytotoxicity at least four times more selective towards the carcinoma cells than the PNT2 cells.

Conclusion: The results obtained from this study have provided some evidence for the ethnomedicinal use of *C. aurantium* against cancer and the acridone alkaloids present in its stem bark, have appeared to be responsible for this effect. Further research to explore the underlying molecular mechanism of the isolated acridone alkaloids is needful.

Keywords: *Citrus aurantium*; Rutaceae; acridone alkaloids; cytotoxicity, MTT assay

Abbreviations

A549, human lung adenocarcinoma; ANOVA, one-way analysis of variance; ATCC, American type culture collection; $CDCl_3$, chloroform; DMSO, dimethyl sulfoxide; ECACC, European collection of authenticated cell cultures; HepG2, human liver adenocarcinoma; HPLC, high performance liquid chromatography; HR-EI-MS, high resolution electrospray ionization mass spectrometry; IC_{50}; 50% inhibitory concentration; MCF7, human breast adenocarcinoma; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium; NCI, national cancer institute; NMR, nuclear magnetic resonance; OD, optical density; PBS, phosphate buffered-saline; PC3, human prostate adenocarcinoma; PNT2, normal human immortalised prostate cell line; SI, selectivity index; SS, stock solution; VLC, vacuum liquid chromatography; MeOH, methanol; DCM, dichloromethane; *n*-hex, *n*-hexane; SEM, standard error of mean; COSY, correlated spectroscopy; HMBC, heteronuclear multiple bond correlation; NOESY, nuclear Overhauser effect spectroscopy; DEPTQ, distortionless enhancement by polarisation transfer; FBS, foetal bovine serum; FHI, forest herbarium Ibadan; IR, infrared spectroscopy; *m/z*, *m*ass to charge ratio; WHO, world health organisation; Hz, hertz; CC, column chromatography; HSQC, homonuclear single quantum correlation; UV, ultraviolet spectroscopy

1. Introduction

Citrus aurantium L. (Rutaceae), commonly known as bitter orange or sour orange (local name in Yoruba, South West Nigeria, "*Osan gaingain*"), is used in traditional medicine to treat cancer, diabetes, malaria, typhoid and worm infestations (Arias and Ramón-Laca, 2005). The fruit extract of *C. aurantium* is used traditionally for the management anorexia and obesity. A protoalkaloid, *p*-synephrine is the most important bioactive compound present in the fruits of this plant (Stohs et al., 2011). This compound, due to its β-adrenergic activity, is responsible for the reduction in appetite and weight gain associated with the folklore use of *C. aurantium.* Hordenine, *N*-methyltyramine, octopamine and tyramine are other adrenergic amines present in small amount, also contribute to the weight reduction activity of *C. aurantium* (Fugh-Berman and Myers, 2004; Pellati and Benvenuti, 2007). There have been conflicting reports concerning the safety of *C. aurantium* extracts and *p*-synephrine. While some studies associated the anti-obesity effects of *C. aurantium* with reduction of locomotor activity and cardiovascular toxicity in mice and rats (Calapai et al., 1999; Arbo et al., 2008; Arbo et al., 2009), several clinical studies have shown that *C. aurantium* extract (at a dose of approximately 49 mg *p*-synephrine daily) displayed no adverse effects on the blood pressure, blood chemistries, electrocardiogram, heart rate, hepatic function and renal function (Kaats et al., 2013). Previous ethnopharmacological studies have revealed that *C. aurantium* possesses several useful biological activities which are summarised in Table 1.

Typical of the *Citrus* species, several flavonoids with antioxidant and anti-inflammatory activities have been isolated from various parts of *C. aurantium* (Rio et al., 1992; Barreca et al., 2011; Zhao et al., 2012). The seeds of *C. aurantium* are rich in limonoid glucosides; notably isolimonic acid, ichangin, limonin glucoside, deacetylnomilin glucoside and obacunone glucoside (Bennett et al., 1991). The triterpenoid, ichanexic acid, isolated from the ground

seeds of *C. aurantium* had significant chemopreventive activities on human colon cancer cells (Jayaprakasha et al., 2008).

In our search for cytotoxic agents from natural sources, we have recently carried out an ethnobotanical survey of medicinal plants used for cancer treatment amongst the Ijebu ethnic group of southwestern Nigeria, and several traditional healers mentioned *C. aurantium*, as part of herbal recipes for the treatment of cancer (Segun et al., 2018). Other studies within the same region documented the anticancer ethnomedicinal use of *C. aurantium* (Soladoye et al., 2010; Olujimi et al., 2014). Although *C. aurantium* is not indigenous to Nigeria, it has become a widely used medicinal plant in the country.

This study was carried out to determine the *in vitro* cytotoxicity of the crude extract of the stem bark of *C. aurantium*, identify and isolate the bioactive constituents and to establish the cytotoxicity of such constituents. In the present study, the cytotoxicity of the stem bark of *C. aurantium* on four human cancer cell lines: breast (MCF7), liver (HepG2), lung (A549) and prostate (PC3) carcinoma cells in addition to the isolation and structural elucidation of six known acridone cytotoxic alkaloids (**1**-**6**) are reported.

2. Material and methods

2.1 Instrumentation

Ultra Violet (UV) spectra were obtained on an Analytik Jena UV Visible spectrophotometer (Germany) in methanol (MeOH). Infra-red (IR) spectra were recorded on a PerkinElmer FTIR Spectrum BX spectrometer. Proton (^{1}H) and ^{13}C NMR spectra (1D and 2D experiments) were recorded on the Bruker Avance III Spectrometer at 600 and 150 MHz, respectively. Chemical shifts were reported in δ (ppm) using $CDCl_3$ as the internal standard and the coupling constants (*J*) are measured in Hertz (Hz). Mass spectrometric (HR-EI-MS) data were measured using the Thermo Scientific LTQ Orbitrap XL mass spectrometer. Analytical TLC was performed using pre-coated silica gel 60 F_{254} aluminium foil (Sigma-Aldrich, Germany) and spots were

visualised 254 nm after spraying with *p*-anisaldehyde-sulphuric acid reagent and heated at 70 °C. The HPLC analysis was performed on an Agilent 1260 infinity HPLC System coupled with an Agilent 1260 diode array detector using an Ace-5 C18 column (150 × 21.2 mm, 5 µm particle size, Hichrom Ltd) and HPLC grade solvents were purchased from Fischer Scientific (Loughborough, UK). Absorbance measurements for the MTT assay were taken on a CLARIO star microplate reader (BMG labtech, Germany).

2.2 Cell culture materials

Human adenocarcinoma cell lines: breast (MCF7), liver (HepG2), lung (A549) and prostate (PC3) were purchased from the American Type Culture Collection (ATCC), (LGC Standards, Middlesex, UK), and the normal human immortalised prostate cell line (PNT2) was obtained from the European Collection of Authenticated cell cultures (ECACC), (Public Health England, Salisbury, UK). Cell culture materials including Dulbecco's Modified Eagle Medium (DMEM), foetal bovine serum (FBS) and trypsin were obtained from Biosera (Nauaille, France). Chemicals including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), vinblastine, trypan blue, phosphate buffered-saline (PBS) tablets and penicillin-streptomycin antibiotics suspension were purchased from Sigma-Aldrich (Dorset, UK).

2.3 Plant material

Fresh stem bark of *C. aurantium* was purchased at the Bode herb market, Ibadan, Nigeria in July 2016. The material was identified and authenticated at the Forest Herbarium Ibadan, Nigeria, where a voucher specimen (FHI 111229) was deposited. The botanical name of the plant was verified on the plant list database (http://www.theplantlist.org/; accessed 29/10/2016).

