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Research Article

A New Cytotoxic Glycocerebroside from *Rauwolfia macrophylla* Stapf.

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ABSTRACT

The present study investigated secondary metabolites from stem bark of a cameroonian medicinal plant *Rauwolfia macrophylla* Stapf. and their anti-proliferative activities against human leukaemia cell lines, THP-1 and mouse macrophage cell line Raw 264.7. One new glycocereboside, Rauvocerebroside **(1)** with six other known compounds,; 11-methoxy-19-epiajmalicine **(2)**, 19-epiajmalicine **(3)**, Sucrose **(4)** **methyl-3,4,5-trimethoxycinnamate (5),**, Betulinic acid **(6)** and Lupeol **(7)** were isolated. Their structural elucidation was done using 1D and 2D NMR, IR and MS spectroscopy data together with published reviews. Analyses of the cytotoxicity of compounds **(1)**, **(2)** and (3) displayed good activity of (1) on THP-1 (IC_{50} 20.70 µM) and Raw 264.7 (IC_{50} 26.34 µM) cancer cell lines. Compounds **(2)** and **(3)**, showed moderate activity on THP-1 cells (IC_{50} 43.23 µM for **(2)** and 34.25 µM for **(3)** and a low activity on Raw 264.7 cells. These results suggest that compounds **(1)**, **(2)** and **(3)** could be potential anticancer agent.

Keywords: *Rauvolfia macrophylla*; Glycocerebroside; Anti-proliferative; Cytotoxicity.

INTRODUCTION

Plants are undeniably very important for human life since they are indispensable for our nutrition, are good shelter and used in traditional medicine to maintain good health [1]. *Rauvolfia macrophylla* Stapf. is a plant widely used to treat common diseases in Cameroon's folk medicine [2]. It is found in Tropical Regions on Earth and belong to Apocynaceae family. This family of flowering plants is made up of 5 subfamilies, 164 genera and 1500 species [3]. *Rauvolfia* genus, named after Leanhard Rauwolf a German physician specialised in plant collection [3]. includes about 120 species among which *R. macrophylla* [4]. This genus is very rich in alkaloids and also contain other classes of secondary metabolites known for their broad range of biological properties including cytotoxic and antimalarial [5]. The stem bark and roots of *Rauvolfia* are mainly used to treat malaria, snake bite, stomachache, hepatitis, epilepsy, and edema, effortless childbirth in Africa's traditional medicine and for antiarrhythmic disorders in India [1,6]. In Ebolowa, South Region of Cameroon, aqueous decoction of the stem bark and roots is administered to women with ovarian cysts in addition to the cited traditional uses. Previous studies report anti-leishmania and antimalarial activities and proof of antimicrobial, antioxidant activities and the acetylcholinesterase inhibition of three compounds: ergosterol, 2´'-deoxyribolactone and hexylitaconic acid [1]. To the best of our knowledge, not much has been reported on the secondary metabolites isolated or biological activities of *R. macrophylla* of Cameroon. In this study, we isolated and characterized secondary metabolites from the stem bark of *R. macrophylla* and investigated the anti-proliferative activity of some isolated compounds on two cancer cell lines.

MATERIALS AND METHODS

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Plant Material

The stem bark of *Rauvolfia macrophylla* Stapf. was collected from Ebolowa, South Region of Cameroon in October 2017. It was identified by Dr. Tchiengue Barthélemy, botanist at the National Herbarium, Yaoundé-Cameroon. Sample has been deposited under reference number 43413HNC.

General Materials

Column chromatography was carried out on silica gel (70–230 and 230–400 mesh, E. Merck, Darmstadt, Germany) as the stationary phase. Thin layer chromatography was performed on percolated 0.5 mm thick aluminium sheets (Merck Si gel 60 F_{254} and visualized under UV lamp by spraying with 10% sulphuric acid solution followed by heating on a hot plate. 1D and 2D NMR spectra were recorded on a Bruker DRX-400 and DRX-500 instruments, 1H and 13C-NMR probes operating at 500 and 125 MHz, respectively, with TMS as an internal standard. The FAB-SM was measured on a Kratos MS 80 RFA mass spectrophotometer with pyridine-d_5 as solvent. IR spectra were recorded using Jasco IR-700 infrared spectrophotometer. Solvents and reagents were purified by standard procedures as necessary. The THP-1 and Raw 264.7 cell lines purchased from the American Type Collection Culture (ATCC) were used for *in vitro* cytotoxicity tests. The WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) were purchased from Roche Diagnostics, Germany.

