TWO NEW OLEANANE-TYPE GLYCOSIDES FROM *Napoleonaea imperialis* P. BEAUV RIND

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ABSTRACT

Napoleonaea imperialis P. Beauv (family Lecythidaceae) commonly called Napoleon's hat is a medicinal plant found in South-Eastern Nigeria. The rind was separated from the fruit and macerated using methanol. The crude methanol extract was partitioned to yield n-hexane fraction (7.4%), dichloromethane fraction (23.7%) and methanol fraction (68.7%). The dichloromethane fraction was chromatographed and further purified to afford two compounds whose structures were elucidated using ^{1}H, ^{13}C and two-dimension NMR experiments. Isolated compounds, Napoleonaside G and Napoleonaside R, were characterized as 3-O-[D-glucopyranosyl(1→2)-D-glucopyranosyl]-21,28-diangeloyloxy-24-hydroxy-olean-11,13(18)-diene and 3-O-[D-glucopyranosyl(1→4)-D-glucopyranosyl-2"-angeloyl]-21-angeloyloxy-24,28-dihydroxy-olean-11,13(18)-diene, respectively.

Keywords: *Napoleonaea imperialis*, rind, saponin, oleanane, glycoside, napoleonaside G, napoleonaside R

INTRODUCTION

Plants for some decades have become source of developing modern medicines for human health[1]. Plant usage for medicinal purpose depends on phytochemicals for its biological activities[2, 3]. Flavonoids, alkaloids, saponins, coumarins, terpenes, tannins and glycosides are phytochemicals mostly present in plants[4].

Rind is the outer covering of a fruit regarded as waste material, which is often discarded after consumption [5]. They are reported to have phytochemicals of pharmaceutical interest and important sources of flavour and aroma, utilized as dietary fibre, animal feed and biofuel[6-10]. Environmental pollution can also be reduced by converting waste materials into useful products[11].

Napoleonaea imperialis, commonly called Napoleon's Hat, belongs to the family Lecythidacaea (The Brazil Nut Family), is a non-timber medicinal plant found in South-Eastern Nigeria and grows abundantly in bush fallows and secondary bushes[12]. The bark and fruit rind are used to treat respiratory tract infections while the twig has antimicrobial potential, hence they are used as traditional chew stick for oral hygiene[13, 14]. The rind and seed of *N. imperialis* have antibacterial potency when evaluated against Gram-positive and Gram-negative bacteria[13]. Neral and geranial are reported to be the major volatile compounds of the essential oil of *N. imperialis* rind, while triterpenoidal saponins have been isolated from the seed of *N. imperialis*[15-17].

MATERIALS AND METHODS

General

All solvents used in this work were redistilled. Analytical thin layer chromatography (TLC) was conducted on silica gel (Merck F_{253}) precoated aluminium plates. Vacuum liquid chromatography and column chromatography were conducted using silica gel 70-230 and 60-200 mesh ASTM (60H Merck) respectively. Detection of spots on developed TLC plates was done under UV light at wavelength of 254 nm and using spray reagent (10 % H_2SO_4-MeOH) followed by heating at 105 ℃ for 5 min. One dimension TLC was carried out using 5x5 cm TLC plates and developed with various solvent systems.

All NMR data were acquired at 25 ℃ on a Bruker 600 MHz spectrometer (^{1}H, 600.06 MHz) equipped with a 5 mm cryoprobe. The sample (~6 mg) was solubilized in 550 µL methanol (CD_3OD). The solution was then transferred to a 5 mm NMR tube. ^{1}H {^{13}C} NMR experiment was acquired with spectral width of 9090.9 Hz, 32768 data points, 8 scans and 1.5 s relaxation delay. ^{13}C NMR experiments was acquired with spectral width of 35714.3 Hz, 32768 data points, 3840 scans and 1.5 s relaxation delay. 2D NMR data were acquired with ^{1}H spectral widths of 4424.8 Hz (COSY, TOCSY and NOESY), ^{1}H/^{13}C spectral widths of 4424.8 Hz / 22123.9 Hz (HSQC and HSQC-TOCSY) and ^{1}H/^{13}C spectral widths of 4424.8 Hz / 22123.9 Hz (HMBC). COSY data was acquired with 2 scans and 1536 * 768 (t1 * t2) points, TOCSY data was acquired with 8 scans and 1024 * 384 (t1 * t2) points, NOESY data was acquired with 20 scans and 1280 * 512 (t1 * t2) points, HSQC data was acquired with 8 scans and 1024 * 768 (t1 * t2) points, HSQC-TOCSY data was acquired with 16 scans and 871 * 768 (t1 * t2) points and HMBC data was acquired with 44 scans and 1536 * 256 (t1 * t2) points. Chemical shifts were referenced to the methanol signals at 3.35, 4.78 ppm (^{1}H) and 49.3 ppm (^{13}C). All spectra were processed and analysed with MestreNova (version 14.0.1-23559).

