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Antibacterial triterpenes from *Syzygium guineense* (Myrtaceae)

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Abstract

Antibacterial bioassay-guided fractionation of *Syzygium guineense* leaf extracts afforded 10 triterpenes, namely betulinic acid **1**, oleanolic acid **2**, a mixture of 2-hydroxyoleanolic acid **3a**, 2-hydroxyursolic acid **3b**, arjunolic acid **4a**, asiatic acid **4b**, a mixture of terminolic acid **5a**, 6-hydroxyasiatic acid **5b**, and a mixture of arjunolic acid 28-β-glucopyranosyl ester **6a** and the asiatic acid 28-β-glucopyranosyl ester **6b**. Isolated compounds were submitted to an antibacterial assay system against gram-positive and -negative bacteria and human pathogen bacteria. Compounds **4a** and **4b** showed the most significant antibacterial activity against *Escherichia coli*, *Bacillus subtilis* and *Shigella sonnei*. The fraction **5a**–**5b** was the least active, whereas compounds **1**, **2** and the mixtures of **3a**–**3b** and **6a**–**6b** were inactive in the assays.

Keywords: *Syzygium guineense*; Triterpenes; Antibacterial activity

1. Introduction

Syzygium guineense (Myrtaceae) is a small tree with edible fruits (Ambé, 2001). It is widespread in Subsaharan Africa (Uganda, Swaziland and Cameroon) where the bark is traditionally used to treat stomachache and diarrhea (Tsakala et al., 1996; Hamil et al., 2000; Oluwole et al., 2002). Previous work on this species reported the antibacterial activity of hydrosoluble dry extracts (Tsakala et al., 1996), but until today no phytochemical studies has been carried out to identify the active metabolites. In our systematic search for new and/or bioactive metabolites from plants, we investigated the methanolic extract from the leaves of *Syzygium guineense*. In this paper, we report the antibacterial bioassay-guided identification and characterisation from the leaves of *Syzygium guineense* of 10 triterpenes derivatives, namely, betulinic acid **1**, oleanolic acid **2**, a mixture of 2-hydroxyoleanolic acid **3a** and 2-hydroxyursolic acid **3b**, arjunolic acid **4a**, asiatic acid **4b**, a mixture of terminolic acid **5a** and 6-hydroxyasiatic acid **5b**, and a mixture of arjunolic acid 28-β-glucopyranosyl ester **6a** and asiatic acid 28-β-glucopyranosyl ester **6b**. Some structure–activity relationships are also discussed.

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2. Materials and methods

2.1. Plant material

Leaves of *Syzygium guineense* were collected in August 2002 in Fongo-Tongo, Menoua subdivision, western Cameroon. The plant material was identified by Dr. Satabier, former director of the National Herbarium of Cameroon. Voucher specimens have been deposited in the Cameroon National Herbarium.

2.2. Identification of pure compounds

Structures of isolated compounds were elucidated by spectroscopic methods using an Agilent 1100 ESI/LCMSD/Trap

for mass spectra measurement. ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), DEPTs and 2D NMR spectra (COSY, NOESY, HMQC, and HMBC) were recorded in CD_3OD on a Bruker AVANCE 400 spectrometer.

2.3. Extraction and isolation

The dried and finely ground leaves of *Syzygium guineense* (1.5 kg) were extracted at room temperature with dichloromethane (CH_2Cl_2)/methanol (MeOH) (1/1 v/v) for one day and concentrated under vacuum to afford 180 g of crude extract. Extract (100 g) was dissolved in water and successively extracted with hexane, ethyl acetate (EtOAc) and *n*-butanol to yield, respectively, 20, 45, and 18 g. The resultant extracts were tested for antibacterial activity via the disc diffusion method (Hadacek and Greger, 2000) against *Escherichia coli* and *Bacillus subtilis*.