Extraction and Isolation of Compounds

The air-dried and powdered stem bark of *Rauvolfia macrophylla* Stapf. (600 g) was extracted with methanol (12 L) by maceration at room temperature for 6 days. The filtrate was obtained using Whattman filter paper No 1 and concentrated using rotatory evaporator at reduced pressure and was left open at room temperature to evaporate the residual solvent to obtain 280 g of methanol crude extract. The methanol extract (250 g) was fractionated by open column chromatography on a gradient of solvent systems (from n-hexane, n-hexane/EtOAc, to EtOAc/MeOH; 200 mL) and regrouped by analytical TLC to obtain 7 main fractions (A1 to A7). Purification of A3 (50.7 g) by CC on an n-hexane/EtOAc solvent gradient afforded 6 subfractions (A3-1 to A3-6). Subfraction A3-4 (20.3 g) in an isocratic (15% Hex/EtOAc) CC yielded compound 6 (1.3 g), while compound 1 (3 mg) was isolated following a Sephadex LH-20 (100% MeOH) column from same subfraction. Compound 7 crystallized out in A4 (26.7 g) and was washed out while the filtrate underwent a CC on a gradient of solvent to yield compound 5 (13 mg) at 30% Hex/EtOAc. Still on an n-hexane/EtOAc gradient, the purification of A5 (32.5 g) on successive CC and an isocratic (28% Hex/EtOAc) column yielded compounds 2 (1.8 g), and 3 (2.3 g) respectively. Compound 4 (107 g) crystallized out in large amount from A6.

Cytotoxicity

Cell culture and treatment: The cytotoxicity was performed against the human monocytic leukemia cell line (THP-1) and the mouse macrophages Raw 264.7 cell line. Raw 264.7 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) medium and THP-1 on RPMI 1640 culture medium containing 2 mM L-glutamine supplemented with 0.1% 2-mercaptoethanol. All the culture media were supplemented with 10% FCS and 1% antibiotics comprising 100 IU/mL penicillin and 100 µL/mL streptomycin. Cells were cultured in 75 cm^2 flasks and maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Cell viability assay: The cytotoxicity of compounds was evaluated using WST-1 assay according to the manufacturer´s instructions. Cells were seeded (10^4 cells/100 µL per well) in 96-well plates in triplicate and incubated overnight. The next day, cells were exposed to different concentrations (1 µg/mL, 10 µg/mL, 30 µg/mL, 60 µg/mL and 100 µg/mL) of compounds and incubated for 48 hours. Then the medium in each well was aspirated and a solution of WST-1 diluted 1:10 with fresh medium was added to each well. Plates were further incubated at 37 °C for 60 minutes and the absorbance was recorded at 450 nm/690 nm using Synergy Multi-Mode Microplate Reader (BioTek). The results were expressed as percentage of the absorbance value obtained in control, which was considered 100% and were graphically presented as a percentage of cell viability versus the compounds concentration using GraphPad Prism software. Therefore, the compound concentrations required to inhibit the growth of cancer cells by 50% (IC_{50}) was determined.

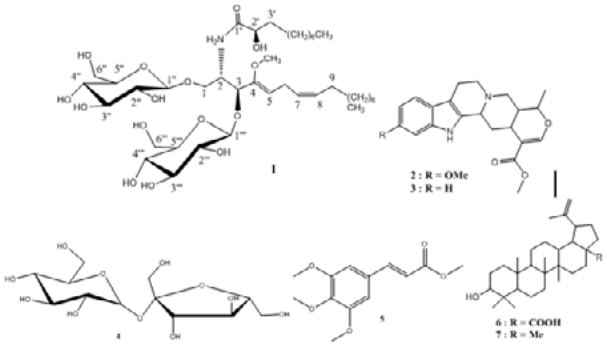

Figure 1: structure of compounds 1-7.

