SUPPLEMENTARY MATERIAL

Antimicrobial and α -glucosidase inhibitory activities of chemical constituents from *Gardenia aqualla* (Rubiaceae)

Jean Noël Nyemb ^{1,2*}, Roland Tchuente Tchuenguem ³, Alessandro Venditti ^{4*}, Alembert Tiabou Tchinda ⁵, Céline Henoumont ⁶, Emmanuel Talla ⁷, Sophie Laurent ⁶, and Jamshed Iqbal ²

Abstract:

An aliphatic alkene namely pentapentacontene (**4**) was isolated for the first time from a natural source, *Gardenia aqualla*, along with fourteen other compounds including nonacosanol (**1**), tetratriacontanol (**2**), octatriacontanol (**3**), β -sitosterol (**5**), stigmasterol (**6**), daucosanol (**7**), ursolic acid (**8**), uvaol (**9**), $3\beta,19\alpha,23\beta,24\alpha$ -tetrahydroxyurs-12-en-28-oic acid (**10**), lupenone (**11**), oleanolic acid (**12**), vanillin (**13**), vanillic acid (**14**) and D-mannitol (**15**). α -glucosidase inhibitory assay revealed that MeOH and EtOAc extracts of leaves had the best activity with IC₅₀ of 9.65 and 20.03 μ g/ml respectively. All the tested compounds showed dose dependent inhibition of α -glucosidase and some of them were found to be comparable to acarbose. Compound **10** was the most potent with IC₅₀ = 1.72 μ M. It also showed the most interesting antibacterial activity, against the isolate strain of *S. typhi* and *P. aeruginosa* and also exhibited the most significant antifungal activities against all the tested yeasts.

Keywords: *Gardenia aqualla*, Antimicrobial activity, α -glucosidase, Triterpenoids, pentapentacontene

4. Experimental

4.1 General experimental procedures

Mass spectral data (TOF-ESI-MS⁺) were obtained from a Q-TOF Ultima spectrometer (Waters). ¹H (500 MHz) and ¹³C NMR (125 MHz) spectra were run on a Bruker Avance AV-500 spectrometer using TMS as internal standard, chemical shifts being quoted in parts per million (ppm). The solvents used to dissolve compounds for the NMR experiment were DMSO-*d*₆, CD₃OD or CDCl₃; For column chromatography separations, silica gel Merck 60 (230-400 Mesh) was used. Thin-layer chromatography was performed on pre-coated Kieselgel 60 F₂₅₄ aluminum sheets (Merck) and spots were detected under ultraviolet fluorescence and then sprayed with 10% H₂O-H₂SO₄ reagent, followed by heating at 105°C for 10 mins.

4.2 Plant material

Seeds and leaves of *Gardenia aqualla* Stapf & Hutch, were collected in September 2014 from the locality called Borongo in the Subdivision of Ngaoundere 3rd, Vina Division in the Adamawa Region of Cameroon. The authentication was done at the National Herbarium of Cameroon in Yaounde where a voucher specimen was deposited under the reference number 36894/HNC.

4.3 Extraction and isolation

The air-dried powders of each part of the plant were extracted three times with ethyl acetate (EtOAc), then with methanol (MeOH) at room temperature, with a 1:3 (w/v) ratio of powder-solvent. Thus 3 000 g of leaves were extracted by maceration successively with 9 L of EtOAc then with 9 L of MeOH; By the same process 400 g of seeds was extracted with both solvent EtOAc and MeOH using 1200 ml of each solvent. After filtration, the solvent was evaporated under reduced pressure to dryness leading 63.72 g (2.12%) of EtOAc, 104.5 g (3.48%) of MeOH extracts of leaves, while seeds gave 78.55 g (19.64%) and 49.38 g (12.35%) of EtOAc and MeOH extracts respectively.

