

Cytotoxic properties of the stem bark of *Citrus reticulata* Blanco (Rutaceae)

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Abstract

The bioassay-guided fractionation of the *n*-hexane extract of *Citrus reticulata* Blanco (Rutaceae) stem bark yielded scoparone (1), xanthyletin (2), lupeol (3), β -amyirin (4), stigmasterol (5), β -sitosterol (6) and palmitic acid. The structures of these compounds were determined by comprehensive spectroscopic analyses, i.e., 1D and 2D NMR and EI-MS, and by comparison with the reported data. Extracts, fractions and isolated compounds 1-6 were assessed for cytotoxicity by the MTT assay against three human cancer cell lines, i.e., human lung adenocarcinoma cell line A549, human breast adenocarcinoma cell line MCF7 and human Caucasian prostate adenocarcinoma cell line PC3. Significant activity of the *n*-hexane and the dichloromethane extracts was observed against the breast cancer cell line MCF7 with IC₅₀s of 45.6 and 54.7 μ g/mL, respectively. Moreover, the 70% ethyl acetate in *n*-hexane chromatographic fraction showed significant activity displaying IC₅₀ values of 53.0, 52.4 and 49.1 μ g/mL against the cancer cell lines A549, MCF7 and PC3, respectively. Encouragingly, an IC₅₀ of 510.0 μ g/mL against the human normal prostate cell line PNT2 indicated very low toxicity, and hence favourable selectivity indices for the 70% ethyl acetate in *n*-hexane fraction in the range of 9.6-10.4 towards cell lines A549, MCF7 and PC3. Since compounds isolated from the above fraction only delivered IC₅₀ values in the range of 18.2-96.3, 9.2-34.1 and 7.5-97.2 μ g/mL against A549, MCF7 and PC3 cell lines, respectively, synergistic action between compounds is suggested. Bioassay results valorize the anticancer effectivity of the stem bark of this plant in Cameroonian pharmacopeia.

Keywords: *Citrus reticulata*, Rutaceae, bioassay-guided fractionation, cytotoxic activities, selectivity indices

Introduction

Citrus reticulata Blanco (Rutaceae) or mandarin originates from China and was introduced to Africa and Europe *via* the Northern African route between the fifteenth and nineteenth century through trade with Portuguese and British Asian colonies (Ramón-Laca *et al.*, 2003). In Cameroon, the plant conquered all habitats and can nowadays even be found disseminated in the Cameroonian rainforest. The medicinal potential of its bark against tumours had been recognized early on by medicinal practitioners.

Since fruits of *Citrus* species are used worldwide in traditional medicine for a wide range of conditions, including cancer prevention, treatment of flu, inflammation and more generally in healing and strengthening of the body, their pharmacological potential had been widely investigated during recent decades, resulting in hundreds of publications. The hydroxylated polymethoxyflavones 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone and 5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone isolated from the peel display strong inhibitory activity against the proliferation of the human promyelocytic leukaemia cell line HL60 and induce apoptosis (Li *et al.*, 2007). Hesperidin, also isolated from the peel, showed pronounced activity against larynx, breast and liver carcinoma cell lines (Al-Ashaal and El-Sheltawy, 2011). Inhibition of human lung cancer cell lines H1299, H441 and H460 and induction of apoptosis was reported for nobiletin, 3,5,6,7,8,3',4'-heptamethoxyflavone and 5-hydroxy-3,7,8,3',4'-pentamethoxyflavone isolated from a peel extract (Xiao *et al.*, 2009). Limonin and naringin produced by *Citrus* fruits displayed bone calcium concentration preservation and increased the antioxidant status in animal model (Mandadi *et al.*, 2009).

