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Triterpenoids and Semisynthetic Derivatives with Antimicrobial Activities from the Leaves of *Caloncoba glauca* (Flacourtiaceae)

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Abstract

Chemical investigation of the MeOH extract from the leaves of *C. glauca* yielded nine known triterpenoids (**1-9**) belonging to the cycloartane and friedelane series. Two of these compounds namely glaucartanoic acid A (**1**) and 3*β*,21*β*-dihydroxy-30-nor-(D:A)-friedoolean-20(29)-en-27-oic acid (**5**) were subjected to chemical derivatizations and afforded five new derivatives: diacetylglaucartanoic acid A (**1a**), 24-acetylglaucartanoic acid A (**1b**), glaucartanoic acid A methyl ester (**1c**), 24-methoxyglaucartanoic acid A methyl ester (**1d**), and 3*β*,21*β*-diacetoxy-30-nor-(D:A)-friedoolean-20(29)-en-27-oic acid (**5a**). Their structures were assigned based on their NMR and MS data and by comparison with literature values. The MeOH extract, isolated compounds and some new semi-synthetic derivatives were subjected to *in vitro* antimicrobial assays against a panel of pathogenic microorganisms, including Gram-positive and Gram-negative bacteria, and fungi using broth microdilution method. The MeOH extract displayed activity towards all the tested pathogenic bacterial and fungal strains with good activity (MIC < 100 µg/mL) against *Staphylococcus aureus* ATCC25923 and *Shigella flexneri* SDINT. Compounds **3** and **5** showed the most potent antimicrobial effect.

Keywords

Caloncoba glauca, Triterpenoids, Chemical Derivatizations, Antimicrobial

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#Of late memory.

Activity, Structure-Activity Relationship

1. Introduction

Antimicrobial resistance has emerged as one of the major public health concerns of the 21st century that threatens the effective prevention and treatment of an ever-increasing range of infections caused by bacteria, parasites, viruses and fungi that are no longer susceptible to the common medicines used for their treatment [1]. The rapid emergence of resistant microorganisms is occurring worldwide, undermining many other advances in health and medicine. The situation is most dire in less developed countries, where the uncontrolled use of antibiotics, often coupled with a lack of proper healthcare infrastructure, leads to high morbidity rates [2]. Thus, the need of searching and developing new therapeutic agents capable of overcoming the resistance mechanisms of microbes is imperative [3], and plants with medicinal properties constitute a rich source of biologically active compounds. Triterpenoids, a class of compounds widely distributed in nature have shown diverse biological activities, including antimicrobial properties [4] [5]. Structure-activity studies have revealed that alterations in key carbons may enhance biological activity and reduce cytotoxicity [6]. Previous chemical studies of some *Caloncoba* species have proved this genus to be a rich source of triterpenoids [7]-[15]. *Caloncoba glauca* (P. Beauv.) Gilg (Flacourtiaceae) is a tree up to 15 m tall found in tropical Africa [16]. Its leaves are used as purgative and against inflammations and skin diseases [11]. Many microbial species including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida tropicalis*, *Candida albicans* and *Cryptococcus neoformans* are capable of causing various skin and other organs infections. Skin lesions due to *C. neoformans* are present in almost 5% of patients with cryptococcal meningitis [17] and the frequency is greater in liver transplant recipients receiving tacrolimus [18] or in patients infected with serotype D [19]. In most cases, skin lesions are due to hematogenous spread (*i.e.*, secondary cutaneous cryptococcosis). This prompted us, in connection with our search for bioactive compounds [13] [20] [21] [22], to reinvestigate the leaves of *C. glauca* in view of evaluating its antimicrobial properties. In the present paper, we describe the structure elucidation of five new semi-synthetic cycloartanes (**1a - 1d**) and friedelane-type (**5a**) triterpenoids obtained from the chemical transformations of two out of the nine triterpenoids isolated, and the antimicrobial activity of both the naturally occurring triterpenoids and the semi-synthetic derivatives. The structure-activity relationship is also discussed.

2. Material and Methods

2.1. Plant Material

C. glauca was selected on the basis of its utilization in traditional medicine. Its leaves were harvested in Bangang-Wabane village, Cameroon, in May 2016. The

sample was authenticated by Mr. Francois NANA, a botanist of the National Herbarium of Cameroon (Yaounde, Cameroon), where a voucher specimen (55064/HNC) was deposited.