40 g of the MeOH extract of seeds were separated by silica gel (400 g, 70-230 mesh, ASTM) column chromatography, eluted with increasing gradient of *n*-Hexane-EtOAc (1:0→0:1) and EtOAc-MeOH (1:0→0:1) solvent mixtures. 418 sub-fractions of 300 ml were collected and pooled together according to their chromatographic profiles on TLC, to yield twelve fractions, S1-S12. Four compounds were obtained from the main column, before the pooling of the sub-fractions: compound **12** (4 mg) obtained as a white powder in the sub-fraction 66-71 eluted with *n*-Hexane-EtOAc (7:3); compound **14** (3 mg) precipitated as a white powder from sub-fractions

83-90 also obtained with *n*-Hexane-EtOAc (7:3); compound **7** (3 mg) filtered from sub-fractions 179-185 (*n*-Hex-EtOAc, 0:1) as a white powder, and compound **15** (204 mg) as a white solid, obtained from the sub-fractions 236-260 eluted with EtOAc-MeOH (8:2). Further purification of fraction S2 (0.15 g) led to the isolation of compound **13** (7 mg) as a white powder at *n*-Hexane-EtOAc (8:2) through column chromatography (CC) of silica gel 60 (3 g).

EtOAc leaves extract (50 g) was subjected to a silica gel (500 g, 70-230 mesh, ASTM) column chromatography using a gradient solvent system of *n*-Hexane-EtOAc (1:0→0:1). 307 sub-fractions of 300 ml were collected and grouped according to their TLC profile to afford 10 fractions A-J. Compounds **4** (68 mg), **3** (90 mg), **10** (19 mg) and the mixture **5+6** (74 mg) were obtained from the main column respectively from sub-fractions 5-23 eluted at *n*-Hexane-EtOAc (1:0), 56-70 eluted at *n*-Hexane-EtOAc (0.5:9.5), 263-264 eluted at *n*-Hex-EtOAc (3:7) and 76-85 eluted at *n*-Hexane-EtOAc (0.5:9.5). Silica gel column chromatography of fraction A (1.42 g) eluting with a mixture of *n*-Hexane-EtOAc (7.5:2.5) gave compound **2** (6.81 mg).

MeOH extract (40 g) was also separated by silica gel (400 g, 70-230 mesh, ASTM) column using a gradient of *n*-Hexane-EtOAc (1:0→0:1) to obtain compounds **1** (6 mg), **5+6** (14 mg) and **8** (55 mg). Sub-fractions (411) of 300 ml were collected and pooled together according to their TLC profile affording five fractions K-O. From the main column, compound **1** was obtained as a white powder from sub-fractions 43-47 eluted at *n*-Hexane-EtOAc (9:1); **5+6** was filtered from sub-fractions 58-61 eluted at *n*-Hexane-EtOAc (0:1) and compound **8** precipitated from sub-fractions 113-119 eluted at *n*-Hexane-EtOAc (8.5:1.5). Fraction M (0.32 g) was applied to a silica gel (3.5 g) column and eluted with a mixture of *n*-Hexane-CH₂Cl₂ to give compound **11** (75 mg) as a white powder at *n*-Hexane-CH₂Cl₂ (9:1). Compound **9** (8 mg) was obtained after further purification of fraction N (1.56 g) through a silica gel column chromatography using *n*-Hexane-EtOAc (8.5:1.5) as solvent system.

4.4 Antimicrobial assay

4.4.1 Microorganisms and culture media

Antimicrobial activities of isolated compounds were evaluated against 4 four bacterial strains (*Salmonella typhi* ATCC6539, *Salmonella Typhimurium*, *Pseudomonas aeruginosa* ATCC9721, *Escherichia coli*) and four yeasts (*Candida albicans* ATCC9002, *Candida parapsilosis* ATCC22019 and two clinical isolates *Candida krusei* and *Candida albicans*). Among these microorganisms *E. coli*, *C. albicans* and *C. krusei* were obtained from the microorganism bank of the Ayub Theaching Hospital of Abbottabad (Pakistan) while *S. typhimurium* was obtained from the Laboratory of Bacteriology and Mycology of the “Centre Pasteur” of Yaounde,

Cameroon. These microorganisms were maintained on agar slant in refrigerator at 4°C. The reference strains were obtained from American Type Culture Collection (ATCC). Mueller Hinton Agar (MHA) for the activation of tested Gram-negative bacteria while Mueller Hinton Broth (MHB) was used for the *in vitro* antibacterial assay as culture media.