Conversely however, very little is known about the pharmacological potential of secondary metabolites produced by seeds, leaves, roots and the bark of the *Citrus* species. In our ongoing investigation of the medical potential of plants of the Rutaceae family, several compounds isolated from the roots of *C. reticulata* have been reported to display strong

oxidative burst inhibition and suppression of the phagocytosis response upon activation with serum opsonized **zymosan** in the range of 0.2-10.5 μM (Fomani *et al.*, 2016). In continuation of our research on *C. reticulata*, this paper deals with the isolation, structure elucidation and pharmacological evaluation of secondary metabolites produced by its stem bark.

Materials and methods

General experimental procedures

Ultraviolet spectra were recorded on a Hitachi UV 3200 spectrophotometer in MeOH. Infrared spectra were recorded on a JASCO 302-A spectrophotometer. EI-MS were recorded on a Finnigan MAT 95 spectrometer (70 eV) with perfluorokerosene as reference substance for EI-HR-MS. The ^1H - and ^{13}C -NMR spectra were recorded at 600 MHz and 150 MHz, respectively on Bruker AMX 600 NMR spectrometers. Methyl, methylene and methane carbons were distinguished by DEPT experiments. Homonuclear ^1H connectivities were determined by using the COSY experiment. ^1H - ^{13}C one-bond connectivities were determined with HSQC gradient pulse factor selection. Two- and three-bond connectivities were determined by HMBC experiments. Chemical shifts are reported in δ (ppm) using TMS as internal standard and coupling constants (J) were measured in Hz. Column chromatography was carried out on silica gel (70-230 mesh, Merck). Thin Layer Chromatography (TLC) was performed on Merck precoated silica gel 60 F₂₅₄ aluminium foil, and spots were detected using ceric sulphate spray reagent. Phenolic compounds were detected using FeCl_3 reagent. The purity of the compounds was investigated by means of ^1H -NMR and ESIMS. The degree of purity of the positive control was $\geq 98\%$, while that of the isolated compounds was 95%. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Dorset, UK). Solvents were purchased from Fischer Scientific (Loughborough, UK). All cell culture reagents were purchased from Labtech International Ltd (East Sussex, UK). The human lung adenocarcinoma cell line A549, human breast adenocarcinoma cell line MCF7 and human

Caucasian prostate adenocarcinoma cell line PC3 were purchased from the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, USA. The normal human prostate cell line PNT2 was obtained from the European Collection of Authenticated cell cultures (ECACC), (Salisbury, UK). All reagents used were of analytical grade.

Plant material

Bark of *C. reticulata* was collected at the Batouri locality in Eastern Cameroon in December 2015 and identified by Mr. Nana Victor of the National Herbarium, Yaoundé, Cameroon, where a voucher specimen (25856SRF/Cam) was deposited.

Extraction and bioassay guided isolation

Soxhlet extraction of 1.2 kg of the air dried and powdered bark of *C. reticulata* yielded - after evaporation under reduced pressure - 19.3, 25.4 and 38.2 g of *n*-hexane (Hex), dichloromethane (DCM) and methanol (MeOH) extract. Soxhlet extraction for each solvent was continued for ten cycles. The extracts were submitted to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity/viability assay employing human cancer cell lines A549, MCF7, PC3 and normal cell PNT2. Tests were run in triplicate and activities compared to the positive control etoposide, a semi-synthetic derivative of podophyllotoxin, a compound originating from the roots of *Podophyllum peltatum* (Berberidaceae). The Hex extract was found to be the most active against all three cancer cell lines and thus subjected to Vacuum Liquid Chromatography (VLC) on TLC grade silica gel as the stationary phase. Elution was performed with mixtures of *n*-hexane and ethyl acetate (EA), namely 20% of EA in Hex, 50% of EA in Hex and 70% of EA in Hex to give three fractions, which were collected and evaporated under reduced pressure to obtain 2.5, 6.3 and 8.2 g, respectively. Subsequent MTT assays showed best results for the 70% of EA in Hex fraction, which was then submitted to column chromatography over silica gel 60 (230-400 mesh, 3.5 x 3.5 cm)