Extraction and partitioning

Fresh fruits of *N. imperialis* were harvested from Owerri, Imo state, Nigeria. The rinds were separated from the seed, chopped, air-dried and pulverized at room temperature (25 ℃) and weighed. Extraction of pulverized rind (617.2 g) was done using maceration with methanol for 48 hours. The extract was concentrated to dryness using a rotary evaporator (Labrota 4002) at 40 ℃ to afford a brown-sticky crude extract (148.12 g). The resulting crude methanol extract was dissolved in methanol (200 ml) and subsequently partitioned successively between 400 ml *n*-hexane and 400 ml of dichloromethane in a separatory funnel. Each of the partitioned fraction was concentrated to yield *n*-hexane fraction (11.01 g, 7.4%), dichloromethane fraction (35.16 g, 23.7%) and methanol fraction (101.75 g, 68.7 %). These fractions were stored in the refrigerator until required for further analysis.

Vacuum Liquid Chromatography

Dichloromethane fraction (32.97 g) was weighed, and 20 g of silica gel added to it. The silica gel was properly mixed with the fraction and a light brown powder was obtained. The mixture was poured into a sintered glass funnel (9.8 cm x 10.4 cm) already packed with 130 g of silica gel and was evenly distributed to the height of 4.5 cm. Gradient elution using different combinations of *n*-hexane (*n*-Hex), ethyl acetate (EtOAc) and methanol (MeOH) was conducted, and fractions (200 ml each) collected. A total of 25 fractions were obtained and combined based on TLC analysis to afford 13 major fractions (F_1 - F_{13}).

The dry fractions were weighed and stored in glass vials.

Purification through Column Chromatography

F_{10} and F_{11} which eluted with EtOAc-MeOH (9:1) from VLC yielded a light brown solid (3.26 g) and brown powder (3.68 g) respectively after evaporation of solvents; and were subjected to purification using column chromatography. All fractions were monitored with thin layer chromatography. A packed column containing 2.3 g of F_{10} was eluted with EtOAc-*n*-Hex (6:4), EtOAc-*n*-Hex (9.5:0.5), 100% EtOAc and EtOAc-MeOH (9.5:0.5); and 20 ml per fraction collected to give a total of 99 fractions. Fractions 53-71 which eluted with EtOAc-MeOH (9.5:0.5) were combined and solvent evaporated to give isolate A (80 mg).

F_{11} (2.5 g) was loaded in a column and eluted with EtOAc-*n*-Hex (8:2), EtOAc-*n*-Hex (9.5:0.5), EtOAc-MeOH (9.5:0.5) and EtOAc-MeOH (9.2:0.8); and 20 ml per fraction collected to give a total of 78 fractions.

Fractions 60-76 which eluted with EtOAc-MeOH (9.2:0.8) were combined and solvent evaporated to give golden crystals (84 mg) which was subjected to further purification. For the purification of the golden crystals, a column of 30 cm length and 2.4 cm diameter was packed using EtOAc-*n*-hex (1:1) as solvent system. The column was eluted with 100% EtOAc and EtOAc-MeOH (9.5:0.5); and 5 ml per fraction collected to give a total of 41 fractions. Fractions 16-39 eluted with EtOAc-MeOH (9.05:0.5) were combined and solvent evaporated to give isolate B (45 mg).

Isolates A and B (5 mg each) were separately dissolved in dichloromethane (I ml) and tested with freshly prepared Liebermann-Burchard reagent (1 ml). Purple colouration was observed for both reactions.

RESULTS AND DISCUSSION

Isolates A and B showed positive reaction to Liebermann-Burchard test with purple colouration (Table 1) indicating the presence of triterpenoidal saponins [17, 18]. Visibility of the two compounds under UV light of wavelength 252 nm (Table 1) revealed the existence of a conjugated diene while the purple spots seen after developed plates were sprayed with 10% sulphuric acid in methanol reagent and heated indicated the presence of terpenoids [19-21].