RESULTS AND DISCUSSION

The methanol crude extract of *R. macrphilla* stem bark (250 g) subjected to successive chromatography and separation techniques to isolate seven compounds amongst which one new compound (1) and six other known compounds (Figure 1) including two indole alkaloids: 11-methoxy-19-epiajmalicine (2) and 19-epiajmalicine (3) [7]; sucrose (4) [8]; methyl-3,4,5-trimethoxycinnamate (5) [7], butulinic acid (6) [9] and Lupeol (7) [10].

Compound 1 was isolated as a white amorphous powder with an optical rotation of $[\alpha]_D^{25}$=-3.1 (c 0.2, MeOH), the HR-ESI-MS, $[M+Na]^+$ at m/z 816.4708 (calcd. 816.4721), which is in harmony with the molecular formula $C_{39}H_{71}NO_{15}$. The IR spectrum of (1)

showed a strong absorption broad band at 3382.1 cm^{-1}, together with two bands at 1081.51 cm-1 and 1035.51 cm^{-1} assigned to (-OH and –NH) and (C-OH and C-NH) groups respectively. Other bands are observed at 1624.31 cm^{-1} and 1464.91 cm^{-1} characteristic of a secondary amide derivative and a double bound respectively. This information was supported by 1H-NMR, 13C-NMR and DEPT (90 and 135) spectra. 1H and 13C-NMR spectra indicate the presence of two β-D-glucopyranosyl moiety, at δ_H 4.43 ppm [1H-1'', d, J=6.4, anomeric proton; δ_C 106.2(C1''), 74.0(C2''), 76.2(C3''), 69.8(C4''), 76.1(C5''), and 61.3(C6'')] and at δ_H4.19 ppm [1H-1''', d, J=7.7, anomeric proton; δ_C 102.9(C1'''), 74.3 (C2''), 76.2(C3''), 69.6(C4''), 76.1(C5'') and 61.3(C6''')]. The signal of an amidomethine (δ_H 4.70, m, H-2; δ_C 50.1(C-2)) together with two oxygenated methines (δ_H 3.14, d, J=1.5, H-3; δ_C 73.30 (C-3) and δ_H 3.90, m, H-1'; δ_C 72.0 (C-1')) were pointed out. In addition, the signal of an oxygenated methylene (δ_H 3.96, d, J=17,9; δ_H 3.72, d, J=17,8; δ_C 68.5(C-1) and a methoxy group (δ_H 3.7, 3H, s; δ_C 55.9(C-4)) were also pick out. Once more the signal of three olefinic protons were observed at δ_H 6.05 (1H, brd, J=6. 3, H-5; δ_C93.9 (C-5), 5.27 (1H, dt, J=6.8-10.7, H-7; δC 129.2(C-7) and 5.29 (1H, dt, J=6.7-10.7, H-8; δ_C 130.1(C-8). Based on the DEPT spectrum and 13C NMR, the signal of two quaternary carbons was assigned at 175.6 ppm (C-1') and 153.0 ppm (C-4) which are respectively attributable to a carboxyl and an olefinic carbon. Moreover, COSY, together with HMBC spectra supports the position of some functionality (Table 1). So, ^{2,3,4}J correlations were observed between H-3'' and C-1''/C-2''; and H-1'' with C-1 which assumed that, the carboxyl group is on the second chain as well as the first glucoside on the first chain [8]. Then, other ^{3}JHC correlations between H-1 and C-1''/C-3, H-2'' with C-1' and H-1'' with C-3 assume de position of the second glucoside with the first chain, as well as the connection between the first and the second chain. In addition, ^{2,3,4}J correlation were observed between the proton of the methoxy group with C-4, C-5, between H-5 and C-4, C7 and C-8 which have never been described so far. The relative α/β orientation of 1 was based on it NOESY spectrum (Figure 2), were H-1b and H-2 indicate their α orientation while H-1a, H-3 and H-2'' indicate their β orientation [11]. Thus, 1 was identified as a glycocerebroside with systematic name1,3-bis-O-β-D-glucopyranosyl-(2S,3S,4Z,7Z)-4-methoxy-2-[(2'*R*')-2-hydroxydecanoylamino]-4,7-hexadecadiene-1,3-diol and to which we assign the trivial name Rauvocerebroside. Complete data 1H and 13C NMR spectra is presented in Table 1.