2.4 Extraction and isolation of active compounds

The air-dried and powdered stem bark of *C. aurantium* (1 kg) was extracted with MeOH (5 L x 2) at room temperature (25-34 °C) with continuous stirring for 72 h. The combined filtrate

was concentrated under reduced pressure at 40 °C to give a dark brown crude extract (138 g). A portion of this crude extract (80 g) was dissolved in MeOH (140 mL) and suspended in H_2O (60 mL) and partitioned successively with *n*-hexane (200 mL x 5), dichloromethane (DCM) (200 mL x 5) and methanol (MeOH) (200 mL x 5). The DCM fraction (5 g) was subjected to vacuum liquid chromatography (VLC) on silica gel (5 x 30 cm; < 55 in size) and eluted with gradient mixtures of DCM/MeOH (10:1 to 5:5, each 250 mL). A total of 10 sub-fractions were collected and pooled into four main fractions (Fractions A-D), based on analytical TLC profiles of the fractions.

Fraction C (2 g) was chromatographed over a silica column gel (5 x 30 cm; 63–200 µm) and successively eluted with *n*-hexane/EtOAc (3:7 to 1:10) and EtOAc/MeOH (1:10 to 5:5) by gradient elution. A total of 180 sub fractions of (100 mL each), were collected and pooled into 16 sub-fractions (Sub-Fractions 1 - 16). Sub-fraction 3 (121 mg) was purified on reversed phase preparative HPLC (mobile phase H_2O/MeOH containing 0.1 trifluoroacetic acid (TFA); 0-5 min: 30-50% MeOH, 5-20 min: 50-80% MeOH, 20-30 min: 80-100% MeOH, 30-35 min: 100% MeOH, 35-40 min: 30% MeOH) to afford citrusinine-I (**1,** 7.7 mg; t_R=12.3 min), citracridone-I (**2,** 9.7 mg; t_R=19.3 min) and 5-hydroxynoracronycine (**3,** 7.3 mg; t_R=21.8 min). Each time, UV detection were at 220, 254, and 320 nm and the flow rate was maintained at 10 mL/min. Sub-fraction 4 (81 mg) was purified on RP-Prep HPLC (mobile phase H_2O/MeOH containing 0.1 TFA; 0-30 min: 40-100% MeOH, 30-35 min: 100% MeOH, 35-40 min: 40% MeOH) to afford natsucitrine-I (**4,** 6.4 mg; t_R=15.1 min). Sub-Frac 5 (86 mg) was purified on RP-Prep HPLC (mobile phase H_2O/MeOH containing 0.1 TFA; 0-3 min: 30-75% MeOH, 3-30 min: 75-100% MeOH, 30-33 min: 100% MeOH, 33-38 min: 30% MeOH) to afford glycofolinine **(5,** 7.1 mg, t_R=14.1 min). Sub-fraction 6 (91 mg) was purified on RP-PREP HPLC (mobile phase H_2O/ACN containing 0.1 TFA; 0-3 min: 30-50% ACN, 3-30 min: 50-

100% ACN, 30-33 min: 100% ACN, 33-38 min: 30% ACN) to afford citracridone-III **(6,** 9.9 mg, t_R=13.2 min).

2.5 Cytotoxicity studies

2.5.1 Cell lines and cell culture

Human cancer cell lines: A549 (lung), HepG2 (liver), MCF7 (breast) and PC3 (prostate) alongside the normal prostate epithelium cells (PNT2) were cultured in T75 flasks containing DMEM supplemented with 10% FBS and 1% penicillin-streptomycin antibiotics suspension. The cells were maintained in a humidified atmosphere, containing 5% CO_2 at 37 °C and passaged bi-weekly at a 1:3 dilution using 0.05% trypsin - 0.02% EDTA in PBS. Changes in cell morphology, corresponding to the effects of test compounds, were monitored through an inverted microscope (40x and 100x, Olympus CKX41, UK). Cells were counted using an automated haemocytometer (C-Chip NanoEnTek, USA).

2.5.2 Preparation of extracts and compounds

Plants extracts and fractions were solubilised in 100% dimethyl sulfoxide (DMSO), to give a stock solution (SS) of 100 mg/mL. Uniformity of the solution was ensured by stirring with vortex agitation (1 min) prior to use. From the SS, serial dilutions were made in the cell culture media to obtain working concentrations of 100, 20, 4, 0.8 and 0.16 µg/mL. For the pure compounds, a SS of 10 M was prepared in 10% DMSO solution, thereafter, serial dilutions were made to give working concentrations of 100, 50, 25, 12.5 and 6.25 µM for treatment with MCF7, HepG2, A549 and PC3, and working concentrations of 800, 400, 200, 100 and 50 µM for treatment with PNT2. For vinblastine (positive control), a SS of 10 µM was prepared in 10% DMSO solution, thereafter, serial dilutions were made to give working concentrations of 100, 50, 25, 12.5, 6.25 nM. Dilutions of the SS were made in cell culture media with the final concentration of DMSO not exceeding 0.1%, which had previously been found not to be cytotoxic (Basar et al., 2015).

2.5.3 Colorimetric MTT *in vitro* assay

Cytotoxicity testing was carried out using the MTT assay, which determines the number of viable cells in culture (Gerlier and Thomasset, 1986). After repeated initial experiments, the cell densities for optimal growth were found to be 2 x 10^4, 4 x 10^4, 1.2 x 10^4, 2 x 10^4 and 4 x 10^4 cells/well for MCF7, HepG2, A549, PC3 and PNT2, respectively. The exponential phases of the cell lines were seeded into flat-bottomed, 24-well plate (Corning Costar® TC-treated, Merck, UK) and incubated at 37°C/5% CO_2 for 24 h to allow the cells to adhere to the surface of the plate. After reaching about 90% confluence at 24 h, the old medium was removed by vacuum suctioning, and the cells treated with varying concentrations (1 mL each) of the extract(s), fraction(s) and compound(s), and incubated for 72 h. The first five wells of the 24-well plate (A1-A5) contained the various concentrations of the test extract/fractions/compounds, while the last well (A6) contained only 1 mL of the DMSO-growth medium (negative control). The experiment was conducted in quadruplicate for each test dilution, so that wells A1-D1, A2-D2, A3-D3, A4-D4 and A5-D5 contained different concentrations of the extract/fractions/compounds. At the expiration of the incubation period, the MTT assay was performed to measure the cell viability. The protocol described earlier in the literature was adopted with slight modifications (Ogbole et al., 2017). Briefly, the old medium was removed from each well by vacuum suctioning and replaced with 1 mL of 500 µg/mL MTT dye in PBS. The plates were incubated for 2 h at 37°C/5% CO_2 to allow the production of formazan crystals. After incubation, the medium was removed by vacuum suctioning and 500 µL of propanol was added, under light protection, to dissolve the dark blue formazan crystals. The plates were placed on a shaker for 10 min to enhance the dissolution of preformed formazan crystals. The dilution in each 24-well plate was transferred into a 96 well plate, so that the solution in A1 (24-well) was transferred to A1-A4 (96-well), B1 (24-well) was transferred to B1-B4 (96-well) and so on. The absorbance of the solution was measured at