Table 1. Profile of isolates A and B

Isolate	Spot under UV	Spot after MeOH-H_2SO_4 reagent +heat	TLC mobile phase	R_f value	Liebermann-Burchard test	Percentage yield
A (Crystal)	Purple	Purple	9:1 EtOAc- MeOH	0.6	Purple	0.2
B (Crystal)	Purple	Purple	9:1 Acetone- MeOH	0.9	Purple	0.1

Structures of isolates A (**1**) and B (**2**) were determined using ^1H and ^{13}C NMR experiments aided by connectivity observed in two-dimension NMR experiments (Tables 2 and 3).

^{13}C NMR spectrum of isolate A showed fifty-two carbon signals. Two carbon signals at δ170.6 (C-1*) and δ168.5 (C-1**) were identified as carbonyl (esters) carbons. Ten ring residue signals, δ74.7 (C-2'), δ73.9 (C-3'), δ71.0 (C-4'), δ71.6 (C-5'), δ61.70 (C-6'),

δ76.6 (C-2''), δ71.8 (C-3''), δ91.2 (C-4''), δ72.7 (C-5''), δ66.4 (C-6'') belonging to the sugar moiety as well as two anomeric carbons, δ105.0 (C-1') and δ104.2 (C-1''), were also identified. ^{1}H NMR spectrum of isolate A showed two anomeric protons at δ4.57 (H-1') and δ4.77 (H-1''). The assignments of isolate A ^{1}H and ^{13}C signals using HSQC and verified with ^{1}H-^{1}H COSY (Table 2) show eight quaternary carbon signals, δ47.5(C-4), δ44.5 (C-8), δ35.7 (C-10), δ139.7 (C-13), δ42.5 (C-14), δ42.2 (C-17), δ139.1 (C-18) and δ37.5 (C-20); six primary carbon (methyl groups) signals, δ16.2 (C-23), δ16.2 (C-25), δ16.5 (C-26), δ14.1 (C-27), δ18.5 (C-29) and δ19.9 (C-30); ten secondary carbon (methylenes) signals, δ39.2 (C-1), δ26.9 (C-2), δ27.1 (C-6), δ31.6 (C-7), δ33.0 (C-15), δ34.5 (C-16), δ24.9 (C-19), δ34.0 (C-22), δ64.2 (C-24) and δ66.2 (C-28); six tertiary carbon signals at δ80.5 (C-3), δ40.3 (C-5), δ41.0 (C-9), δ125.8 (C-11), δ124.2 (C-12) and δ68.8 (C-21) were assigned to the aglycone. An ester carbonyl carbon, 170.6 (C-1*), quaternary carbon, δ142.1 (C-2*), primary carbons, δ22.10 (C-3*) and δ15.8 (C-5*), and tertiary carbon δ130.8 (C-4*) indicated the presence of an angeloyl group (angeloyl 1), while the ester carbonyl carbon signal at δ168.5 (C-1**), quaternary carbon δ128.7 (C-2**), primary carbons δ20.09 (C-3**), 15.8 (C-5**) and tertiary carbon δ140.0 (C-4**) showed the presence of another angeloyl group (angeloyl 2). From HSQC spectrum, eight proton signals observed at δ4.52 (H-^{a}O), δ4.54 (H-^{b}O), δ3.81 (H-^{c}O), δ4.75 (H-^{d}O), δ4.48 (H-^{e}O), δ3.76 (H-^{f}O), δ3.61 (H-^{g}O) and δ3.78 (H-^{h}O) were not attached to any carbon in the HSQC spectrum and were assigned as the sugar hydroxy protons.

Olefinic carbon signals at δ125.8 (C-11), δ124.2 (C-12) supported by proton signals at δ5.40 (H-11) and δ5.32 (H-12) and two quaternary carbons at δ139.1 (C-13) and δ139.7 (C-18) indicate the existence of a heteroannular diene in the structure of isolate A. These signals are similar to the diene peaks of saikosaponin [22]. The additional quaternary carbons at δ47.5 (C-4), δ44.5 (C-8), δ35.7 (C-10), δ42.5 (C-14), δ42.2 (C-17) and δ37.5 (C-20) have been reported for saikosaponin isolated from *Bupleurum chinense*, and derivatives of saikosaponin [22, 23]. Signals of the angeloyl groups δ170.6 (C-1*), δ168.5 (C-1**), δ142.1 (C-2*), δ128.7 (C-2**), δ1.98 (H-3*), δ1.82 (H-3**), δ6.12 (H-4*) δ6.22 (H-4**), δ2.10 (H-5*) and δ1.95 (H-5**) were similar to angeloyl reported by other researchers [17, 24]. The proton signals at δ1.98 (H-3*) and δ1.82 (H-3**) of the angeloyl groups correlated to δ4.21(H-21) and δ4.28 (H-28) of the aglycone respectively (Figure 1) indicating the positions where the angeloyl groups are attached to the aglycone. Therefore, the aglycone was characterized as 21,28-diangeloyloxy-24-hydroxy-olean-11,13(18)-diene. The anomeric proton δ4.57 (H-1') was found to correlate to δ3.58 (H-3) which was directly correlated to δ80.5 (C-3) of the aglycone while the anomeric proton δ4.77 (H-1'') correlated with δ3.21 (H-2') of the sugar moiety. These correlations show the position of attachment of the sugar moiety to the aglycone and the connectivity of all the protons in the sugar moiety. The sugar moiety of isolate A was characterized as 3-O-[D-glucopyranosyl(1→2)]-D-glucopyranoside. The proposed name for isolate A (**1**), Napoleonaside G, is 3-O-[D-glucopyranosyl(1→2)-D-glucopyranosyl]-21,28-diangeloyloxy-24-hydroxy-olean-11,13(18)-diene.