4.4.2 Antibacterial activity: MIC and MBC determination

The minimal inhibitory concentration (MIC) values were determined by the broth microdilution method using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay as previously reported by Nyemb et al. (2018). Samples were dissolved in DMSO and the assay was carried out in 96-well plates. Eight successive 2-fold dilutions of each compound were mixed with the appropriate MHB to provide a concentration range ranging from 128 to 1 µg/ml. Each of these aliquots was then inoculated with 100 µl of suspension of the selected bacterial strains: *Salmonella typhi* ATCC6539, *Salmonella typhi*, *Pseudomonas aeruginosa* ATCC9721 and *Escherichia coli*. The plates were then incubated at 37°C for 24 h. Ciprofloxacin was used as positive control while DMSO was used as negative control. MIC was defined as the lowest concentration of samples that prevent visible growth of the microorganism in the microdilution wells.

After MIC determination, the minimum bactericidal concentration (MBC) was determined from the well which did not show any growth after incubation during MIC assay, as previously reported by Nyemb et al. (2018). MBC was the lowest concentration of samples that prevented the color change after addition of MTT.

4.4.3 Antifungal activity

Antifungal assay was performed on some isolated compounds against four strains of *Candida*: *C. albicans* ATCC9002, *C. albicans*, *C. parapsilosis* ATCC22019, and *C. krusei*. MICs and the minimum fungicidal concentration (MFCs) were determined by the broth microdilution method as previously reported by Dzoyem et al. (2014) using Ketoconazole as positive control.

4.5 α -glucosidase inhibitory activity assay

The inhibition of α -glucosidase enzyme was performed on a FluoStar Omega plate reader according to the standard method (Nyemb et al., 2018b) with slight modification. In a 96-wells microtiter plate (Corning Costar, Cambridge, MA, USA), 10 µl of tested compounds (100 µg/ml in DMSO 10%), 70 µl of sodium phosphate buffer (70 mM, pH 6.8) and 10 µl of enzyme solution (2.5 U/ml) were mixed and pre-incubated for 3-5 mins at 37°C. The reaction was

initiated after the pre-incubation time by adding 10 μ l of α -*p*NPG (4-Nitrophenyl- β -D-glucopyranoside) as a substrate. After an additional 30 min of incubation at the same temperature, the reaction was stopped by adding 40 μ l of a solution of Na₂CO₃ (200 mM). The amount of released *p*-nitrophenyl product was measured at 405 nm. Both α -glucosidase and *p*-PNG were dissolved in 70 mM NaH₂PO₄ (pH 6.8). The α -glucosidase activity of the samples showing more than 50% of inhibition was expressed as IC₅₀ that is the amount of tested sample required to decrease 50% of α -glucosidase activity. It was determined by the further dilution of the main concentration of the concerned sample, into 8 different concentrations and calculated using Graph PRISM software version 5.0 (San Diego, CA, USA) as the mean $\pm$ S.E.M. of three assays. The percentage inhibition of each sample was calculated by the following formula:

$$\text{Inhibition (\%)} = [(A_{St} - A_{S0}) / (A_{Ct} - A_{C0})] \times 100$$

A_{St} and A_{S0} being respectively the absorbance of the sample at time *t* and at zero time; A_{Ct} and A_{C0} represent the absorbance of the blank control at *t* time and zero time respectively.