570 nm on a Clario Star microplate reader. The average optical density (OD) obtained from the negative control wells was set arbitrarily at 100% and the average OD of treatment wells was calculated relative to the negative control. In addition, the same process was carried out for vinblastine (positive control) using the concentration range mentioned above. The cell viability was calculated as follows:

$$\% \text{ Inhibition } = \frac{\text{Average OD(control)} - \text{Average OD(treated)}}{\text{Average OD(control)}} \text{ x } 100$$

2.6 Statistical analysis

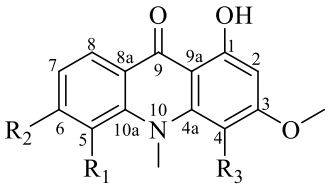
The results obtained were expressed as mean ± standard errors of means (SEM). The experiments were conducted in quadruplicate on three different occasions. Statistical analysis was done using GraphPad (version 5.01, GraphPad Prism Software Inc., San Diego, CA). One-way analysis of variance (ANOVA) followed by Tukey's test (each treatment compared to control) were used to test for the statistical differences between the groups. Differences between means were considered statistically significant when $P<0.05$. Inhibitory concentrations at (IC_{50}) were obtained from graphs plotted using non-linear regression equation log (inhibitor) versus response - variable slope.

3. Results and discussion

3.1 Structure determination

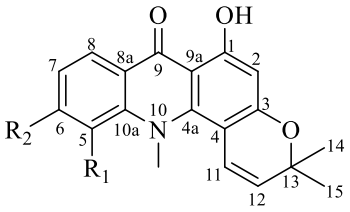
As the DCM fraction of *C. aurantium* was the most cytotoxic, the activity-guided fractionation of this fraction was performed using several chromatographic techniques, including vacuum liquid chromatography (VLC), column chromatography and HPLC to afford compounds **1-6.** Spectroscopic analysis, mainly, ^{1}H NMR, ^{13}C NMR, ^{13}C DEPTQ, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC and ^{1}H-^{1}H NOESY and mass spectrometry (MS) were used to unambiguously elucidate the structures of the isolated compounds. All spectral data were compared with the published literature data and the compounds were identified as citrusinine-I (**1**) (Wu and Furukawa,

1983), citracridone-I (**2**) (Wu et al., 1983), 5-hydroxynoracronycine (**3**) (Wu and Furukawa, 1983; Yamamoto et al., 1989), natsucitrine-I (**4**) (Juoichi et al., 1985), glycofolinine (**5**) (Ono et al., 1995) and citracridone-III (**6**) (Yamamoto et al., 1989). This is the first report of the isolation of these compounds from *C. aurantium*, though they have been reported from other *Citrus spp* (*C. sinensis* (L.) Osbeck, Wu and Furukawa, 1983; *C. reticulata* Blanco, Wu et al., 1983; *C. natsudaidai* (Yu. Tanaka) Hayata, Juoichi et al., 1985; *C. sinensis* (L.) Osbeck,Yamamoto et al., 1989).



2

1. R_1 = OH; R_2 = H; R_3 = OCH_3
4. R_1 = OCH_3; R_2 = OH; R_3 = H
5. R_1 = OCH_3; R_2 = OH; R_3 = OCH_3



2. R_1 = OCH_3; R_2 = OH
3. R_1 = OH; R_2 = H
6. R_1 = OH; R_2 = OH

Figure 1: Structures of compounds **1**-**6**

Citrusinine-I (**1**): pale yellow amorphous powder; UV (MeOH) λ_{max} (log ε) 208 (0.13), 266 (015), 320 (0.07), 418 (0.02) nm; IR (KBr) ν_{max} 3241, 2966, 1625, 1588, 1563, 1462, 1384, 1128, 1103 cm^{-1}; positive HRESIMS *m/z* 302.1030 $[M+H]^+$ (calcd for $C_{16}H_{16}NO_5$, 302.1028); ^{1}H-NMR and ^{13}C-NMR (600/150 MHz, in $CDCl_3$) shown on Tables 2 and 3, respectively.

Citracridone-I (**2**): orange amorphous powder; UV (MeOH) λ_{max} (log ε) 202 (0.57), 268 (0.79), 328 (0.27) nm; IR (KBr) ν_{max} 3199, 2967, 2643, 1681, 1625, 1587, 1566, 1174, 1129 cm^{-1}; positive HRESIMS *m/z* 354.1338 $[M+H]^+$ (calcd for $C_{20}H_{20}NO_5$, 354.1336); ^{1}H-NMR and ^{13}C-NMR (600/150 MHz, in $CDCl_3$) shown on Tables 2 and 3, respectively.

5-hydroxynoracronycine (3): yellow amorphous powder; UV (MeOH) λ_{max} (log ε) 208 (0.08), 266 (0.09), 284 (0.09), 322 (0.04), 418 (0.01) nm; IR (KBr) ν_{max} 3250, 2960, 1625, 1588, 1563, 1359, 1128, 1103 cm^{-1}; positive HRESIMS *m/z* 324.1233 $[M+H]^+$ (calcd for $C_{19}H_{18}NO_4$, 324.1230); ^{1}H-NMR and ^{13}C-NMR (600/150 MHz, in $CDCl_3$) shown on Tables 2 and 3, respectively.

Natsucitrine-I (4): yellow amorphous powder; UV (MeOH) λ_{max} (log ε) 268 (1.28), 330 (0.33), 384 (0.17) nm; IR (KBr) ν_{max} 3197, 2967, 2646, 1680, 1625, 1556, 1481, 1174, 1129 cm^{-1}; positive HRESIMS *m/z* 302.1025 $[M+H]^+$ (calcd for $C_{16}H_{16}NO_5$, 302.1023); ^{1}H-NMR and ^{13}C-NMR (600/150 MHz, in $CDCl_3$) shown on Tables 2 and 3, respectively.

Glycofolinine (5): yellow amorphous powder; UV (MeOH) λ_{max} (log ε) 272 (2.36), 346 (0.65) nm; IR (KBr) ν_{max} 3194, 2967, 2646, 1680, 1625, 1566, 1480, 1174, 1129 cm^{-1}; positive HRESIMS *m/z* 332.1131 $[M+H]^+$ (calcd for $C_{17}H_{18}NO_6$, 332.1129); ^{1}H-NMR and ^{13}C-NMR (600/150 MHz, in $CDCl_3$) shown on Tables 2 and 3, respectively.

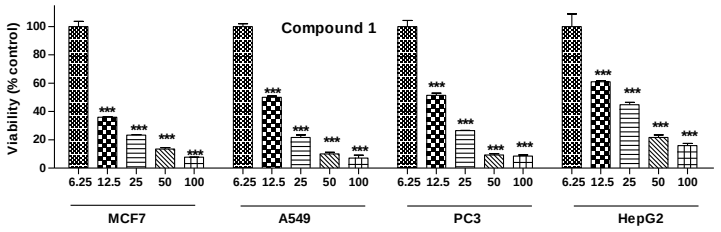
Citracridone-III (6): yellow amorphous powder; UV (MeOH) λ_{max} (log ε) 204 (2.01), 272.0 (0.32) nm; IR (KBr) ν_{max} 3241, 2966, 1625, 1588, 1564, 1462, 1384, 1128, 1103 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR (600/150 MHz, in $CDCl_3$) shown on Tables 2 and 3, respectively.