2.2. Extraction and Isolation

The air-dried and powdered leaves of *C. glauca* (1.2 kg) were extracted with methanol (3 × 10 L) for 72 h at room temperature to yield a crude extract (90 g) after evaporation of solvent. Part of this extract (85 g) was subjected to column chromatography (8 cm × 60 cm) over silica gel (300 - 400 mesh) eluted with gradients of *n*-Hex/EtOAc (90:10, 70:30, 50:50, 30:70) then EtOAc/MeOH (100:0, 90:10, 70:30, 50:50, 0:100) and 68 fractions of 300 mL each were collected. These fractions were combined on the basis of their TLC (Thin Layer Chromatography) profiles into five major fractions A-E: A (9 g, 1 - 14), B (14 g, 15 - 28), C (16 g, 29 - 43), D (12 g, 44 - 53) and E (23 g, 54 - 68). Fraction A with a fatty appearance was not further investigated. Fraction B (14 g) was separated on a silica gel (100 - 200 mesh) column chromatography using a gradient of *n*-Hex/Me_2CO (100:0 to 70:30) to yield caloncobalactone C (**8**, 12 mg). Fraction C (16 g) was separated on a silica gel (100 - 200 mesh) column eluted with gradients of *n*-Hex/EtOAc (90:10 to 0:100) then EtOAc/MeOH (95:5 to 0:100) and subfractions C.I to C.IV were obtained. Successive column chromatography of subfraction C.I (2.5 g) over silica gel (100 - 200 mesh) and Sephadex LH-20 eluted respectively with gradients of *n*-Hex/Me_2CO (90:10 to 100:0) and isocratic CH_2Cl_2/MeOH (1:1) solvents mixtures afforded glaucartanoic acid B (**4**, 9.5 mg). Repeated purifications of sub fraction C.II (4 g) on silica gel (100 - 200 mesh) column chromatography eluted with CH_2Cl_2/Me_2CO (100:0 to 50:50) afforded caloncobic acid A (**6**, 14 mg). Subfraction C.III (3.5 g) was subjected successively to a silica gel (100 - 200 mesh) column chromatography eluted with CH_2Cl_2/Me_2CO (100:0 to 70:30) and passage over Sephadex LH-20 (CH_2Cl_2/MeOH, 1:1) to give glaucalactone (**3**, 19 mg). Sub fraction C.IV (1.5 g) precipitated in *n*-Hex/Me_2CO (80:20) and the deriving powder was purified over a Sephadex LH-20 column chromatography eluted with CH_2Cl_2/MeOH, (1:1) to afford glaucalactone B (**9**, 8.4 mg). Fraction D (12 g) was chromatographed on a silica gel (100 - 200 mesh) column eluted with gradients of *n*-Hex/EtOAc (90:10 to 0:100) then EtOAc/MeOH (95:5 to 0:100) to give three subfractions: D.I, D.II and D.III. Subfraction D.II (7 g) was subjected to a silica gel (100 - 200 mesh) column chromatography eluted with *n*-Hex/Me_2CO (from 90:10 to 0:100) and afforded sub fractions D.II.1 - D.II.3. Successive chromatography of subfraction D.II.2 (2.3 g) over a silica gel column eluted with *n*-Hex/Me_2CO (70:30 to 100:0) and Sephadex LH-20 eluted with CH_2Cl_2/MeOH (1:1) yielded glaucatarnoic acid A (**1**, 170 mg) and 3β,21β-dihydroxy-30-nor-(D:A)-friedoolean-20(29)-en-27-oic acid (**5**, 30 mg). Subfraction D.II.3 (1.8 g) was purified similarly to D.II.2 to furnish caloncobalactone (**2**, 14.3 mg). Fraction E (23 g) precipitated in EtOAc. Purification of the filtered powder over a silica gel column chromatography eluted with EtOAc/MeOH (95:5) followed by gel permeation over a Sephadex LH-20

column using CH_2Cl_2/MeOH, (1:1) gave caloncobic acid C (**7**, 8.3 mg).

2.3. Chemical Transformations and Purifications

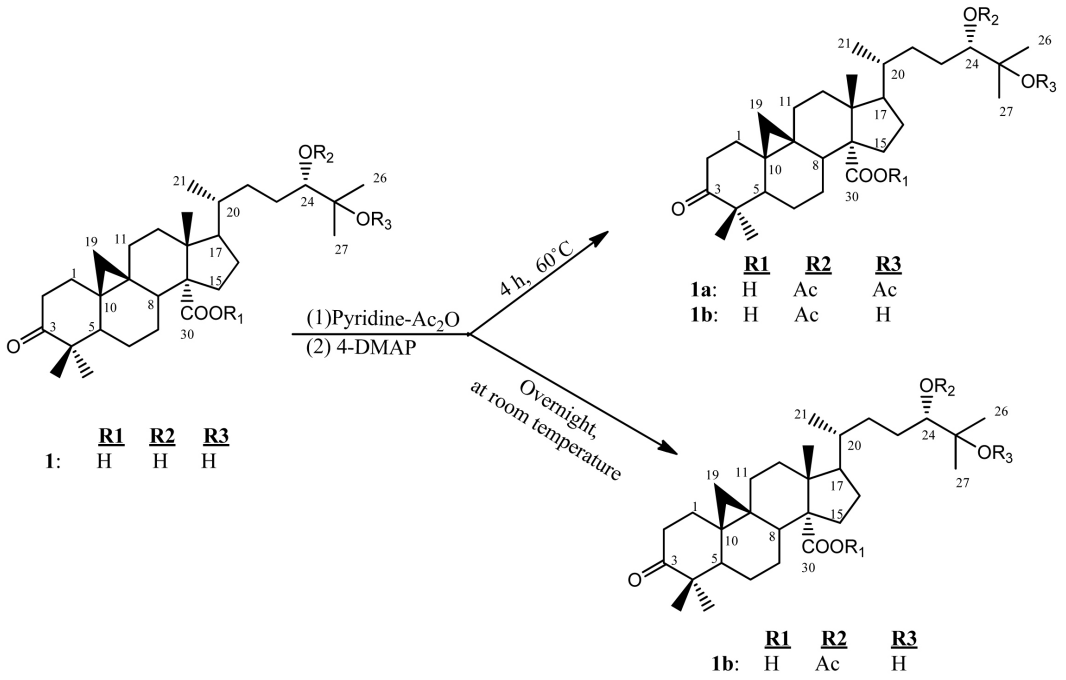
2.3.1. Acetylation of Glaucartarnoic Acid A (1)

A sample (15 mg, 30.7 mmol) of glaucartanoic acid A (**1**) was dissolved in pyridine-Ac_2O (3 ml, 1:1). Catalytic amount of 4-DMAP was added and the mixture was kept overnight in darkness at room temperature (Scheme 1). The reaction mixture was poured in cold water at 5˚C and extracted with EtOAc. The organic layer was washed with 2 M HCl, then 1 M $NaHCO_3$ and dried using a rotary evaporator. Column chromatography over silica gel (*n*-Hex/Me_2CO, 9:1) gave 24-acetylglaucartanoic acid A (**1b**, 12.7 mg, 80%).

This reaction was repeated with the same amount of reactants but was heated at 60˚C for 4 hours (Scheme 1). The reaction mixture worked-up in the same conditions yielded diacetylglaucartanoic acid A (**1a**, 14.3 mg, 81%) and a very little amount of **1b**.

Diacetylglaucartanoic acid A (1a): White powder (from Me_2CO); $[\alpha]_D^{23}$ − 49.4 (*c* 0.11, MeOH); **^{1}H** (400 MHz, CD_3OD) and **^{13}C** (100 MHz, CD_3OD) NMR data, see Table 1 and Table 2 respectively; positive **ESIMS** m/z (rel. int.) 596 $[M + H + Na]^+$ (6), 573 $[M + H]^+$ (5), 513 $[M - AcO]^+$ (100), 453 $[M - H - 2AcO]^+$ (5); **HRESIMS** m/z 595.3612 $[M + Na]^+$ (calcd for $C_{34}H_{52}O_7Na$, 595.3610).