position	^{1}H, m (J in Hz)	^{13}C	HMBC	COSY
NH	8.55, d(9.1)			
1a	3.96, d(17.9)	68.5		
1b	3.72, brd(17.8)			
2	4.19, m	50.1	C1' C4	H1-H2-H3
3	3.40, m	73.3		
4		153		
5	6.05, brd(6.3)	93.9	C4, C7, C8	H5-H6
6	1.80, m	27.1		
7	5.27, dt(6.7-10.7)	129.2		H7-H8
8	5.29, dt(6.7-10.7)	130.1		
9	1.90, m	27		
10.0-14.0	1.20 - 1.90, m	25.5-32.4		
15	1.10, m	22.5		
16	0.85, t(6.8)	13.8		
OCH3	3.69, s	55.9	C4, C5	
1'		175.6		
2'	3.90, m	72		
3'	1.10, m	34.2	C1', C2'	H3'-H2'
4'	1.10, m	31.7		
5'-8'	1.20-1.90, m	27-29.5		
9'	1.10, m	22.5		
10'	0.85, t(6.8)	13.8		
1''	4.43, d(6.4)	106.2	C1	
2''	3.45, m	74		
3''	3.31, m	76.2		
4''	3.42, m	69.6		
5''	3.40, m	76.1		
6''a	3.56, brd(11.6)	61.3		
6''b	3.68, dd(11.8,3.16)	61.3		
1'''	4.19, d(7.7)	102.9	C3	
2'''	3.45, m	14.3		
3'''	3.38, m	76.2		
4''	3.42, m	69.9		
5'''	3.40, m	76.1		
6'''a	3.56, brd(11.6)	61.3		
6'''b	3.68, dd(11.8,3.16)	61.3		

Table 1: ^{1}H and ^{13}C NMR Data of compound1 (pyridine-d_5 500MHz and 125MHz respectively).

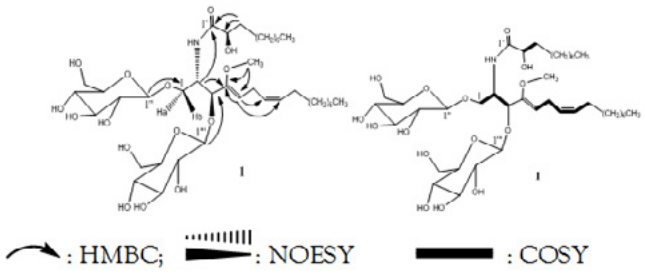

Figure 2: HMBC, NOESY and COSY correlations.

The THP-1 and Raw 264.7 cancer cell lines were used to examine the cytotoxic activity of compounds 1, 2, and 3 via WST-1 assay. The curve of dose-response (Figure 3) revealed good cytotoxic effects of compound 1 against the two cancer cells line with an IC_{50} value of 20.70 μM and 26.34 μM respectively for THP-1 and Raw 264.7 cell lines.

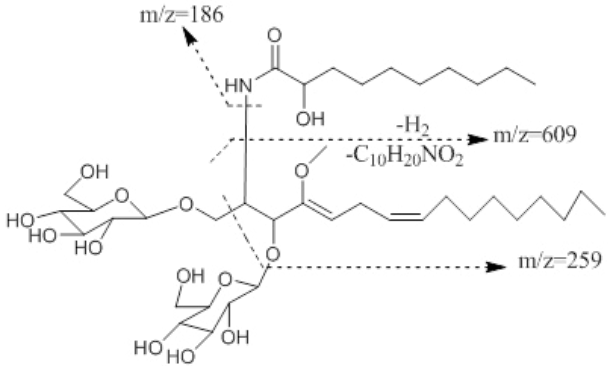

Figure 3: Some fragmentations of 1 (FAB pos.)