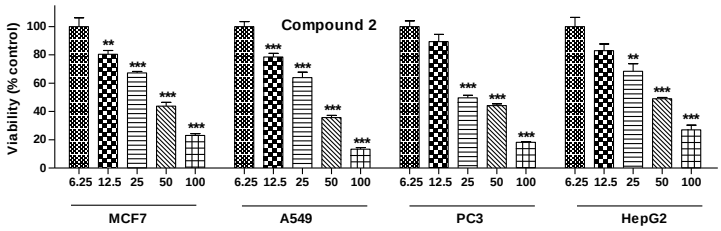
3.2 Cytotoxicity assay

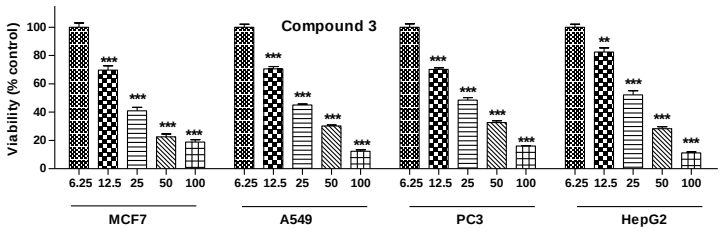
The effects of the extract, fractions and isolated compounds of *C. aurantium* was tested on MCF7, HepG2, A549 and PC3 cell lines. These cell lines were selected for this study, because of high and increased in incidence rates of these cancers. The need to develop effective and safe chemotherapeutic agents as novel treatments cannot be over emphasised. Breast, liver and lung cancers account for 60% of the cancer cases among women globally, while the prostate cancer is the most diagnosed cancer type in males (Torre et al., 2017). The DCM and MeOH fractions of the stem bark of *C. aurantium* were found to possess activity below 100 µg/mL

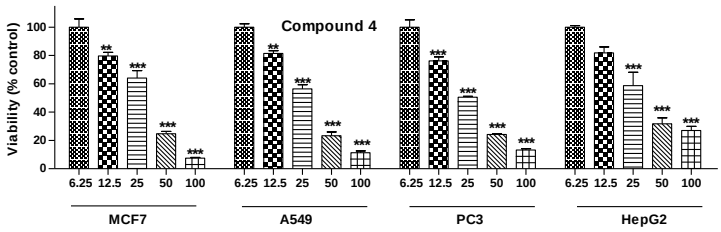
against the cancer cells while the *n*-hexane fraction was inactive at 100 µg/mL against the carcinoma cell lines (Table 4). However, only the DCM fraction had an activity that met the specification of the National Cancer Institute (NCI), which requires a crude plant extract/fraction to possess IC_{50} value < 30 µg/mL before it could be considered as active (Rajkapoor et al., 2007).

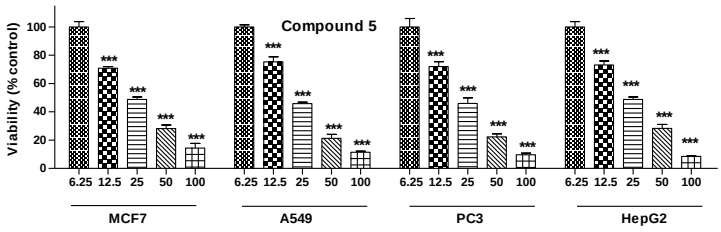
Therefore, the DCM fraction was investigated further in this work. The DCM fraction showed highest activity against A549 cells (IC_{50} = 3.88 µg/mL) and displayed the least activity on HepG2 cells (IC_{50} = 5.73 µg/mL). The DCM fraction was subjected to further purification from which six acridone alkaloids were isolated. The MTT cell viability assay was used to determine the cytotoxicity of the fractions and the isolated compounds. This assay measures the ability of the mitochondrial enzyme, succinate dehydrogenase, to reduce the soluble tetrazolium containing yellow dye (MTT) into purple colour formazan crystals. Carcinoma cells rapidly divide and so display high degree of metabolic activity and aid a high degree of MTT reduction. The higher the number of viable cells in a culture, the higher the quantity of formazan that will be produced, and this gives an indication of the degree of cytotoxicity of the test extract/fraction/compound. Vinblastine, a reference compound used in several cytotoxicity evaluations, was included as a positive control. All test compounds elicited dose-dependent activity across the panel of human cancer cells (Figure 2), although with varying potencies as revealed by their IC_{50} values (Table 5). In addition, all the test compounds exhibited time-dependent activity as the extent of morphological damage to the cells observed in the photomicrographs increased with prolonged exposure period. As the treatment time increased, cells showed progressive loss of normal elongated shape, shrunk to smaller round cells with a resultant loss of cell viability.


Compound 1
Viability (% control)
0
20
40
60
80
100
6.25 12.5 25 50 100
MCF7
A549
PC3
HepG2


Compound 2
Viability (% control)
0
20
40
60
80
100
6.25 12.5 25 50 100
MCF7
A549
PC3
HepG2


Compound 3
Viability (% control)
0
20
40
60
80
100
6.25 12.5 25 50 100
MCF7
A549
PC3
HepG2


Compound 4
Viability (% control)
0
20
40
60
80
100
6.25 12.5 25 50 100
MCF7
A549
PC3
HepG2


Compound 5
Viability (% control)
0
20
40
60
80
100
6.25 12.5 25 50 100
MCF7
A549
PC3
HepG2

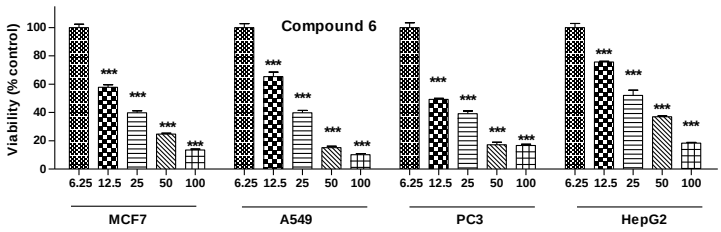

Compound 6
Viability (% control)
0
20
40
60
80
100
6.25 12.5 25 50 100
MCF7
A549
PC3
HepG2

Fig 2: Concentration-dependent compound-induced reduction in the viability of MCF-7, HepG2, A549 and PC3. Cells were treated with a range of concentrations of the compounds for 72 h and the viability was assessed using MTT assay. Vinblastine was used as a reference standard, with IC_{50} of approximately 20 – 40 nM across the panel of carcinoma cells. According to the IC_{50} value, the rank order of increasing potencies of the compounds is citracridone-I (**2**) > citracridone-III (**6**) > 5-hydroxynoracronycine (**3**) > glycofolinine (**5**) > natsucitrine-I (**4**) > citrusinine-I (**1**). Values shown are means ± SEM (Standard error of the mean) of three different experiments. **P< 0.01 and ***P< 0.001, compared to the negative control (DMSO only), evaluated using the Tukey multiple comparison. X-axis: concentration in μM.