using a gradient system of *n*-hexane and CH₂Cl₂. A total of 53 sub-fractions (ca. 100 mL each) were collected and pooled on the basis of TLC analysis leading to three series (A-C). Fraction A [1.5 g, combination of sub-fractions 1-15, eluted with a mixture of *n*-hexane-CH₂Cl₂ (4:1)] yielded palmitic acid (18.2 mg) and lupeol (**3**) (21.5 mg), while fraction B [2.6 g, constituted by sub-fractions 16-31, eluted with a mixture of *n*-hexane-CH₂Cl₂ (3:2)] led to β -amyrin (**4**) (11.2 mg), stigmasterol (**5**) (15.3 mg) and β -sitosterol (**6**) (16.4 mg). Fraction C [3.1 g, combination of sub-fractions 31-53, eluted with *n*-hexane-CH₂Cl₂ (1:1) to 100% CH₂Cl₂] gave scoparone (**1**) (15.5 mg) and xanthyletin (**2**) (8.1 mg) (Fig. 1). Finally, compounds **1-6** were submitted to the MTT assay, and bioactivity results evaluated together with those previously received from crude extracts and fractions, in comparison to the positive control etoposide.

Scoparone (1): (Afek and Szejnberg, 1988).

Xanthyletin (2): (Tillekeratne *et al.*, 1989).

Lupeol (3): (Fomani *et al.*, 2016).

β -amyrin (4): (Longue *et al.*, 2015).

Stigmasterol (5): (Fomani *et al.*, 2016).

β -sitosterol (6): (Fomani *et al.*, 2016).

Cell lines, cell cultures and the MTT assay

The potential cytotoxicity of *Citrus reticulata* stem bark extracts, their VLC fractions and the isolated compounds were studied against cancer cell lines A549 (human lung adenocarcinoma), MCF7 (human breast adenocarcinoma), PC3 (human prostate adenocarcinoma) and the normal cell line PNT2 (human prostate) using the MTT assay (Mosmann, 1983; Basar *et al.*, 2015). All cell lines were cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum and 1% antibiotic-antimycotic solution (100 x).

The cells were cultured at 37 °C in 95% humidity and 5% CO₂. For the MTT assays, the cells were washed using phosphate buffered saline and harvested by trypsinization. Cells were then seeded into 24 well plates at a density of 1.2 x 10⁴ cells/well in a working volume of 1 mL/well and allowed to grow for 24h before the commencement of each experiment. The cells were then treated for 24h with different concentrations of test samples (the *C. reticulata* extracts and VLC fractions at 0, 0.8, 4, 20, 100, 500 µg/mL and the compounds at 0, 0.4, 2, 10, 50, 75 and 100 µg/mL). Dilution of stock solutions was made in culture medium yielding final sample concentrations with a final dimethyl sulfoxide (DMSO) concentration of 0.1%, including the control. Each sample concentration was used to treat four wells of cells in each 24-well plate. After the 24h treatment period, the toxicity of the samples on each cell line was quantified. To achieve this, the medium in each well was replaced by MTT solution (500 µg/mL in medium) and incubated for 2h. Viability of cells was assessed by their ability to reduce the yellow dye MTT to a blue formazan product (Popescu *et al.*, 2015). The MTT reagent was removed, the formazan crystals were dissolved in isopropanol, and the absorbance at 560 nm determined using a microplate reader (CLARIO Star Microplate Reader, BMG Labtech, UK). The average absorbance value obtained from zero treatment control (0.1% DMSO) wells was arbitrarily set at 100% for each plate, and the absorbance value for the average of wells of cells treated with each test sample concentration was expressed as a percentage of this control. Each assay was performed on a minimum of three separate occasions, and the IC₅₀ values for each sample on each cell line were calculated using Microsoft Excel version 2013. The anti-lung cancer drug etoposide was used as positive control.