24-acetylglaucartanoic acid A (1b): White powder (from Me_2CO); $[\alpha]_D^{23}$ − 36.9 (*c* 0.12, MeOH); **^{1}H** (400 MHz, CD_3OD) and **^{13}C** (100 MHz, CD_3OD) NMR data, see Table 1 and Table 2 respectively; positive **ESIMS** m/z (rel. int.) 531 $[M + H]^+$ (5), 553 $[M + Na]^+$ (8), 513 $[M + H - H_2O]^+$ (100); **HRESIMS** m/z 553.3505 $[M + Na]^+$ (calcd for $C_{32}H_{50}O_6Na$, 553.3505).



Scheme 1. Semisynthesis of **1a** and **1b** from glaucartanoic acid A (**1**).

Table 1. ^{1}H-NMR data of compounds **1a-1d** and **5a**.

Position	1a* (in MeOD)	1b* (in MeOD)	1c** (in MeOD)	1d** (in MeOD)	5a* (in $CDCl_3$-MeOD)
1	1.89 m; 1.66 m	1.85 m; 1.66 m	1.87^{ov}; 1.66^{ov}	1.89 m; 1.66 m	1.42^{ov} (2H)
2	2.24 m; 2.79 td (13.8, 6.4)	2.24 m; 2.79 td (13.8, 6.4)	2.26 m; 2.80 td (13.9, 6.3)	2.23 m; 2.80 td (13.9, 6.4)	1.84 m; 1.46 m
3					4.83 brs
4					1.30 m
5	1.71 dd (12.4, 3.4)	1.71 dd (12.4, 3.1)	1.67^{ov}	1.69 dd (6.2, 2.5)	
6	1.07 m; 1.50 m	1.11^{ov}; 1.51^{ov}	1.07 m; 1.51^{ov}	1.07 m; 1.50^{ov}	1.70 m; 0.96^{ov}
7	1.52 m; 1.19 m	1.22 m; 1.51^{ov}	0.98 m; 1.50^{ov}	0.97 m; 1.50^{ov}	1.52 m; 1.32^{ov}
8	1.84 brd (5.1)	1.83 brd (6.4)	1.86^{ov}	1.87 dd (10.4, 3.7)	1.48 m
9					
10					0.82 brd (6.3)
11	1.38 m; 2.26 m	1.36 brdd (10.0, 3.2); 2.29 m	1.38 m; 2.24 m	1.40^{ov}; 2.28 m	1.60 m; 1.24 m
12	1.76 brdd (11.6, 7.5); 1.86 m	1.76 m; 1.89 brdd (12.6, 3.2)	1.58 m; 1.88^{ov}	1.56 m; 1.92 m	1.42 m; 2.22 m
13					
14					
15	2.16 m; 1.27 m	2.13 m; 1.27 m	2.15^{ov}; 1.32 m	2.16 m; 1.32 m	2.59 brdd (13.1, 5.3); 1.21 m
16	1.34 m; 2.04 m	1.30 m; 2.05 m	1.44 m; 2.14^{ov}	1.43 m; 2.14 m	1.68 m; 1.32^{ov}
17	1.50 m	1.53^{ov}	1.40 m	1.42 m	
18	1.15 s	1.15 s	1.16^{ov}	1.15^{ov}	1.75 m
19	0.56 d (4.3); 0.91^{ov}	0.56 d (4.2); 0.91^{ov}	0.57 d (4.0); 0.92^{ov}	0.56 d (4.4); 0.92^{ov}	2.65^{ov} (2H)
20	1.54 m	1.53^{ov}	1.51^{ov}	1.52^{ov}	
21	0.91^{ov}	0.91^{ov}	0.91^{ov}	0.91^{ov}	5.30 brd (3.7)
22	1.04 m; 1.42 m	1.05 m; 1.43 m	1.30 m; 1.50^{ov}	1.28 m; 1.51^{ov}	1.17 m; 2.41 dd (15.1, 5.1)
23	1.60 m; 1.57 m	1.63 m; 1.59 m	1.35 m; 1.50^{ov}	1.40^{ov} (2H)	0.77 d (7.0)
24	5.27 t (6.4)	4.78 dd (10.2, 1.9)	3.22 brd (10.4)	2.88 dd (8.8, 1.9)	0.90 s
25					0.93 s
26	1.47 s	1.16^{ov}	1.16^{ov}	1.16^{ov}	0.97 s
27	1.42 s	1.16^{ov}	1.13 s	1.12 s	
28	1.01 s	1.01 s	1.01 s	1.01 s	1.20 s
29	1.12 s	1.12^{ov}	1.11 s	1.11 s	5.00 brs; 4.92 brs
30					
24-$OCOCH_3$	2.08 s	2.08 s			
25-$OCOCH_3$	1.94 s				
24-OMe				3.49 s	
30-OMe			3.64 s	3.64 s	
3-$OCOCH_3$					2.02 s
21-$OCOCH_3$					1.99 s

^{ov}Overlapped signals within a column; *Recorded with a 400 MHz instrument; **Recorded with a 500 MHz instrument.

Table 2. ^{13}C-NMR data of compounds **1a-1d** and **5a**.