Table 2. ^{13}C and ^{1}H chemical shift data of isolate A (Napoleonaside G) in CD_3OD

Aglycone			Sugar moiety		
Position	**δ^{13}C**	**δ^{1}H**	**Position**	**δ^{13}C**	**δ^{1}H**
1	39.2	1.67 (1H, m), 0.94 (1H, m)	1'	105.0	4.57 (1H, d, 3.0Hz)
2	26.9	1.98 (1H, s), 1.82 (1H, s)	2'	74.7	3.21 (1H, m)
3	80.5	3.58 (1H, m)	3'	73.9	5.06 (1H, m)
4	47.5	-	4'	71.0	3.82 (1H, t, 3.0Hz)
5	40.3	2.54 (1H, t, 3.0Hz)	5'	71.6	5.40 (1H, m)
6	27.1	1.45 (1H, m), 1.36 (1H, d, 6.0Hz)	6'	61.7	3.79 (1H, t, 1.5Hz), 3.72 (1H, q, 3.0Hz)
7	31.6	1.67 (1H, m), 1.45 (1H, m)	1"	104.2	4.77 (1H, d, 3.0Hz)
8	44.5	-	2"	76.6	3.48 (1H, t, 1.5Hz)
9	41.0	2.36 (1H, d, 6.0Hz)	3"	71.8	3.88 (1H, d, 3.0Hz)
10	35.7	-	4"	91.2	4.15 (1H, s)
11	125.8	5.40 (1H, m)	5"	72.7	3.58 (1H, m)
12	124.2	5.32 (1H, m)	6"	66.4	3.42 (1H, m), 3.21 (1H, m)
13	139.7	-	^{a}O		4.52 (1H, t, 1.5Hz)
14	42.5	-	^{b}O		4.54 (1H, s)
15	33.0	1.30 (1H, d, 3.0Hz), 1.98 (1H, s)	^{c}O		3.81 (1H, s)
16	34.5	1.78 (1H, t, 1.5Hz), 1.36 (1H, d, 6.0Hz)	^{d}O		4.75 (1H, s)
17	42.2	-	^{e}O		4.48 (1H, s)
18	139.1	-	^{f}O		3.76 (1H, d, 3.0Hz)
19	24.9	1.92 (1H, s), 1.90 (1H, d, 3Hz)	^{g}O		3.61 (1H, d, 3.0Hz)
20	37.5	-	^{h}O		3.78 (1H, t, 1.5Hz)
21	68.8	4.21 (1H, m)	Angeloyl 1		
22	34.0	1.67 (1H, s), 1.36 (1H, d, 6.0Hz)	1*	170.6	-
23	16.2	1.21 (3H, s)	2*	142.1	-
24	64.2	4.09 (1H, t, 3Hz), 3.21 (1H, d, 3Hz)	3*	15.8	1.98 (3H, s)
25	16.2	1.15 (3H, s)	4*	130.8	6.12 (1H, q, 6.0Hz)
26	16.5	1.06 (3H, s)	5*	22.1	2.10 (3H, s)
27	14.1	0.94 (3H, s)	Angeloyl 2		
28	66.2	4.28 (1H, s), 3.37 (1H, m)	1**	168.5	-
29	18.5	1.03 (3H, s)	2**	128.7	-
30	19.9	0.98 (3H, s)	3**	20.1	1.82 (3H, s)
			4**	140.0	6.22 (1H, q, 6.0Hz)
			5**	15.8	1.95 (3H, s)