Statistical analysis

All experiments were carried out in triplicate on separate occasions. Data were expressed as means \pm standard error of the mean. The graphs were plotted using nonlinear regression with the use of Microsoft Excel version 2013.

Results and discussion

Soxhlet extraction of 1.2 kg of bark material of *C. reticulata* Blanco led - after evaporation under vacuum - to 19.32 g of Hex, 25.45 g of DCM and 38.16 g of MeOH extract. These extracts were tested against cancer cell lines A549, MCF7 and PC3, and normal cell line PNT2 using the MTT assay. Etoposide was used as positive control. While the MeOH extract was found to be inactive against above cancer cell lines, displaying IC₅₀ values > 500 $\mu\text{g/mL}$, the DCM extract showed IC₅₀ values of (A549) 220.5, (MCF7) 54.7 and (PC3) 220.2 $\mu\text{g/mL}$, respectively. The Hex extract was the most active with IC₅₀ values of (A549) 100.2, (MCF7) 45.6 and (PC3) 100.5 $\mu\text{g/mL}$ and was selected for fractionation by VLC technique. Three fractions, namely 20% of EA in Hex, 50% of EA in Hex and 70% of EA in Hex were obtained and submitted to MTT assay. The 20% of EA in Hex fraction resulted in IC₅₀ values of (A549) 210.3, (MCF7) 210.1 and (PC3) 445.4 $\mu\text{g/mL}$; the 50% fraction in (A549) 70.1, (MCF7) 205.5 and (PC3) 49.4 $\mu\text{g/mL}$ and, most interestingly, the 70% of EA in Hex fraction in IC₅₀ values of (A549) 53.0, (MCF7) 52.4 and (A549) 49.1 $\mu\text{g/mL}$. The positive control etoposide delivered IC₅₀ values of (A549) 3.5, (MCF7) 10.2 and (PC3) 7.9 $\mu\text{g/mL}$, while the negative control consisting of media with DMSO did not display activity as was expected. It should be noted that the 70% of EA in Hex fraction is only 5-15 fold less active than the anticancer drug etoposide, depending on the cell line tested (Table 1; Figs. 2a-f, supporting information).

In the following, the 70% EA in Hex fraction was subjected to column chromatography on silica gel and preparative TLC to afford seven known compounds identified as scoparone (1), xanthyletin (2), lupeol (3), β -amyryn (4), stigmasterol (5), β -sitosterol (6) and palmitic acid.

The structures of compounds were determined by comprehensive spectroscopic analyses (1D and 2D NMR and EI-MS) and by comparison with reported data. Other than expected, compounds isolated from the 70% EA in Hex fraction delivered moderate to low activities in MTT assay: while scoparone (**1**) gave IC₅₀ values of 18.2, 20.3 and 18.7 µg/mL against cell line A549, MCF7 and PC3, respectively, stigmasterol (**5**) showed values of 21.1, 9.2 and 7.5 µg/mL, respectively. The latter values were comparable to those reported previously with 20.0, 8.8 and 7.8 µg/mL, respectively (Malek *et al.*, 2009; Hsu *et al.*, 2011; Shen *et al.*, 2012). In addition, lupeol (**3**) displayed IC₅₀ values of 21.0, 32.1 and 30.1 µg/mL against cell line A549, MCF7 and PC3, respectively, and β-amyrin (**4**) and xanthyletin (**2**) were moderately to low active with IC₅₀ values of 74.2, 34.1, 70.1 and 80.1, 19.3, 97.2 µg/mL, respectively. Finally, β-sitosterol (**6**) displayed low activity with IC₅₀ of 96.3 µg/mL against cell line A549 and was shown to be not active against cell line MCF7 and PC3 displaying IC₅₀ values > 250 µg/mL corresponding well to IC₅₀ values of 87 µg/mL against cell line A549 and 72 µg/mL against cell line PC3 reported previously (Malek *et al.*, 2009) (Table 2; Figs. 2a-f, supporting information).