Position	1a* (in MeOD)	1b* (in MeOD)	1c** (in MeOD)	1d** (in MeOD)	5a* (in $CDCl_3$-MeOD)
1	34.6	34.6	34.7	34.6	16.9
2	38.2	38.2	38.2	38.2	32.6
3	219.2	219.3	219.0	219.1	75.7
4	51.1	51.1	51.1	51.1	48.5
5	49.3	49.3	49.4	49.4	37.6
6	21.9	21.9	21.9	21.9	41.8
7	27.9	27.9	28.1	28.1	18.0
8	46.8	46.8	47.0	46.9	52.3
9	21.7	21.7	21.6	21.6	38.2
10	28.5	28.5	28.5	28.6	61.7
11	29.4	29.4	29.4	29.4	39.0
12	34.9	34.9	35.0	35.0	27.7
13	48.5	48.5	48.9	48.9	52.5
14	63.8	63.8	64.4	64.4	41.5
15	32.6	32.6	32.5	32.4	28.7
16	30.4	30.4	30.6	30.5	37.0
17	53.6	53.6	54.1	53.8	31.9
18	18.4	18.5	18.3	18.3	44.1
19	30.6	30.6	30.8	30.8	27.1
20	35.8	35.9	36.6	36.8	141.9
21	18.5	18.6	18.8	18.8	74.9
22	33.0	33.3	34.3	34.5	40.1
23	26.4	26.6	28.7	28.5	11.6
24	77.8	80.7	78.6	90.7	16.1
25	84.2	72.8	73.9	74.4	20.3
26	22.7	25.6	25.8	25.8	18.3
27	22.5	25.9	24.8	25.7	179.3
28	22.6	22.7	22.6	22.6	33.9
29	21.4	21.4	21.4	21.4	117.1
30	180.1	180.1	178.4	178.4	
24-O<u>C</u>OCH$_3$	172.5	172.9			
24-OCO<u>C</u>H$_3$	20.9	21.1			
25-O<u>C</u>OCH$_3$	172.1				
25-OCO<u>C</u>H$_3$	22.2				
24-OMe				61.4	
30-OMe			51.5	51.5	

Continued

3-OCO<u>C</u>H$_3$	21.5
3-O<u>C</u>OCH$_3$	172.4
21-OCO<u>C</u>H$_3$	21.8
21-O<u>C</u>OCH$_3$	171.8

*Recorded with a 400 MHz instrument; **Recorded with a 500 MHz instrument.

2.3.2. Methylation of Glaucatarnoic Acid A (1)

Compound **1** (50 mg, 102.4 mmol) was dissolved in DMF (5 mL) and dry NaH (2.5 mg) was added, followed by MeI (25 mg) (Scheme 2). The mixture was allowed to reflux over a water bath at 60˚C for 24 hours. The solvent was distilled off and the residue poured into water. The oil formed from the reaction mixture was extracted with EtOAc, then washed and dried. Evaporation of the organic solvent left a residue which was separated over a column of silica gel (*n*-Hex/Me_2CO, 9:1) to yield glaucartanoic acid A methyl ester (**1c**, 5.2 mg, 10%) and 24-methoxyglaucartanoic acid A methyl ester (**1d**, 26.7 mg, 51%).

Glaucartanoic acid A methyl ester (1c): White powder (from Me_2CO); $[\alpha]_D^{23}$ − 17.7 (*c* 0.10, MeOH); **^{1}H** (500 MHz, CD_3OD) and **^{13}C** (125 MHz, CD_3OD) NMR data, see Table 1 and Table 2 respectively; positive **ESIMS** m/z (rel. int.) 503 $[M + H]^+$ (6), 525 $[M + Na]^+$ (100); **HRESIMS** m/z 503.3733 $[M + H]^+$ (calcd for $C_{31}H_{51}O_5$, 503.3736).

24-methoxyglaucartanoic acid A methyl ester (1d): White powder (from Me_2CO); $[\alpha]_D^{23}$ − 29.7 (*c* 0.10, MeOH); **^{1}H** (500 MHz, CD_3OD) and **^{13}C** (125 MHz, CD_3OD) NMR data, see Table 1 and Table 2 respectively; positive **ESIMS** m/z (rel. int.) 517 $[M + H]^+$ (7), 539 $[M + Na]^+$ (70), 540 $[M + H + Na]^+$ (12); **HRESIMS** m/z 517.3896 $[M + H]^+$ (calcd for $C_{32}H_{53}O_5$, 517.3893).

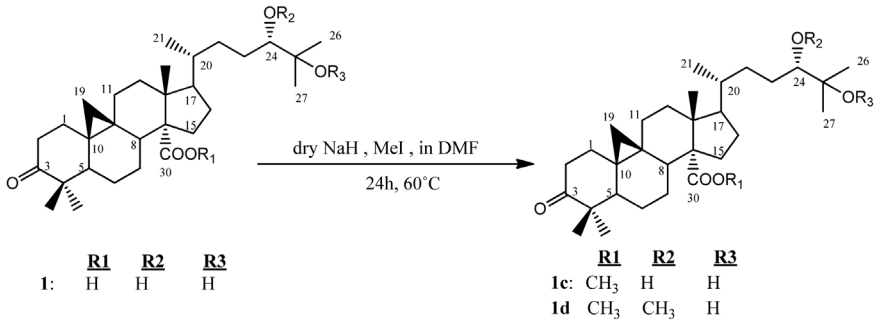
2.3.3. Acetylation of 3*β*,21*β*-Dihydroxy-30-Nor-(D:A)-Friedoolean-20 (29)-En-27-Oic Acid (5)

Compound **5** (10 mg, 21.83 mmol) was treated at room temperature as described above for **1** and afforded the diacetyl derivative **5a** (8.9 mg, 76%) (Scheme 3).

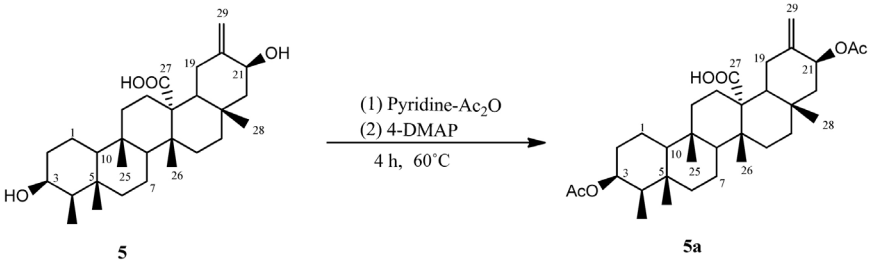
3*β*,21*β*-diacetoxy-30-nor-(D:A)-friedoolean-20(29)-en-27-oic acid (5a): White powder (from Me_2CO); $[\alpha]_D^{23}$ + 18.4 (*c* 0.10, $CHCl_3$); **^{1}H** (400 MHz, $CDCl_3$-CD_3OD) and **^{13}C** (100 MHz, $CDCl_3$-CD_3OD) NMR data, see Table 1 and Table 2 respectively; positive **ESIMS** m/z (rel. int.) 565 $[M + Na]^+$ (50), 483 $[M - OAc]^+$ (100), 423 $[M - H - 2OAc]^+$ (12); **HRESIMS** m/z 565.3503 $[M + Na]^+$ (calcd for $C_{33}H_{50}O_6Na$, 565.3505).