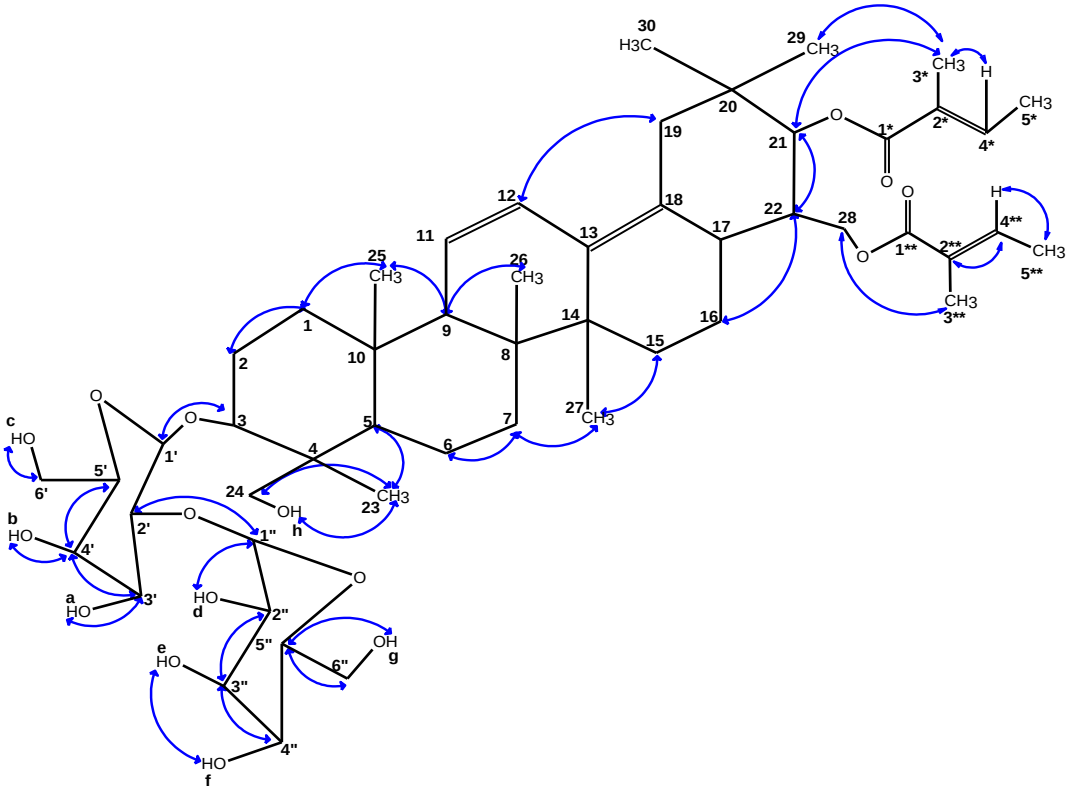
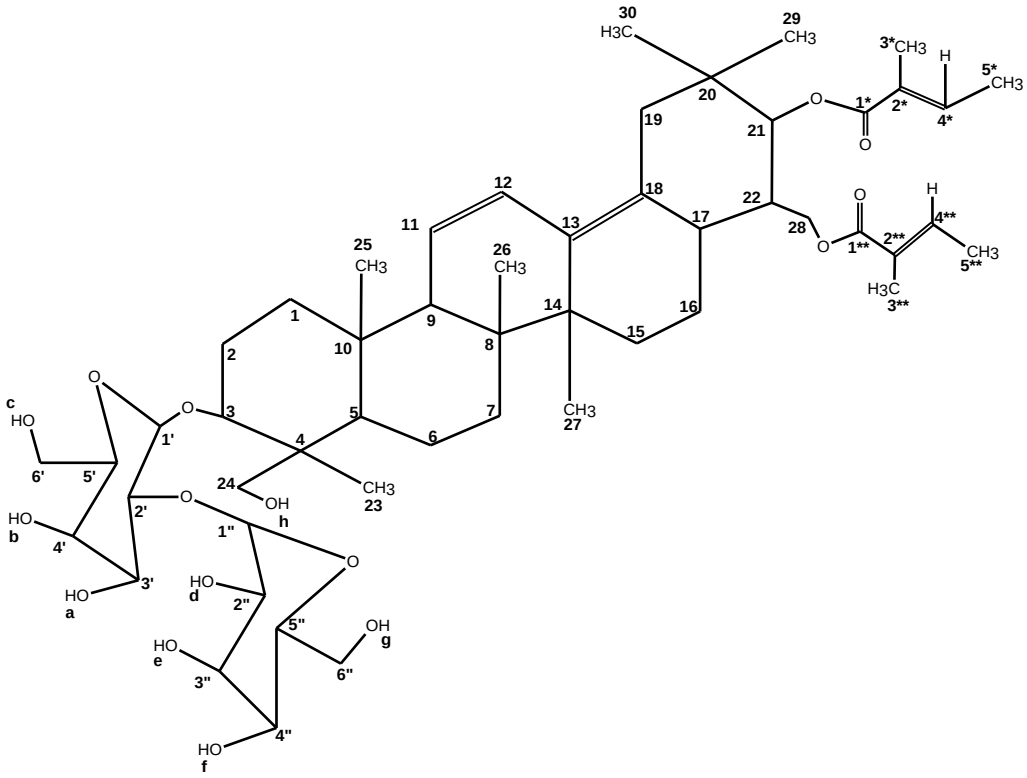