A comparison of IC₅₀ values of crude extracts, fractions and isolated compounds clearly shows that the 70% EA in Hex fraction not only displays significant activities against cell lines A549, MCF7 and PC3, but is only around 3 times less active than scoparone (**1**) and stigmasterol (**5**), the two most active compounds reported here from this fraction. While the 70% EA in Hex fraction is in addition only 6 times less active than positive control etoposide against human prostate cancer cell line PC3 this fraction displayed low toxicity against the human normal prostate cell line PNT2 with IC₅₀ = 510.0 µg/mL, resulting in very favourable selectivity indices of 9.6, 9.7 and 10.4 µg/mL towards cell lines A549, MCF7 and PC3, respectively (Table 3).

It should be mentioned that in natural products chemistry and pharmacology, a medicinal plant extract displaying inhibitory activity of $IC_{50} < 500 \mu\text{g/mL}$ is regarded as active, and IC_{50} values of 300-400 $\mu\text{g/mL}$ characterise an extract **worth** to identify its active ingredients. Interestingly, IC_{50} values around 50 $\mu\text{g/mL}$ determined for the 70% EA in Hex fraction combined with moderate to low activities in the IC_{50} range of 7.5-97.2 $\mu\text{g/mL}$ measured for secondary metabolites present in this fraction, strongly indicate synergistic effects.

Etoposide is used as one of the drugs of choice in the treatment of human lung adenocarcinoma and was shown here to give an IC_{50} of 3.5 $\mu\text{g/mL}$ against human lung cancer cell line A549, compared to the 70% EA in Hex fraction, 50% EA in Hex fraction and the hexane crude extract from the stem bark of *C. reticulata* showing IC_{50} values of 53.0, 70.1 and 100.2 $\mu\text{g/mL}$, respectively.

Conclusions

In vitro assays show that the stem bark of *C. reticulata* widely used in Cameroon against tumours is probably a good crude drug treatment against lung, breast and prostate cancer **when extracted with hexane and in addition against breast cancer when extracted with dichloromethane**. Moreover, first assays display very low toxicity of a **hexane** extract fraction against a human normal prostate cell line. However, **additional *in vitro* and *in vivo* toxicity testing** will be necessary, before **a recommendation of above preparations** can be given. A positive outcome might initiate formulation studies for the development of a safer anticancer plant product from the stem bark of this plant, to be manufactured in developing countries. While the anti-lung cancer drug etoposide is mostly given in combination therapy to reduce the therapeutical dosis, it is suggested that the 70% **ethyl acetate in hexane** fraction from the stem bark of *C. reticulata* might turn out to be a candidate for supplemental treatment, subject to further investigation of its overall synergistic and biochemical mechanisms of action towards cancer cells as well as its toxicity *in vivo*.

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Conflict of interest

The authors declare no conflict of interest.