2.4. Chromatography Methods

Column chromatography was performed on silica gel G (100 - 200 and 300 - 400 mesh, Qingdao Haiyang Chemical Co., Ltd., People's Republic of China) and Sephadex LH-20 (40 - 70 μm, Amersham Pharmacia Biotech AB, Sweden). TLC was carried out on precoated silica gel plates (Qingdao Haiyang Chemical Co.), and spots were visualized by heating the plates at 90˚C after they were dipped into a 10% ethanolic H_2SO_4 solution. Solvents were distilled prior to use.



Scheme 2. Semisynthesis of **1c** and **1d** from glaucartanoic acid A (**1**).



Scheme 3. Semisynthesis of **5a** from 3β,21β-dihydroxy-30-nor-(D:A)-friedoolean-20 (29)-en-27-oic acid (**5**).

2.5. Analytical Analyses

Optical rotations were measured with a JASCO P-1020 digital polarimeter. The NMR spectra were recorded on Bruker AV-400 or DRX-500 NMR spectrometers. Chemical shifts (δ) are expressed in ppm with reference to TMS, and coupling constants (*J*) are given in Hz. ESIMS and HRESIMS were carried out on an API Qstar time-of-flight spectrometer.

2.6. Antimicrobial Assay

2.6.1. Microorganisms and Growth Conditions

The studied microorganisms consisted of sensitive and multidrug resistant Gram-positive bacteria (*Staphylococcus aureus* ATCC25923, *methicillin sensitive S. aureus* MSSA1, *methicillin resistant S. aureus* MRSA3, *methicillin resistant S. aureus* MRSA4), Gram-negative bacteria (*Shigella flexneri* SDINT, *Pseudomonas aeruginosa* ATCC27853) and three strains of yeasts (*Candida tropicalis* PK233, *Candida albicans* ATCC10231 and *Cryptococcus neoformans* H99) taken from our laboratory collection. The bacterial and fungal species were grown at 37˚C and maintained on nutrient agar (NA, Conda, Madrid, Spain) and Sabouraud Dextrose Agar (SDA, Conda) slants respectively.

2.6.2. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Microbicidal Concentration (MMC)

INT colorimetric assay [23] was performed to assess the minimal inhibitory concentrations (MICs) of crude extract and compounds against a panel of yeasts, Gram-negative and Gram-positive bacteria. Briefly, test samples were first dissolved in dimethyl sulfoxide (DMSO). The solution obtained was then added to

Mueller Hinton Broth (MHB) for bacteria or Sabouraud Dextrose Broth (SDB) for yeasts and serially diluted twofold (in a 96-well microplate). One hundred microlitres (100 µL) of inoculum (1.5×10^6 CFU/mL for bacteria and 10^5 spores/mL for yeasts) prepared in MHB/SDB was added. The plates were covered and agitated to mix the contents of the wells using a plate shaker and incubated at 35˚C for 24 h (for bacteria) or for 48 h (for yeasts). The final concentration of DMSO was 1% and does not affect the microbial growth. Wells containing MHB/SDB, 100 µL of inoculum, and DMSO at a final concentration of 1% served as a negative control. Ciprofloxacin (Sigma-Aldrich, Steinheim, Germany) and nystatin (Merck, Darmstadt, Germany) were used as reference drugs for bacteria and yeasts respectively. The MIC values of samples were determined by adding 40 µL of a 0.2 mg/mL *p*-iodonitrotetrazolium violet solution followed by incubation at 35˚C for 30 min. Viable microorganisms reduced the colourless dye to pink. MIC was defined as the lowest sample concentrations that prevented this change and exhibited complete inhibition of microbial growth. All assays were performed in triplicate and repeated thrice. For the determination of MMC values, a portion of liquid (5 µL) from each well that showed no growth of microorganism was plated on Mueller Hinton Agar or SDA and incubated at 35˚C for 24 h (for bacteria) or 35˚C for 48 h (for yeasts). The lowest concentrations that yielded no growth after these subcultures were taken as the MMC values.

3. Results and Discussion

3.1. Chemical Analysis

The MeOH extract from the leaves of *C. glauca* was subjected to column chromatography (CC) over silica gel to afford nine known compounds including glaucartanoic acid A (**1**: $C_{30}H_{48}O_5$; m/z 488) [11], caloncobalactone B (**2**: $C_{30}H_{48}O_6$; m/z 504) [12], glaucalactone (**3**: $C_{29}H_{44}O_4$; m/z 456) [12], glaucartanoic acid B (**4**: $C_{30}H_{46}O_5$; m/z 486) [11], 3β,21β-dihydroxy-30-nor-(D:A)-friedoolean-20(29)-en-27-oic acid (**5**: $C_{29}H_{46}O_4$; m/z 458) [8], caloncobic acid A (**6**: $C_{30}H_{46}O_4$; m/z 470) [12], caloncobic acid C (**7**: $C_{30}H_{48}O_6$; m/z 504) [13], caloncobalactone C (**8**: $C_{33}H_{52}O_5$; m/z 528) [13] and glaucalactone B (**9**: $C_{29}H_{46}O_4$; m/z 458) [13]. Chemical derivatizations including acetylation and methylation of compound **1** and acetylation of compound **5** respectively, afforded five previously unreported semisynthetic derivatives (**1a-1b** and **5a**). The structures of these compounds (**Figure 1**) were elucidated by the means of 1D and 2D NMR spectroscopy, MS and comparison of these data with those reported in the literature.