Figure 1. Structure and numbering of isolate A (Napoleonaside G) used in this report. COSY correlations are indicated with blue arrows.



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Assignment of ^{1}H and ^{13}C peaks of isolate B aided by 2D NMR experiments (Table 3) had similarities to isolate A (Table 2). Long range connectivity from HMBC showed a correlation between δ170.7 (C-1*) of the angeloyl group and δ68.8 (C-21) which is directly correlated to δ4.20 (H-21) (Figure 2). The aglycone of isolate B was characterized as 21-angeloyloxy-24,28-dihydroxy-olean-11,13(18)-diene. The correlation between anomeric proton H-1' and C-3 of the aglycone, C-1" and H-2" of the sugar moiety were established from 2D connectivity. The signal at δ91.9 (C-3) which is directly correlated with a δ3.19 (H-3) in HSQC (Table 3) show a long-range correlation with the proton signal δ4.32 (H-1') which is directly correlated with the anomeric carbon δ104.8 (C-1'). ^{13}C signal at δ105.8 (C-1") exhibited a long-range correlation with ^{1}H peak at δ5.03 (H-2"). HMBC of isolate B (Figure 2) shows a strong correlation between δ168.8 (C-1**) of an angeloyl group and δ5.03 (H-2") which is directly correlated with δ74.4 (C-2") of the sugar moiety. This type of correlation of an angeloyl group to a sugar moiety has been reported in pentacyclic triterpenoid saponin isolated from the husks of *Xanthoceras sorbifolium* Bunge and sesquiterpenes from the fruits of *Pittosporum undulatum* [25, 26]. The sugar moiety of isolate B was characterized as 3-O-[D-glucopyranosyl(1→4)-D-glucopyranoside-2"-angeloyl. The proposed name for isolate B (**2**), Napoleonaside R, is 3-O-[D-glucopyranosyl(1→4)-D-glucopyranosyl-2"-angeloyl]-21-angeloyloxy-24,28-dihydroxy-olean-11, 13(18)-diene.

Glycosides with oleanane-type triterpenoid sapogenin are reported to show antioxidant and anti-diabetic potentials, antimicrobial, anti-inflammatory, cytotoxic activities and are used as tools in cancer therapy [27-31].