3.6 Statistical analysis

All assays were performed in triplicate. Statistical analyses were done using GraphPad Prism 6 Software, Inc. (San Diego, CA, USA) for a one-way ANOVA analysis, followed by Student Newman-Keuls' test. Differences at $p < 0.05$ were considered to be statistically significant.

Table S1. ^1H & ^{13}C NMR (CDCl_3 , 500 MHz) data of compound **4**

N ^o	δ_{H} (Mult., J(Hz))	δ_{C}	COSY
	4.86 (<i>ddt</i> ; 10.2 ; 2.3 ; 1.2)		H-1b ; H-2
1	4.92 (<i>ddd</i> ; 17.1 ; 3.6 ; 1.6)	114.1	H-1a ; H-2
2	5.74 (<i>ddt</i> , 16.9 ; 10.2 ; 6.7)	139.3	2H-1 ; 2H-3
3	1.97 (<i>m</i>)	33.8	H-2 ; 2H-4
4-52		28.9-29.7	/
53	1.21-1.30 (<i>m</i>)	31.9	2H-26
54		22.7	2H-26 ; 3H-28
55	0.81 (<i>t</i> ; 7.0)	14.1	2H-27

Table S2. Results of antibacterial activities (MIC and MBC ($\mu\text{g/ml}$)) of compounds

Samples	Bacterial strains							
	<i>E. coli</i>		<i>S. typhi</i> ATCC6539		<i>S. typhi</i>		<i>P. aeruginosa</i> ATCC9721	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1	128	-	-	-	-	-	128	-
3	-	-	128	-	-	-	-	-
4	-	-	-	-	-	-	-	-
5+6	-	-	-	-	128	128	32	-
8	-	-	128	128	128	-	64	-
9	-	-	64	-	128	-	16	64
10	128	-	128	128	32	64	32	-
11	-	-	-	-	-	-	-	-
Ciprofloxacin	0.5	0.5	0.5	1	0.5	0.5	0.5	1

(-): >128 $\mu\text{g/ml}$ **Table S3.** Results of antifungal activities (MIC and MFC ($\mu\text{g/ml}$)) of compounds

Samples	Yeasts							
	<i>C. albicans</i>		<i>C. albicans</i> ATCC9002		<i>C. krusei</i>		<i>C. parapsilosis</i> ATCC22019	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
1	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-
5+6	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-
10	32	32	32	32	32	32	64	64
11	-	-	-	-	-	-	-	-
Ketoconazol	0.25	64	0.5	64	2	64	2	16

(-): >128 $\mu\text{g/ml}$

Table S4. Enzyme inhibition activities of extracts and some isolated compounds

Samples	α -Glucosidase
	IC ₅₀ (μ M) $\pm$ SEM/% Inhibition
Seeds	
EtOAc	n.d.
MeOH	35.08%
Leaves	
EtOAc	20.03 $\pm$ 1.80 ^a
MeOH	9.65 $\pm$ 0.06 ^b
Compounds	
3	42.32 %
4	12.42 %
5+6	29.59 %
8	11.06 $\pm$ 0.14 ^c
9	22.80 $\pm$ 2.31 ^d
10	1.72 $\pm$ 0.03 ^e
11	16.75 %
Acarbose	234.6 $\pm$ 2.01 ^f

Values with different letter superscripts are significantly different (P<0.05, Newman-Keuls test).

The unit is μ g/ml for all extracts

Figure S1. Proposed fragmentation of compound **4** in TOF-ESI-MS⁺ **Figure S2.** TOF-MS-ESI⁺ spectra of compound **4**

Figure S3. ^1H NMR spectra of compound **4** in CDCl_3

Figure S4. ^1H NMR spectra of compound **4** in CDCl_3

Figure S5. ¹³C NMR spectra of compound **4** in CDCl₃

Figure S6. ¹H-¹H COSY NMR spectra of compound **4** in CDCl₃