The acridones examined in this study could be classified into two groups: tricyclic alkaloids (**1** (citrusinine-I)**, 4** (natsucitrine-I) and **5** (glycofolinine)) and tetracyclic alkaloids (**2** (citracridone-I)**, 3** (5-hydroxynoracronycine) and **6** (citracridone-III). Generally, the tetracyclic acridones displayed higher cytotoxicity towards the carcinoma cell lines than the tricyclic acridones. A similar pattern was observed in a previous work (Kawaii et al., 1999). Comparing the IC_{50} value for the cytotoxicity of the acridone alkaloids revealed important structural requirements for the observed activity. Citracridone-I which possess a methoxyl group at the C-5 position and a hydroxyl group at the C-6 position had the highest anti-proliferative activity (IC_{50} value = 12.5 – 14.8 μM) amongst the alkaloids. Citracridone-III which has a hydroxyl group attached to the C-5 position, unlike the methoxyl group of citracridone-I, had a reduced antiproliferative activity (IC_{50} value = 18.2 – 20.9 μM). Further investigation of the tetracyclic alkaloids revealed that 5-hydroxynoracronycine which lacked the hydroxyl group present in citracridone-III, had the least cytotoxic activity on the panel of cell carcinoma with IC_{50} value of 24.0 – 30.0 μM. Similar to the tetracyclic acridones, the presence of a methoxyl group and hydroxyl group at C-5 and C-6, respectively increase the cytotoxicity of the tricyclic acridones (glycofolinine > citrusinine-I**;** natsucitrine-I > citrusinine-I). In addition, methoxylation at the C-4 position enhances cytotoxicity (glycofolinine > natsucitrine-I). All the acridone studied in

this report had a hydroxyl group at the C-1 position. A previous study that examined the structure-activity relationships (SAR) of natural occurring acridones revealed that the intramolecular hydrogen bonding between the hydroxyl group on C-1 position and the peri-carbonyl function of the acridone nucleus is necessary for antiproliferative activity as the replacement of the hydroxyl group with methoxyl group resulted in a marked decrease in cytotoxicity (Michael, 2008). All acridones studied displayed low activity towards the HepG2 cells and high activity against MCF7 cells.

Previous antiproliferative studies on citrusinine-I reported varying cytotoxicity towards tumour cells. An earlier report showed that citrusinine-I displayed a potent antiproliferative activity towards HL-60 cell line with an IC_{50} value of 8.6 µM according to the alamar blue assay (Braga et al., 2007), while another study reported an IC_{50} value less than 50 µM on HepG2 and KB cell lines according to the MTT assay (Teng et al., 2005). However, citrusinine-I did not show significant activity on COR-L23, C32, MCF7 and MRC-5 with IC_{50} value above 100 µM in the sulforhodamine-B assay (Braga et al., 2007). These differences might be the result of different cancer cell lines and cell proliferation methods used in the studies. Citracridone-I and 5-hydroxynoracronycine were reported to display moderate cytotoxicity towards AU565, A431, SKBR-3 and T47D cell lines (Phetkul et al., 2014), while 5-hydroxynoracronycine exhibited a moderate antiproliferative activity towards PC3 cell line (Happi et al., 2011). In an *in vitro* assay of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induced Epstein–Barr virus early antigen (EBV-EA) activation assay, citrusinine-I and glycofolinine displayed a better anti-tumour promoting activity than β-carotene with IC_{50} value 300 mol ratio/TPA and 535 mol ratio/TPA respectively (Itoigawa et al., 2003). The cytotoxicity of natsucitrine-I is reported in this work for the first time.

Several studies have reported the antiviral potentials of the acridone alkaloids studied in this paper. Glycofolinine displayed moderate activity against both HSV-1 and HSV-2 in

inactivation treatment and post-treatment with EC_{50} of 151 µM in both treatments (Chansriniyom et al., 2009). Another study revealed that citrusinine displayed an EC_{50} of 0.56 µg/mL and 0.74 µg/mL against HSV-1 and HSV-2, respectively, while citracridone-I exhibited an EC_{50} of 1.3 µg/mL and 4.8 µg/mL against HSV-1 and HSV-2, respectively (Yamamoto et al., 1989). 5-Hydroxynoracronycine displayed a moderate anti-HIV-1 protease activity with an IC_{50} value of 93.1 µM (Panthong et al., 2013) and displayed remarkable inhibitory effects on Epstein-Barr virus activation (Takemura et al., 1995).

The goal of cancer chemotherapy is the ability of chemotherapeutic agents for cancer to differentiate between cancer and normal human cells. Anticancer drugs are expected to exhibit selective toxicity, as this is needful to overcome noxious side effects associated with their usage. To measure the selectivity indices (SI) of the acridones used in this study, the normal human prostate cells (PNT2) was included in the present study. Vinblastine, the positive control, had a SI value ranging from 1.00 to 1.76 across the cancer cell lines, suggesting that the drug cannot sufficiently differentiate between normal and cancer cells, a result in agreement with a previous study (Ashidi et al., 2010). It is interesting to note that the cytotoxicity of these compounds was significantly lower toward the normal human cell line compared to the carcinoma cell lines (Table 6). The table revealed that the compounds exerted at least four times higher cytotoxicity towards the carcinoma cells than the PNT2 cells. Citracridone-I (**2**) had the highest SI value (approx. 7-13) across the panel of carcinoma cells, while citrusinine (**1**) displayed the lowest SI value (approx. 4-5) against the tumour cells. These results suggest that the acridone alkaloids have potentials in the development of low-toxicity chemotherapeutic agents.

Citrus aurantium is widely used by traditional medical practitioners in southwestern Nigeria for the treatment of breast cancer, prostate cancer, wounds and other inflammatory diseases (Segun et. al., 2018). For the management of breast cancer, the stem bark of *C. aurantium,*

Entandrophragma utile (Dawe & Sprague) Sprague (Meliaceae) leaves, *Annona muricata* L. (Annonaceae) leaves and the roots of *Xylopia aethiopica* (Dunal) A. Rich. (Annonaceae) are boiled together in water for two hours. Three cups (approximately 20 mL per cup) of the decoction is taken thrice daily for one month. In addition, the stem bark of *C. aurantium, Garcinia kola* Heckel (Clusiaceae) fruits and *Chrysophyllum albidum* G.Don (Sapotaceae) fruits are grounded in a mortar and mixed with palm oil. The paste obtained is applied on the affected breast twice daily for up to two months. The result of the *in vitro* MTT assay has given credence to the ethnomedicinal use of this plant. However, it is expedient to carry out further *in vivo* toxicity studies to ascertain the safety of this medicinal plant, as a positive outcome from such research might lead to the development of low-toxicity chemotherapeutic agents.

4. Conclusion

This study reported the antiproliferative potential of *C. aurantium* stem bark on four human cancer cell lines. In addition, six acridone alkaloids have been reported from *C. aurantium* stem bark. Additionally, the cytotoxicity of natsucitrine-I on any human cancer cell line is reported for the first time. The six acridone alkaloids isolated from the active DCM fraction had varying cytotoxicity, with citracridone-I displaying highest cytotoxicity across four human carcinoma cell lines. The high selectivity displayed by the compounds suggest that the acridone alkaloids have potential in the development of low-toxicity chemotherapeutic agents. The cytotoxicity of the isolated compounds, may support the ethnomedicinal use of this plant, in the management of cancer and other inflammatory diseases. Further study to explore the underlying molecular mechanism of the isolated acridone alkaloids is needful.

Acknowledgement

The Commonwealth Scholarship Commission is hereby appreciated for a Split-Site PhD scholarship award given to PAS (NGCN-2016-110) to carry out part of this work. This work was taken in part from the PhD Thesis of PAS. The EPSRC National Mass Spectroscopy Services, Swansea University, is gratefully acknowledged for providing the mass spectroscopic data for purified compounds. Mr T.K Odewo (Forest Herbarium Unit, Forestry Research Institute of Nigeria) is greatly appreciated for identification and authentication of the plant material. The authors also thank Mrs Stephanie T. Guetchueng, Mrs Afaf Al-Groshi and Mrs Shaymaa Al-Majmaie (Liverpool John Moores University) for help during this piece of work.