Compound **1a**, obtained from the acetylation reaction of **1** (**Scheme 1**), was isolated as a white powder in acetone. The molecular formula $C_{34}H_{52}O_7$ was deduced from its HR-ESI-MS which displayed the pseudo-molecular ion peak at m/z 595.3612 $[M + Na]^+$. This mass was 84 mass units higher than that of **1**, indicating the addition of two acetyl units as compared to **1**. The positive ion mode ESI-MS of **1a** also confirmed the presence of two acetoxy groups in the molecule by displaying the ion peaks at m/z 513 $[M - OAc]^+$ and 453 $[M - H - 2\ OAc]^+$.

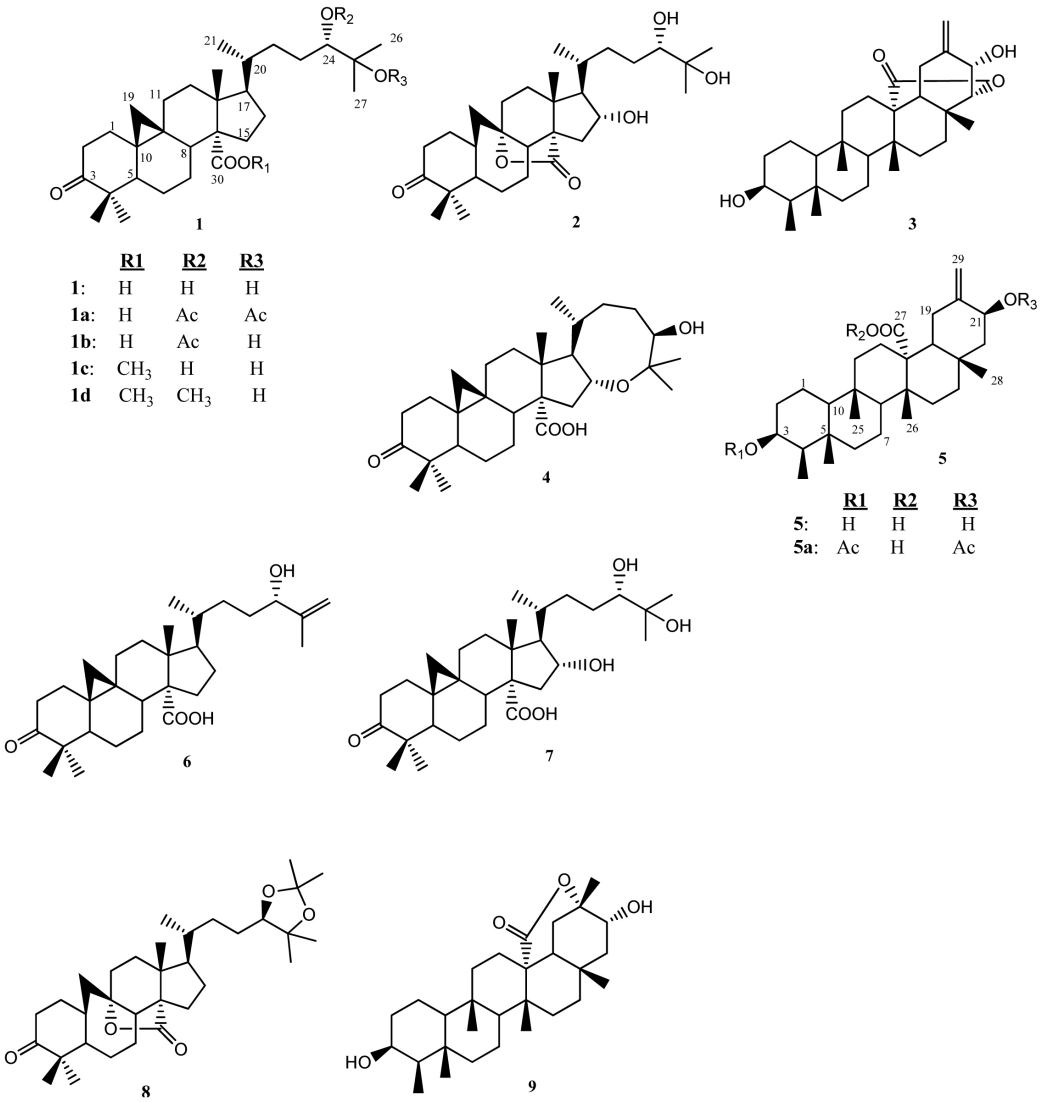


Figure 1. Structures of isolated compounds (**1 - 9**) from the leaves of *Caloncoba glauca* and semisynthetic derivatives (**1a - 1d** and **5a**).

The NMR data of **1a** revealed additional signals compared to those of glaucartanoic acid A (**1**) at δ_H 2.08 and 1.94 (each 3H, s) in the ^{1}H-NMR spectrum (Table 1) and at δ_C 172.5, 172.1, 22.2 and 20.9 in the ^{13}C-NMR spectrum (Table 2) assignable to two acetoxy groups. The downfield shift of the H-24 proton signal from δ_H 3.21 (in compound **1**) to δ_H 5.27 (in **1a**) and of C-25 carbon signal from δ_C 73.0 (in compound **1**) to δ_C 84.2 (in **1a**) indicated the locations of the acetoxy groups at C-24 and C-25 respectively. Compound **1a** was thus elucidated as 24(*S*), 25-diacetoxy-3-oxocycloartan-30-oic acid and trivially named diacetylglaucartanoic acid A.

Compound **1b**, obtained from the acetylation reaction of **1** (Scheme 1), was isolated as a white powder in acetone. Its molecular formula was determined as $C_{32}H_{50}O_6$ from the pseudo-molecular ion peak observed in its HR-ESI-MS at m/z 553.3505 $[M + Na]^+$. This mass was 42 mass units higher than that of **1**, corres-

ponding to the addition of one acetyl unit. The presence of a remaining free hydroxyl group in **1b** was further confirmed by the base peak observed at m/z 513 $[M - H_2O + H]^+$ in its positive ion mode ESI-MS. Its ^1H and ^{13}C NMR data (Table 1 and Table 2) compared to those of compound **1** revealed additional signals at δ_H 2.08 (3H, s) and δ_C 172.9 and 21.1 assignable to the acetoxy group. The downfield shift of the H-24 proton signal from δ_H 3.21 (in **1**) to δ_H 4.78 (in **1b**) together with the deshielding of the C-24 carbon signal at δ_C 80.8 (instead of δ_C 78.6 in **1**) indicated the location of the acetoxy group at C-24. This was further confirmed by the HMBC correlation of H-24 with the carbonyl carbon at δ_C 172.9. Compound **1b** was thus elucidated as a new semi-synthetic derivative named 24-acetylglaucartanoic acid A.