Whereas compounds 2 and 3 point out a moderate activity on THP-1 cells and a low activity against Raw 264.7 cells with respective IC_{50} value of 43.23 μM and 64.19 μM for the compound 2; 34.25 μM and 59.16 μM for compound 4. The positive control (Paclitaxel) showed IC50 values of 3.22 μM and 4.52 μM against THP-1 and Raw 264.7 cells respectively. The analyses of those results show on one hand that THP-1 and Raw 264.7 are more sensitive to compound 1 than compounds 2 and 3 and in the other hand; the THP-1 cell line is the most sensitive to the same compound, 1. With regards to the two other secondary metabolites, compound 3 is more active on those cell lines than compound 2 with emphasis on THP-1. A comparative study done with leutcharic acid and 3-oxo-22-hydroxyhopane tested on the THP-1 cell line [10] showed that, compound 1 is the most active. Many compounds from the *Rauvolfia* genus have been tested for the cytotoxicity activities [12], but up to now this is the first report on cytotoxicity activity of 1, 2 and 3 isolated from *R macrophyla* on THP-1 and Raw 264.7 cell lines. Thus, the anti-leishmania activity described by Weniger et al may be from compounds 1, 2 and 3 presents in the crude extract [12].

Rauvocerebroside (1), $[\alpha]_D^{25}$=-3.1 (c 0.2, MeOH), formula $C_{39}H_{71}NO_{15}$; HR-ESI-MS, $[M+Na]^+$ at m/z 816.4708 (calcd. 816.4721) [FAB(+) m/z 609, 259 and 186]: see Figure 3; IR (Vmax, KBr) IR (Vmax, KBr) 3382.10 cm^{-1}, 1081.51 cm^{-1}, 1035.51 cm^{-1}, 1624.31 cm^{-1}, 1464.91 cm-1: see Figure 4; 1H-NMR (pyridine–d_5, 500 MHz) and 13C-NMR (pyridine–d_5, 125 MHz): see Table 1.

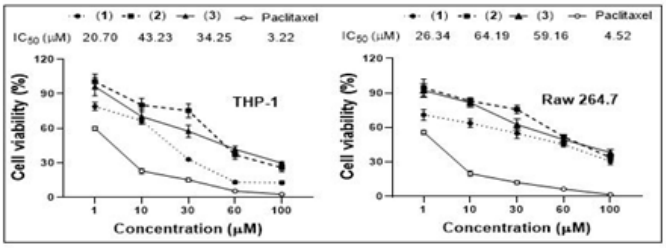

Figure 4: Dose-response activity of compounds 1, 2 and 3 on THP-1 and Raw 264.7 cancer cell lines

CONCLUSION

The Phytochemical studies on dried stem bark of *R. Macrophylla* led to a new glycocerebroside, 1,3-bis-O-*β*-D-glucopyranosyl-(2*S*,3*S*,4*Z*,7*Z*)-4-methoxy-2-[(2'*R**)-2-hydroxydecanoylamino]-4,7-hexadecadiene-1,3-diol trivially named Rauvocerebroside (1), together with six known compounds identified following their spectroscopic data and published review. The cytotoxic activity effected on human cancer THP-1 and mouse cancer Raw 264.7 cell lines showed a good activity of Rauvocerebroside (1) on both cell lines; moderate activity of 11-methoxy-19- epiajmalicine (2) and 19-epiajmalici (3) on THP-1 cell line and a low activity of the two last compounds on Raw 264.7 cells. The overall result testifies that Rauvocerebroside, 11-methoxy-19-epiajmalicine and 19-epiajmalicine could be potential anti-cancer agents. From that result, R. *Macrophylla's* stem bark could be a potential source of anticancer compounds.

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