Authors contribution

PAS and OOO conceived and designed the experiments. PAS carried out the plant extraction and the isolation of compounds. ARE provided materials and supervision for the biological studies. FMDI, OOO and SDS supervised various part of the work. OOO, LN, EOA, ARE and SDS assisted with the interpretation of various data. PAS, EOA and SDS contributed to the writing of the manuscript. All authors read and approved the final version of the manuscript.

References

Ahangarpour, A., Oroojan, A.A., Amirzargar, A., Ghanavati, M., 2011. Antispasmodic effects of *Citrus aurantium* flowers aqueous extract on uterus of non-pregnant rats. Iran. J. Reprod. Med 9, 289.

Ammar, A.H., Bouajila, J., Lebrihi, A., Mathieu, F., Romdhane, M., Zagrouba, F., 2012. Chemical composition and *in vitro* antimicrobial and antioxidant activities of *Citrus aurantium* L. flowers essential oil (Neroli oil). Pak. J. Biol. Sci. 15, 1034-1040.

Arbo, M., Larentis, E., Linck, V., Aboy, A., Pimentel, A., Henriques, A., Dallegrave, E., Garcia, S., Leal, M., Limberger, R., 2008. Concentrations of p-synephrine in fruits and leaves of *Citrus* species (Rutaceae) and the acute toxicity testing of *Citrus aurantium* extract and p-synephrine. Food Chem. Toxicol. 46, 2770-2775.

Arbo, M.D., Schmitt, G.C., Limberger, M.F., Charão, M.F., Moro, Â.M., Ribeiro, G.L., Dallegrave, E., Garcia, S.C., Leal, M.B., Limberger, R.P., 2009. Subchronic toxicity of *Citrus aurantium* L.(Rutaceae) extract and p-synephrine in mice. Regula. Toxicol. Pharmacol. 54, 114-117.

Arias, B.A., Ramón-Laca, L., 2005. Pharmacological properties of citrus and their ancient and medieval uses in the Mediterranean region. J. Ethnopharmacol. 97, 89-95.

Ashidi, J.S., Houghton, P.J., Hylands, P.J., Efferth, T., 2010. Ethnobotanical survey and cytotoxicity testing of plants of South-western Nigeria used to treat cancer, with isolation of cytotoxic constituents from *Cajanus cajan* Millsp. leaves. J. Ethnopharmacol. 128, 501-512.

Barreca, D., Bellocco, E., Caristi, C., Leuzzi, U., Gattuso, G., 2011. Distribution of C-and O-glycosyl flavonoids,(3-hydroxy-3-methylglutaryl) glycosyl flavanones and furocoumarins in *Citrus aurantium* L. juice. Food Chem. 124, 576-582.

Basar, N., Oridupa, O.A., Ritchie, K.J., Nahar, L., Osman, N.M.M., Stafford, A., Kushiev, H., Kan, A., Sarker, S.D., 2015. Comparative cytotoxicity of *Glycyrrhiza glabra* roots from different geographical origins against immortal human keratinocyte (HaCaT), lung adenocarcinoma (A549) and liver carcinoma (HepG2) Cells. Phytother. Res. 29, 944-948.

Bennett, R.D., Miyake, M., Ozaki, Y., Hasegawa, S., 1991. Limonoid glucosides in *Citrus aurantium*. Phytochemistry 30, 3803-3805.

Braga, P., Dos Santos, D., Da Silva, M., Vieira, P., Fernandes, J., Houghton, P., Fang, R., 2007. *In vitro* cytotoxicity activity on several cancer cell lines of acridone alkaloids and N-

phenylethyl-benzamide derivatives from *Swinglea glutinosa* (Bl.) Merr. Nat. Prod. Res. 21, 47-55.

Calapai, G., Firenzuoli, F., Saitta, A., Squadrito, F., Arlotta, M.R., Costantino, G., Inferrera, G., 1999. Antiobesity and cardiovascular toxic effects of *Citrus aurantium* extracts in the rat: a preliminary report. Fitoterapia 70, 586-592.

Chansriniyom, C., Ruangrungsi, N., Lipipun, V., Kumamoto, T., Ishikawa, T., 2009. Isolation of acridone alkaloids and N-[(4-monoterpenyloxy) phenylethyl]-substituted sulfur-containing propanamide derivatives from *Glycosmis parva* and their anti-herpes simplex virus activity. Chem. Pharm. Bull. 57, 1246-1250.

Costa, C.A., Cury, T.C., Cassettari, B.O., Takahira, R.K., Flório, J.C., Costa, M., 2013. *Citrus aurantium* L. essential oil exhibits anxiolytic-like activity mediated by 5-HT 1A-receptors and reduces cholesterol after repeated oral treatment. BMC Comp. Alt. Med. 13, 42.

Fugh-Berman, A., Myers, A., 2004. *Citrus aurantium*, an ingredient of dietary supplements marketed for weight loss: current status of clinical and basic research. Exp. Biol. Med. 229, 698-704.

Gerlier, D., Thomasset, N., 1986. Use of MTT colorimetric assay to measure cell activation. J. Immunol. Methods 94, 57-63.

Happi, E.N., Waffo, A.F.K., Wansi, J.D., Ngadjui, B.T., Sewald, N., 2011. *O*-Prenylated acridone alkaloids from the stems of *Balsamocitrus paniculata* (Rutaceae). Planta Med. 77, 934-938.

Itoigawa, M., Ito, C., Wu, T.-S., Enjo, F., Tokuda, H., Nishino, H., Furukawa, H., 2003. Cancer chemopreventive activity of acridone alkaloids on Epstein–Barr virus activation and two-stage mouse skin carcinogenesis. Cancer Lett. 193, 133-138.

Jayaprakasha, G., Mandadi, K., Poulose, S., Jadegoud, Y., Gowda, G.N., Patil, B.S., 2008. Novel triterpenoid from *Citrus aurantium* L. possesses chemopreventive properties against human colon cancer cells. Bioorg. Med. Chem. 16, 5939-5951.

Juoichi, M., Inoue, M., Fijitani, Y., Furukawa, H., 1985. Natsucitrine-I. II: New acridone alkaloids from *Citrus natsudaida*i Hayata. Heterocycles 23, 1131-1134.

Kaats, G.R., Miller, H., Preuss, H.G., Stohs, S.J., 2013. A 60 day double-blind, placebo-controlled safety study involving *Citrus aurantium* (bitter orange) extract. Food and Chem. Toxicol. 55, 358-362.

Kang, P., Ryu, K.H., Lee, J.M., Kim, H.K., Seol, G.H., 2016. Endothelium- and smooth muscle-dependent vasodilator effects of *Citrus aurantium L. var. amara*: Focus on Ca2+ modulation. Biomed. Pharmacother. 82, 467-471.

Kang, S.R., Park, K.I., Park, H.S., Lee, D.H., Kim, J.A., Nagappan, A., Kim, E.H., Lee, W.S., Shin, S.C., Park, M.K., 2011. Anti-inflammatory effect of flavonoids isolated from Korean *Citrus aurantium* L. on lipopolysaccharide-induced mouse macrophage RAW 264.7 cells by blocking of nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signalling pathways. Food Chem. 129, 1721-1728.

Kawaii, S., Tomono, Y., Katase, E., Ogawa, K., Yano, M., Takemura, Y., Ju-ichi, M., Ito, C., Furukawa, H., 1999. The antiproliferative effect of acridone alkaloids on several cancer cell lines. J. Nat. Prod. 62, 587-589.

Michael, J.P., 2008. Quinoline, quinazoline and acridone alkaloids. Nat. Prod. Rep. 25(1), 166-187.