Compound **1c** was obtained from the methylation reaction of **1** (Scheme 2), and precipitated as a white powder in acetone. Its HR-ESI-MS displayed a pseudo-molecular ion peak at m/z 525.3573 $[M + Na]^+$ compatible with the molecular formula $C_{31}H_{50}O_5$, indicating an additional methyl group as compared to compound **1**. Indeed, the only difference between the NMR data of compound **1**c (Table 1 and Table 2) and those of compound **1** was the appearance of signals attributable to a methoxyl group in the ^1H and ^{13}C NMR spectra of **1c** at δ_H/δ_C 3.64/51.5. The cross-peak observed in its HMBC spectrum between the methoxyl protons and the carbonyl carbon at δ_C 178.4 (C-30) revealed the presence of a methyl ester group. Compound **1c** was thus elucidated as 24(*S*), 25-dihydroxy-3-oxocycloartan-30-carboxylic acid methyl ester and trivially named glaucartanoic acid A methyl ester.

Compound **1d**, obtained from the methylation reaction of **1** (Scheme 2), was isolated as a white powder in acetone. Its HR-ESI-MS displayed a pseudo-molecular ion peak at m/z 539.3820 $[M + Na]^+$ compatible with the molecular formula $C_{32}H_{52}O_5$, indicating one more methoxyl than compound **1c**. The NMR data of **1d** (Table 1 and Table 2) confirmed the presence of two methoxyl groups at δ_H/δ_C 3.64/51.5 and 3.49/61.4 respectively. Detailed comparison of its 1D NMR spectra with those of compound **1** revealed the shifting of signals at 24-position at δ_H/δ_C 2.88/90.7 instead of δ_H/δ_C 3.21/78.6 in **1** indicating the location of one of the methoxyl groups at C-24. This was confirmed by the HMBC spectrum where a long-range connectivity was observed between H-24 (δ_H 2.88) and the methoxyl carbon at δ_C 61.4 (24-OMe). The HMBC correlation also observed between the second methoxyl signal at δ_H 3.64 and the carbonyl carbon at δ_C 178.4 (C-30) proved the existence of a methyl ester functionality at C-30. Compound **1d** was thus characterized as 24(*S*)-methoxy-25-hydroxy-3-oxocycloartan-30-carboxylic acid methyl ester and was trivially named 24-methoxyglaucartanoic acid A methyl ester.

Compound **5a** was obtained from the acetylation reaction of **5** (Scheme 3), and precipitated as a white powder in acetone. Its molecular formula was established as $C_{33}H_{50}O_6$ from the HR-ESI-MS displaying the pseudo-molecular ion peak at m/z 565.3503 $[M + Na]^+$. This molecular formula in accordance with the pseudo-molecular mass furthermore 84 mass units higher than that of **5**, re-

vealed the addition of two acetyl units in the reaction product. The NMR data of **5a** (Table 1 and Table 2) exhibited additional signals of two methyl groups at δ_H/δ_C 2.02 (3H, s)/21.5 and 1.99 (3H, s)/21.8 and for two ester carbonyls at δ_C 172.4 and 171.8 as compared to **5**. Notable differences between the NMR data of compounds **5** and **5a** also involved the downfield shift of signals at C-3 position from δ_H/δ_C 3.65/72.7 in compound **5** to δ_H/δ_C 4.83/75.7 in compound **5a**, and at position C-21 from δ_H/δ_C 4.19/71.5 (in **5**) to δ_H/δ_C 5.30/74.9 (in **5a**). This implied the location of acetoxy groups at C-3 and C-21 positions in compound **5a** which was further elucidated as 3β,21β-diacetoxy-30-nor-(D:A)-friedoolean-20(29)-en-27-oic acid.

3.2. Antimicrobial Activity

The MeOH extract, isolated compounds and some new semisynthetic derivatives were evaluated for their antimicrobial activities against 9 microorganisms including four Gram-positive (*Staphylococcus aureus* ATCC25923, *methicillin sensitive S. aureus* MSSA1, *methicillin resistant S. aureus* MRSA3, *methicillin resistant S. aureus* MRSA4), two Gram-negative (*Shigella flexneri* SDINT, *Pseudomonas aeruginosa*) bacteria and three fungal strains (*Candida albicans* ATCC10231, *Candida tropicalis* PK233 and *Cryptococcus neoformans* H99) (Table 3). The MeOH extract displayed antimicrobial activity towards 9/9 (100%) of tested bacterial and fungal strains with good activity (MIC < 100 µg/mL) [24] against *Staphylococcus aureus* and *Shigella flexneri* SDINT ATCC25923. Among the phytochemicals, compounds **3** and **5** were the most active showing moderate activity (10 < MIC ≤ 100 µg/mL) [24] against 5/9 (55.55%) and 6/9 (66.66%) tested microbial species, respectively. As shown in Table 3, ciprofloxacin and nystatin used as standard drugs were more potent against yeasts, Gram-positive and Gram-negative bacteria than all the tested samples. A microbicidal effect with MMC/MIC ratio ≤ 4 was noted for most of the samples (MeOH extract, **2**, **3**, **5**, **7**, **8**, **1a**, **1b**, **1d** and **5a**) indicating their lethal effect. In general, Gram-negative bacteria were found to be more sensitive to the tested samples when compared with Gram-positive bacteria whereas the bacterial species were resistant as compared to fungal strains. These variations may be due to genetic differences between the microorganisms. The known antimicrobial mechanisms associated to the group of chemicals to which the isolated compounds belong may explain the antimicrobial potency of the MeOH extract. Membrane disruption has been suggested as one of the likely mechanisms of action [25] [26]. This might also explain the antimicrobial activities of isolated triterpenoids and semisynthetic derivatives [25] [26]. Although the fungal strains were more sensitive to the tested compounds than the bacteria strains used in this study, the antibacterial activities of the acetylated (**1a** and **1b**) and the methylated (**1d**) derivatives of cycloartane **1** were improved compared to that of the original substrate mostly on Gram-negative strains. Meanwhile the *O*-acetylation at both the C-3 and C-21 positions of friedelane **5** leading to compound **5a** induced a decrease in the antibacterial activity on both Gram-positive and Gram-negative strains, but an

Table 3. Antimicrobial activity (MIC and MMC in μg/mL) of MeOH extract, isolated compounds, semisynthetic derivatives and reference antimicrobial drugs.