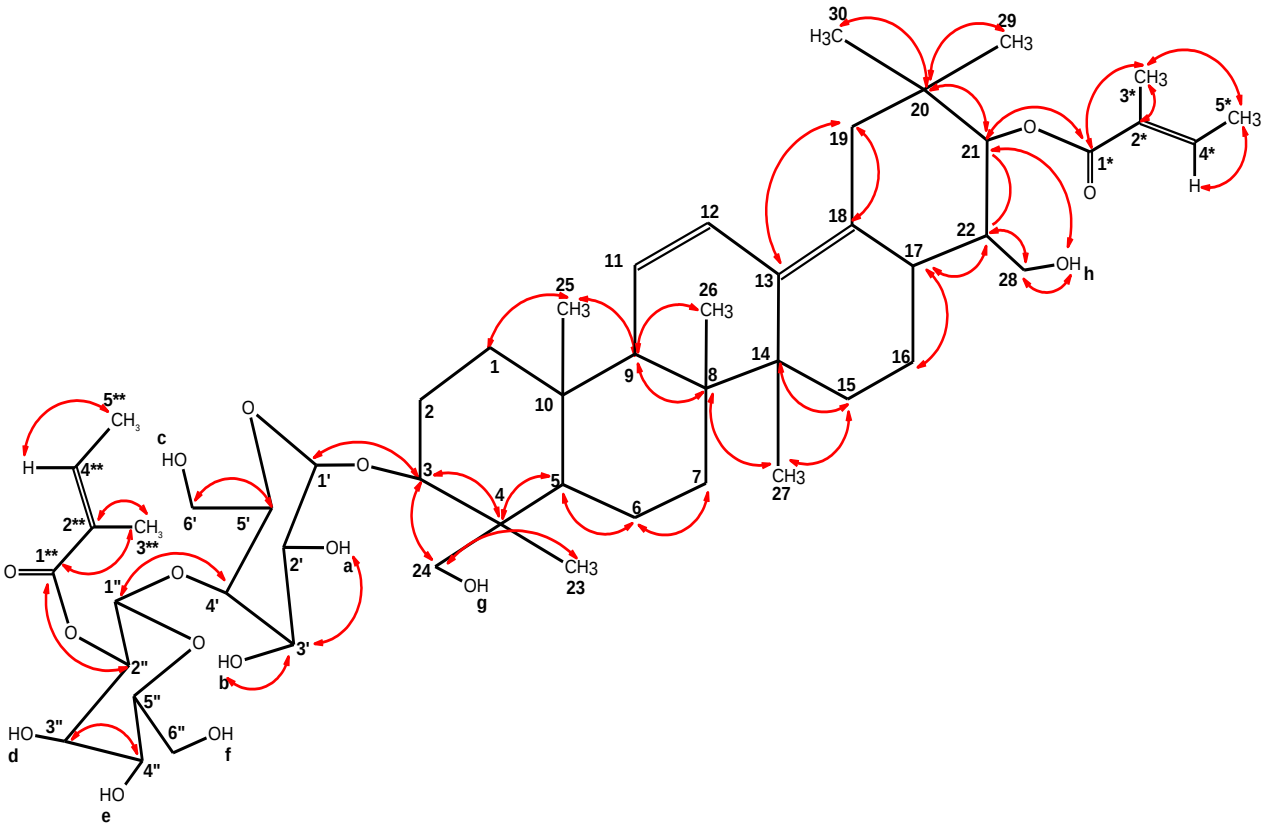
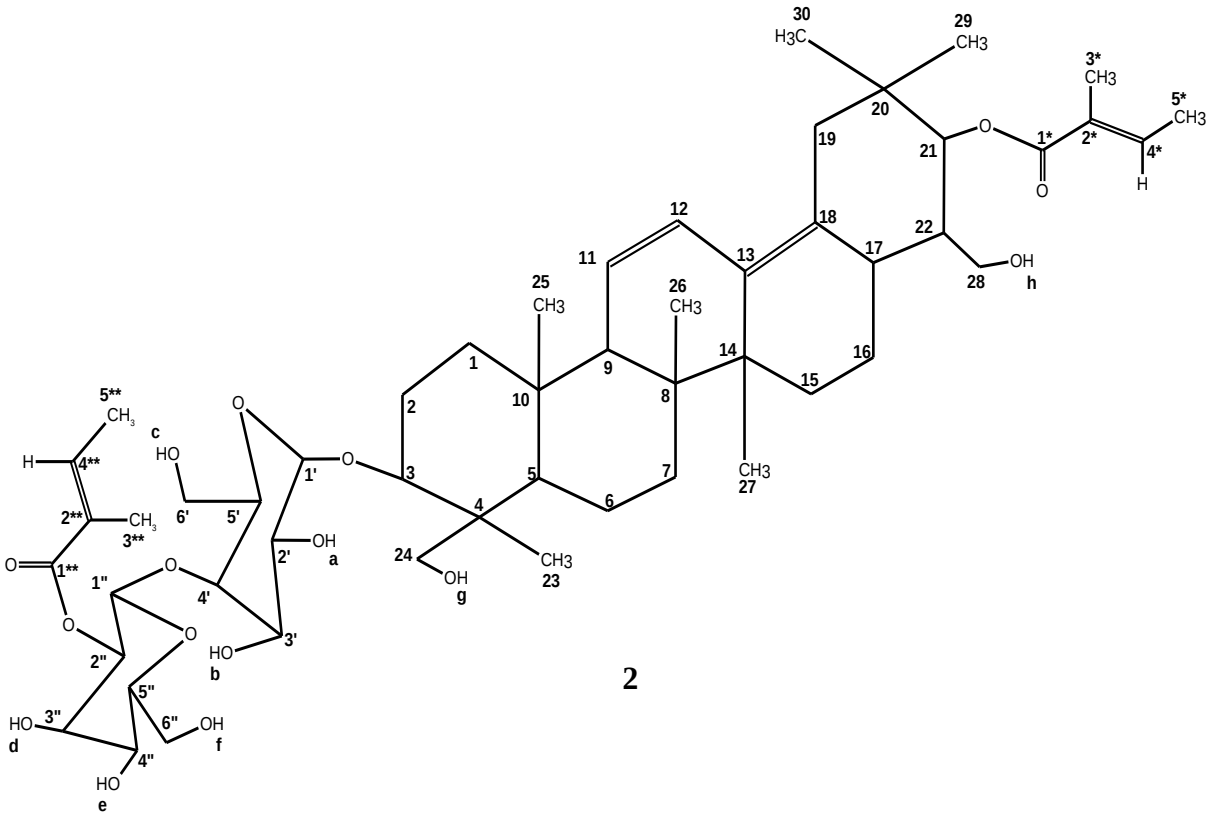