Olujimi, O.O., Bamgbose, O., Arowolo, T., Steiner, O., Goessler, W., 2014. Elemental profiles of herbal plants commonly used for cancer therapy in Ogun State, Nigeria. Part I. Microchem. J. 117, 233-241.

Ogbole, O.O., Segun, P.A., Adeniji, A.J., 2017. *In vitro* cytotoxic activity of medicinal plants from Nigeria ethnomedicine on Rhabdomyosarcoma cancer cell line and HPLC analysis of active extracts. BMC Comp. Alt. Med. 17(1), 494.

Ono, T., Ito, C., Furukawa, H., Wu, T.S., Kuoh, C.S., Hsu, K.S., 1995. Two new acridone alkaloids from *Glycosmis* species. J. Nat. Prod. 58, 1629-1631.

Panthong, K., Srisud, Y., Rukachaisirikul, V., Hutadilok-Towatana, N., Voravuthikunchai, S.P., Tewtrakul, S., 2013. Benzene, coumarin and quinolinone derivatives from roots of *Citrus hystrix*. Phytochemistry 88, 79-84.

Pellati, F., Benvenuti, S., 2007. Chromatographic and electrophoretic methods for the analysis of phenetylamine alkaloids in *Citrus aurantium*. J. Chromatogr. A 1161, 71-88.

Phetkul, U., Phongpaichit, S., Watanapokasin, R., Mahabusarakam, W., 2014. New depside from *Citrus reticulata* Blanco. Nat. Prod. Res. 28, 945-951.

Polo, C. M., Moraes, T. M., Pellizzon, C. H., Marques, M. O., Rocha, L. R. M., Hiruma-Lima, C. A., 2012. Gastric ulcers in middle-aged rats: The healing effect of essential oil from Citrus aurantium L.(Rutaceae). Evid. Based Complementary Altern. Med., 2012.

Rajkapoor, B., Sankari, M., Sumithra, M., Anbu, J., Harikrishnan, N., Gobinath, M., Suba, V., Balaji, R., 2007. Antitumor and cytotoxic effects of *Phyllanthus polyphyllus* on ehrlich ascites carcinoma and human cancer cell lines. Biosci. Biotechnol. and Biochem. 71, 2177-2183.

Rio, J.A., Benavente, O., Castillo, J., Borrego, F., 1992. Neodiosmin, a flavone glycoside of *Citrus aurantium*. Phytochemistry 31, 723-724.

Sanei-Dehkordi, A., Sedaghat, M.M., Vatandoost, H. and Abai, M.R., 2016. Chemical compositions of the peel essential oil of *Citrus aurantium* and its natural larvicidal activity against the malaria vector *Anopheles stephensi* (Diptera: Culicidae) in comparison with *Citrus paradisi*. J. Arthropod Borne Dis. 10(4), 577

Sarrou, E., Chatzopoulou, P., Dimassi-Theriou, K., Therios, I., 2013. Volatile constituents and antioxidant activity of peel, flowers and leaf oils of *Citrus aurantium* L. growing in Greece. Molecules 18(9), 10639-10647.

Segun, P.A., Ogbole, O.O., Ajaiyeoba, E.O., 2018. Medicinal Plants Used in the Management of Cancer among the Ijebus of Southwestern Nigeria. J. Herb. Med. https://doi.org/10.1016/j.hermed.2018.04.002

Soladoye, M.O., Amusa, N., Raji-Esan, S., Chukwuma, E., Taiwo, A., 2010. Ethnobotanical survey of anti-cancer plants in Ogun State, Nigeria. Ann. Biol. Res 1, 261-273.

Stohs, S.J., Preuss, H.G., Shara, M., 2011. The safety of *Citrus aurantium* (bitter orange) and its primary protoalkaloid *p*-synephrine. Phytother. Res. 25, 1421-1428.

Takemura, Y., Ju-ichi, M., Ito, C., Furukawa, H., Tokuda, H., 1995. Studies on the Inhibitory Effects of some Acridone Alkaloids on Epstein-Barr Virus Activation. Planta Med. 61, 366-368.

Teng, W.Y., Huang, Y.L., Shen, C.C., Huang, R.L., Chung, R.S., Chen, C.C., 2005. Cytotoxic acridone alkaloids from the stem bark of *Citrus maxima*. J. Chin. Chem. Soc. 52, 1253-1255.

Torre, L.A., Islami, F., Siegel, R.L., Ward, E.M., Jemal, A., 2017. Global cancer in women: burden and trends. Cancer Epidemiol. 26, 444-457.

Wu, T., Furukawa, H., 1983. Acridone alkaloids. VII. Constituents of *Citrus sinensis Osbeck var. brasiliensis* Tanaka. Isolation and characterization of three new acridone alkaloids, and a new coumarin. Chem. Pharm. Bull. 31, 901-906.

Wu, T., Kuoh, C., Furukawa, H., 1983. Acridone Alkaloids. VI. The Contituents of *Citrus depressa*. Isolation and Structure Elucidation of New Acridone Alkaloids from *Citrus* genus. Chem. Pharm. Bull. 31, 895-900.

Yamamoto, N., Furukawa, H., Ito, Y., Yoshida, S., Maeno, K., Nishiyama, Y., 1989. Anti-herpesvirus activity of citrusinine-I, a new acridone alkaloid, and related compounds. Antiviral Res. 12, 21-36.

Zhao, H.Y., Yang, L., Wei, J., Huang, M., Jiang, J.G., 2012. Bioactivity evaluations of ingredients extracted from the flowers of *Citrus aurantium L. var. amara* Engl. Food Chem. 135, 2175-2181.

Table 1: Pharmacological activities of *Citrus aurantium*

Pharmacological Activities	Part used	Model	Activity	References
Antibacterial	Flowers	Agar-well diffusion assay	Displayed marked antibacterial activity against *Pseudomonas aeruginosa*, *Escherichia coli* and *Listeria monocytogenes*	Ammar et al., 2012
Anti-inflammatory	Fruits	Pro-inflammatory mediator inhibition	Reduction in protein and mRNA levels of COX-2 and iNOS in LPS-stimulated RAW 264.7	Kang et al., 2011
Anti-oxidant	Peel, flowers and leaf	DPPH assay	Oil of leaves displayed high scavenging activity (94.36%)	Sarrou et al., 2013
Antiulcer	Fruit peels	Acetic acid-induced gastric lesion assay.	Significant reduction in lesion area (250mg/kg/day; 76%) within gastric compared to the positive control (cimetidine; 100 mg/kg/day) that only promoted only a 23% cure rate.	Polo et al., 2012
Anxiolytic	Fruits	Light/dark box experiment	Displayed anxiolytic effects after acute (5 mg/kg) or 14-day repeated (1 mg/kg/day) treatments	Costa et al., 2013
Larvicidal	Fruit peel	Larvicidal bioassay	Remarkable larvicidal activity effect against *Anopheles stephensi* with LC_{50} value of 31.20 ppm	Sanei-Dehkordi et al., 2016
Smooth muscle relaxant	Flower	Barium chloride-induced contraction assay	Aqueous extract (1-8 mg/mL) induced antispasmodic effect mainly via calcium influx blockade	Ahangarpour et al., 2011
Vasodilatory	Flower and fruit peel	Vascular contractility assay	Exerts relaxation effect on precontracted mouse aortas through inhibition of Ca^{2+} influx *via* a cation channel	Kang et al., 2016

Table 2: ^{1}H (600 MHz) NMR assignments for **1**-**6** in $CDCl_3$.