		Bacteria						Yeasts		
Extract/Compounds	Inhibition Parameters	*S. aureus* ATCC	MSSA1	MRSA3	MRSA4	SF	*PA*	*CA* ATCC10231	*CT*PK233	*CN*H99
MeOH extract	MIC	64	128	128	128	64	128	2048	2048	1024
	MMC	64	128	256	256	64	128	4096	4096	2048
	MMC/MIC	1	1	2	2	1	1	2	2	2
1	MIC	>256	>256	>256	>256	>256	>256	64	128	32
	MMC	>256	>256	>256	>256	>256	>256	256	256	128
	MMC/MIC	/	/	/	/	/	/	4	2	4
2	MIC	256	>256	>256	>256	256	128	256	128	128
	MMC	256	>256	>256	>256	256	128	256	256	256
	MMC/MIC	1	/	/	/	1	1	1	2	2
3	MIC	32	64	256	256	32	32	256	128	64
	MMC	64	128	256	256	32	32	256	128	64
	MMC/MIC	2	2	1	1	1	1	1	1	1
4	MIC	>256	>256	>256	>256	>256	>256	128	128	64
	MMC	>256	>256	>256	>256	>256	>256	256	256	128
	MMC/MIC	/	/	/	/	/	/	2	2	2
5	MIC	32	64	64	64	32	32	>256	128	128
	MMC	32	64	64	64	32	32	>256	128	128
	MMC/MIC	1	1	1	1	1	1	/	1	1
6	MIC	256	>256	>256	>256	256	256	128	128	64
	MMC	>256	>256	>256	>256	256	256	256	256	256
	MMC/MIC	/	/	/	/	1	1	2	2	4
7	MIC	128	>256	>256	>256	64	64	256	128	64
	MMC	256	>256	>256	>256	128	128	256	128	128
	MMC/MIC	2	/	/	/	2	2	1	1	2
8	MIC	128	>256	>256	>256	128	64	128	128	64
	MMC	128	>256	>256	>256	256	128	256	256	128
	MMC/MIC	1	/	/	/	2	2	2	2	2
9	MIC	128	>256	>256	>256	64	64	256	128	32
	MMC	>256	>256	>256	>256	256	128	256	128	128
	MMC/MIC	/	/	/	/	4	2	1	1	4
1a	MIC	128	>256	>256	>256	128	64	256	32	32
	MMC	256	>256	>256	>256	256	256	256	64	64
	MMC/MIC	2	/	/	/	2	4	1	2	2

Continued

1b	MIC	128	>256	>256	256	64	64	64	128	128
	MMC	128	>256	>256	256	64	64	256	256	128
	MMC/MIC	1	/	/	1	1	1	4	2	1
1d	MIC	128	>256	>256	>256	128	128	256	256	64
	MMC	256	>256	>256	>256	256	128	256	256	128
	MMC/MIC	2	/	/	/	2	1	1	1	2
5a	MIC	128	256	256	>256	128	64	256	128	64
	MMC	256	256	256	>256	128	128	256	256	128
	MMC/MIC	2	1	1	/	1	2	1	2	2
Ref*	MIC	2	1	1	2	16	2	2	0.5	1
	MMC	2	1	1	2	16	2	2	1	1
	MMC/MIC	1	1	1	1	1	1	1	2	1

SA ATCC25923: *Staphylococcus aureus* ATCC25923; MSSA1: *methicillin sensitive S. aureus* MSSA1; MRSA3: *methicillin resistant S. aureus* MRSA3; MRSA4: *methicillin resistant S. aureus* MRSA4; SF: *Shigella flexneri*; PA: *Pseudomonas aeruginosa*; *CA* ATCC10231: *Candida albicans* ATCC10231; *CT* PK233: *Candida tropicalis* PK233; *CN* H99: *Cryptococcus neoformans* H99; MIC: minimum inhibitory concentration; MMC: Minimum microbicidal concentration; /: not determined; *: Ciprofloxacin and nystatin were used as reference drugs for bacteria and yeasts respectively.

increase in the antifungal activity. The lower antibacterial activity mainly on MRSA3 and MRSA4 strains observed for compound **3** with respect to its analogue **5** could arise from lactonization of the C-27 carboxylic group in compound **3**. The difference in the antimicrobial activity of compounds having the same basic skeleton highlights the contribution of esterified carboxylic acid group and acetyl substituents in influencing the activity of this series of compounds.

4. Conclusion

Phytochemical investigation of the MeOH extract from the leaves of *C. glauca* led to the isolation of nine known triterpenoids (**1-9**). Chemical transformations carried out on glaucartanoic acid A (**1**) and 3β,21β-dihydroxy-30-nor-(D:A)-friedoolean-20(29)-en-27-oic acid (**5**) respectively, afforded five new derivatives (**1a**-**1d** and **5a**). The MeOH extract displayed antimicrobial activity towards all the tested pathogenic bacterial and fungal strains with good activity (MIC < 100 µg/mL) against *Staphylococcus aureus* ATCC25923 and *Shigella flexneri* SDINT. Compounds **3** and **5** belonging to the friedelane type showed the most potent antimicrobial effect. These results could justify the traditional use of *C. glauca* in the treatment of skin diseases caused by some of the tested microorganisms.

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Conflicts of Interest

The authors declare they have no competing interest.

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Supplementary Material

Supporting information for this article has been uploaded as the electronic supplementary material.