Figure 2. Structure and numbering of isolate B (Napoleonaside R) used in this report. HMBC correlations are indicated with red arrows

Table 3. ^{13}C and ^{1}H chemical shift data of isolate B (Napoleonaside R) in CD_3OD

	Aglycone			Sugar moiety	
Position	**$\delta^{13}C$**	**$\delta^{1}H$**	**Position**	**$\delta^{13}C$**	**$\delta^{1}H$**
1	39.5	1.58 (1H, s), 1.03 (1H, s)	1'	104.8	4.32 (1H, m)
2	26.9	1.82 (1H, s), 1.67 (1H, d, 3.0Hz)	2'	72.9	3.82 (1H, m)
3	91.9	3.19 (1H, t, 3Hz)	3'	75.7	5.23 (1H, m)
4	40.9	-	4'	77.5	3.58 (1H, m)
5	48.1	2.56 (1H, t, 3.0 Hz)	5'	74.0	3.48 (1H, m)
6	27.4	1.48 (1H, s) 1.43 (1H, m)	6'	67.1	3.23 (1H, m), 3.37(1H, m)
7	45.5	1.67 (1H, d, 3.0Hz), 1.58 (1H, s)	1''	105.8	4.57 (m)
8	37.6	-	2''	74.4	5.03 (1H, m)
9	40.9	2.38 (m)	3''	80.4	3.78 (1H, m)
10	47.5	-	4''	71.3	5.40 (1H, q, 3.0Hz)
11	124.5	5.32 (1H, t, 1.5Hz)	5''	74.4	4.01 (1H, m)
12	125.9	5.40 (1H, q, 3.0Hz)	6''	64.4	3.65 (1H, m), 3.48 (1H, m)
13	139.7	-	[a]O		3.66 (1H, d, 3.0Hz)
14	42.6	-	[b]O		3.72 (1H, s)
15	34.6	1.82 (1H, s), 1.38 (1H, s)	[c]O		3.89 (1H, s)
16	20.2	1.67 (1H, d, 3.0Hz), 1.43 (1H, m)	[d]O		4.89 (1H, d, 3.0Hz)
17	42.2	-	[e]O		4.48 (1H, t, 1.5Hz)
18	139.2	-	[f]O		4.72 (1H, m)
19	24.8	1.90 (1H, d, 3.0Hz), 1.92 (1H, s)	[g]O		3.63 (1H, s)
20	37.6	-	[h]O		3.88 (1H, s)
21	68.8	4.20 (1H, q, 3Hz)	Angeloyl 1		
22	31.6	1.67 (1H, d, 3.0Hz), 1.43 (1H, m)	1*	170.7	-
23	16.2	1.19 (3H, s)	2*	141.9	-
24	70.3	4.07 (1H, m), 3.78(1H, s)	3*	22.6	2.10 (3H, s)
25	18.6	1.03 (3H, s)	4*	140.0	6.22 (1H, q, 6.0Hz)
26	19.4	1.05 (3H, s)	5*	15.9	1.98 (3H, s)
27	14.1	0.94 (3H, s)	Angeloyl 2		
28	66.2	4.25 (1H, m), 4.15(1H, m)	1**	168.8	-

29	16.9	0.94 (3H, s)	2**	128.5	-
30	16.9	1.03 (3H, s)	3**	20.5	1.82 (3H, s)
			4**	140.3	6.12 (1H, q, 6.0Hz)
			5**	15.8	1.95 (3H, s)



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CONCLUSION

Two new oleanane-type glycosides, Napoleonaside G and Napoleonaside R, proposed as 3-O-[D-glucopyranosyl(1→2)-D-glucopyranosyl]-21,28-diangeloyloxy-24-hydroxy-olean-11,13(18)-diene and 3-O-[D-glucopyranosyl(1→4)-D-glucopyranosyl-2''-angeloyl]-21-angeloyloxy-24,28-dihydroxy-olean-11,13(18)-diene respectively were successfully isolated from the rind of *Napoleonaea imperialis.*

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