Position	**1** ^{1}H, *J* (Hz)	**2** ^{1}H, *J* (Hz)	**3** ^{1}H, *J* (Hz)	**4** ^{1}H, *J* (Hz)	**5** ^{1}H, *J* (Hz)	**6** ^{1}H, *J* (Hz)
1	14.04, s	14.32, s	14.30, s	14.51, s	14.43, s	14.30, s
2	6.41, s	6.29, s	6.27, s	6.24, s	6.24, s	6.12, s
3	-	-	-	-	-	-
4	-	-	-	6.24, s	-	-
4a	-	-	-	-	-	-
5	-	-	-	-	-	-
6	7.14, dd (7.8, 1.4)	-	7.33, d (7.9)	-	-	-
7	7.18, t (7.8)	7.02, d (8.8)	7.19, t (7.9)	6.90, d (8.6)	6.92, d (8.8)	6.92, d (8.8)
8	7.96, dd (7.8, 1.4)	8.09, d (8.8)	7.94, dd (7.9, 1.2)	8.09, d (8.6)	8.11, d (8.8)	7.73, d (8.8)
8a	-	-	-	-	-	-
9		-	-	-	-	-
9a	-	-	-	-	-	-
10a	-	-	-	-	-	-
11	-	6.57, d (9.7)	6.70, d (9.7)	-	-	6.75, d (9.5)
12	-	5.62, d (9.7)	5.56, d (9.7)	-	-	5.65, d (9.5)
13	-	-	-	-	-	-
14	-	1.54, s	1.53, s	-	-	1.54, s
15	-	1.54, s	1.53, s	-	-	1.54, s
NCH_3	3.84, s	3.73, s	3.81, s	3.92, s	3.98, s	3.82, s
3-OCH_3	3.82, s	-	-	3.84, s	3.96, s	-
4-OCH_3	3.99, s	-	-	-	3.85, s	-
5-OCH_3	-	3.93, s	-	3.70, s	3.70, s	-

Assignments were confirmed by DEPT, HSQC and HMBC experiments and by comparing with literature values

(Wu and Furukawa, 1983; Wu et al., 1983; Juoichi et al., 1985; Yamamoto et al., 1989; Ono et al., 1995)

Table 3: ^{13}C (150 MHz) NMR and DEPTQ assignments for **1-6** in $CDCl_3$.

Position	**1**	**2**	**3**	**4**	**5**	**6**
	^{13}C, DEPTQ	^{13}C, DEPTQ	^{13}C, DEPTQ	^{13}C, DEPTQ	^{13}C, DEPTQ	^{13}C, DEPTQ
1	160.4	164.6	164.6	165.9	159.0	164.0
2	94.0	98.7	98.2	94.4	87.7	97.0
3	159.8	161.1	161.4	165.5	156.1	160.9
4	130.1	102.5	102.3	91.0	130.5	102.5
4a	142.2	147.2	147.8	147.1	143.1	148.0
5	146.2	135.8	137.2	134.2	134.4	134.6
6	120.6	154.4	120.4	154.8	154.7	150.9
7	123.1	112.0	112.0	111.2	111.7	111.7
8	118.9	123.4	123.2	124.0	123.9	123.7
8a	125.1	118.6	117.8	117.3	117.5	117.3
9	182.4	181.5	182.0	180.4	180.6	181.7
9a	106.7	106.8	107.3	105.0	105.7	105.9
10a	137.5	141.5	146.8	138.0	137.9	138.2
11	-	120.4	120.4	-	-	120.7
12	-	124.7	124.0	-	-	117.2
13	-	77.7	77.4	-	-	76.3
14	-	27.2	27.2	-	-	25.9
15	-	27.2	27.2	-	-	25.9
NCH_3	46.3	47.9	48.9	39.2	39.5	47.8
3-OCH_3	60.3	-	-	55.6	56.1	-
4-OCH_3	56.8	-	-	-	60.8	-
5-OCH_3	-	60.1	-	61.7	61.6	-

Assignments were confirmed by DEPT, HSQC and HMBC experiments and by comparing with literature values

(Wu and Furukawa, 1983; Wu et al., 1983; Juoichi et al., 1985; Yamamoto et al., 1989; Ono et al., 1995)

Table 4: Antiproliferative Effects of the Fractions of the Stem Bark of *Citrus aurantium* against Four Human Cancer Cell Lines[a]

	IC_{50} (µg/mL)[b]			
Fraction	MCF7	A549	PC3	HepG2
n-hexane	> 100	> 100	> 100	> 100
DCM	5.12 ±0.54	3.88 ± 0.58	4.72 ± 0.23	5.73 ± 0.99
MeOH	90.6 ± 4.54	88.9 ± 1.23	78.2 ± 2.14	92.7 ± 4.11

[a]MCF7 - human breast cancer cells; A549- Human lung carcinoma cells; PC3 - human prostate carcinoma cells; HepG2 - human hepatocellular carcinoma cells. [b]MTT method, with the cells incubated with the fractions for 72 h (means ± SEM, n = 3). Vinblastine was used as the positive control and reference compound.

Table 5: Antiproliferative Effects of Acridone Alkaloids from the Stem Bark of *Citrus aurantium* against Four Human Cancer Cell Lines[a]

	IC_{50} (μM)[b]				
Compound	MCF7	A549	PC3	HepG2	PNT2
1	44.53 ± 1.45	35.76 ± 1.55	38.23 ± 1.11	50.74 ± 1.11	179 ± 4.11
2	12.65 ± 0.54	14.02 ± 0.11	14.88 ± 0.17	22.42 ± 0.17	151.5 ± 6.47
3	24.03 ± 1.05	25.94 ± 0.61	28.15 ± 0.68	30.09 ± 0.94	190.3 ± 3.81
4	31.11 ± 1.41	29.69 ± 0.44	27.44 ± 1.61	37.45 ± 0.79	205.1 ± 2.39
5	26.87 ± 0.57	25.02 ± 1.92	24.29 ± 0.49	26.38 ± 0.12	222.7 ±6.12
6	20.91 ± 0.17	20.36 ± 0.81	18.23 ± 0.53	32.62 ± 1.02	175.4 ± 2.11
Vinblastine	0.023 ± 0.01	0.037 ± 0.01	0.028 ± 0.01	0.021 ± 0.01	0.037 ± 0.01

[a]MCF7 - human breast cancer cells; A549 - human lung carcinoma cells; PC3 - human prostate carcinoma cells; HepG2 - human hepatocellular carcinoma cells; PNT2 - normal human prostate cells. [b]MTT method, with the cells incubated with the fractions for 72 h (means ± SEM, n = 3). Vinblastine was used as the positive control and reference compound.

Table 6**.** The Selectivity Index (SI) of **1-6** and Vinblastine (VBN)

Compound	MCF7	A549	PC3	HepG2
1	4.01	5.00	4.68	3.52
2	11.97	10.80	10.18	6.75
3	7.91	7.33	6.76	6.32
4	6.59	6.90	7.47	5.47
5	8.28	8.90	9.16	8.44
6	8.38	8.61	9.62	5.37
VBN	1.60	1.00	1.32	1.76

MCF7 - human breast cancer cells; A549 - Human lung carcinoma cells; PC3 - human prostate carcinoma cells; HepG2 - human hepatocellular carcinoma cells. SI is the ratio of the IC_{50} values of the compound on PNT2 cells to those in the cancer cell lines.