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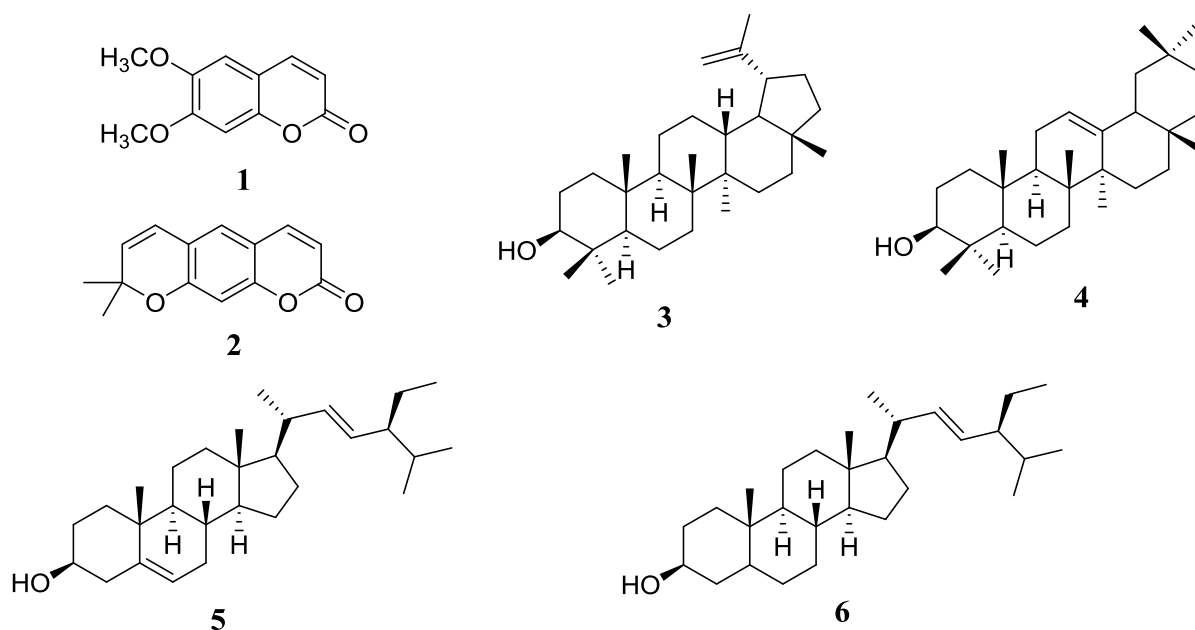


Figure 1. Structures of compounds isolated from *Citrus reticulata* stem bark

Table 1. IC₅₀ values of Hex, DCM and MeOH extracts and EA in Hex fractions of *Citrus reticulata* stem bark for cell lines A549, MCF7 and PC3

Sample Extract	IC ₅₀ in µg/mL		
	A549	MCF7	PC3
Hex	100.2 ± 3.2	45.6 ± 1.2	100.5 ± 4.5
DCM	220.5 ± 2.4	54.7 ± 3.1	220.2 ± 1.8
MeOH	> 500	> 500	> 500
20% EA in Hex	210.3 ± 5.5	210.1 ± 3.6	445.4 ± 4.4
50% EA in Hex	70.1 ± 1.5	205.5 ± 2.5	49.4 ± 5.7
70% EA in Hex	53.0 ± 3.4	52.4 ± 3.6	49.1 ± 1.9
Etoposide	3.5 ± 0.5	10.2 ± 1.2	7.9 ± 1.8

Table 2. IC₅₀ values of compounds isolated from *Citrus reticulata* stem bark for cell lines A549, MCF7 and PC3

Compounds	IC ₅₀ in µg/mL		
	A549	MCF7	PC3
1	18.2 ± 4.2	20.3 ± 1.2	18.7 ± 1.5
2	80.1 ± 4.1	19.3 ± 3.8	97.2 ± 4.0
3	21.0 ± 7.4	32.1 ± 11.2	30.1 ± 5.3
4	74.2 ± 2.2	34.1 ± 1.9	70.1 ± 2.4
5	21.1 ± 0.7	9.2 ± 0.9	7.5 ± 1.1
6	96.3 ± 2.1	>250	>250
Etoposide	3.5 ± 0.5	10.2 ± 1.2	7.9 ± 1.8

Table 3. IC₅₀ values of the 70% EA in Hex fraction of *Citrus reticulata* stem bark for human cancer cell lines A549, MCF7 and PC3 and selectivity indices related to human normal prostate cell line PNT2

Cell line	IC₅₀ in µg/mL	Selectivity index = IC₅₀ of human normal cell line / IC₅₀ of human cancer cell line
Cancer cell line A549	53.0	9.6
Cancer cell line MCF7	52.4	9.7
Cancer cell line PC3	49.1	10.4
Normal cell line PNT2	510.0	-