The active extracts, ethyl acetate and *n*-butanol, were fractionated by column chromatography over silica gel using a gradient of CH_2Cl_2–MeOH as solvents. All fractions were tested for antimicrobial activity by direct bioautography on TLC plates (Hadacek and Greger, 2000) against *Escherichia coli* and *Bacillus subtilis*. Ethyl acetate extract (40 g), the main active fraction, was subjected to a column chromatography (75×5.2) filled with silica gel (63–200, 60 Å) and eluted with a gradient of MeOH in CH_2Cl_2. Sixty-eight fractions of 200 ml were collected and regrouped on the basic of analytical TLC in three fractions. The main active fraction 12 g was subjected to repeated column chromatography (60 cm × 3 cm) filled with silica gel (32–63, 60 Å) eluted with a gradient of MeOH in $CHCl_3$ to yield compound **1** (50 mg, 1.25×10^{-3}%), **2** (70 mg, 1.75×10^{-3}%), **3** (60 mg, 1.5×10^{-3}%) a mixture of two isomers, and **4** (105 mg, 2.62×10^{-3}%). Fraction **4** (100 mg), which was a mixture of two compounds, was separated by the method of Lewis (Lewis and Tucker, 1983), followed by a silica gel column (50 cm × 1.5 cm) using isocratic system $CHCl_3$–MeOH (95–5) to afford **4a** (10 mg, 0.1%) and **4b** (2 mg, 0.02%). Butanolic extract (15 g) was subjected to silica gel column chromatography (60 cm × 3 cm) eluted with a gradient of MeOH in EtOAc. Forty fractions of 200 ml was collected and regrouped in three fractions also on the basic of analytical TLC. The active fraction was purified on silica gel column chromatography using a gradient of MeOH in $CHCl_3$ as eluent to afford **5** (10 mg, 0.66×10^{-3}%) and **6** (12 mg, 0.8×10^{-3}%). Each fraction was a mixture of two isomers. Further purification of these compounds was not achieved because of lack of quantity. The Rf values of compounds **1**–**6** in $CHCl_3$–MeOH (9:1) are 0.59, 0.54, 0.47, 0.35, 0.23, and 0.05, respectively.

2.4. Microorganisms

Three bacteria were used in the bioguided assay. *Escherichia coli* (NEU 1006) and *Bacillus subtilis* (NEU 1) obtained from culture collection at the Institute of Microbiology (Neuchâtel), and *Shigella sonnei* (COP/2004/4212) from the Institut Neuchâtelois de Microbiologie (La Chaux-De-Fonds, Switzerland). Only pure compounds showing activity against *Escherichia coli* and *Bacillus subtilis* were tested against *Shigella sonnei* at the Institut Neuchâtelois de Microbiologie.

2.5. Antibacterial assays

2.5.1. Disk diffusion

The crude extract of *Syzygium guineense* (1 mg) was tested at five different concentrations ranging from 0.0625 to 1 mg/ml in acetone and 40 μl of the solution were applied to 8 mm diameter paper disks. After evaporation of the solvent, two paper disks were placed in Petri dishes of 9 cm diameter containing nutrient agar previously inoculated with 0.2 ml of suspension of bacteria (10^8–10^9 CFU/ml). After 2 days of incubation at 30 °C for *Bacillus subtilis* and 37 °C for *Escherichia coli*, the inhibition zone for the active extract was measured (Hadacek and Greger, 2000).

2.5.2. Bioautography on thin-layer plates

Different concentrations of test compounds were prepared by the method of two-fold serial dilution. Test solutions (10 μl) were applied as small spots on TLC plates (Silica gel G, 20 × 20, 500 μm, Analtech) to give a concentration series of 0.1–30 μg/application zone. The organic solvent was evaporated by steam air and plates were eluted by the adequate solvent to obtain different Rf. The solvent was one more time evaporated by steam air. TLC plates were homogenously sprayed with 10 ml of nutrient agar infected by 1 ml of nutrient broth containing bacteria (10^8–10^9 CFU/ml). Plates were incubated for 2 days at the corresponding temperature of each bacterium in the dark. The appearance of blank after spraying the plate with a solution of thiazolyl blue tetrazolium bromide indicated antibacterial activity (Hadacek and Greger, 2000). Minimum inhibitory concentrations (MIC) against *Escherichia coli*, *Bacillus subtilis*, and *Shigella sonnei* were determined from the lowest test compound concentration causing recognisable bacterial growth inhibition on nutrient agar. In these two methods of assays, chloramphenicol (Sigma) was used as a positive control and acetone as a negative control.

3. Results

The ethyl acetate and *n*-butanol extracts from leaves of *S. guineese* which exhibited antibacterial activity were subjected to chromatographic isolation and purification to yield 10 triterpenes. From the ethyl acetate extract alone, six compounds were identified. Negative ion ESI/MS combined to 1D NMR, ^{1}H, ^{13}C NMR and 2D NMR, COSY, HMQC, and HMBC identified the compounds as betulinic acid **1** and oleanolic acid **2** with *m*/*z* 455 $(M–H)^-$ and the molecular formula of $C_{30}H_{48}O_3$ (Shashi and Asish, 1994), a mixture

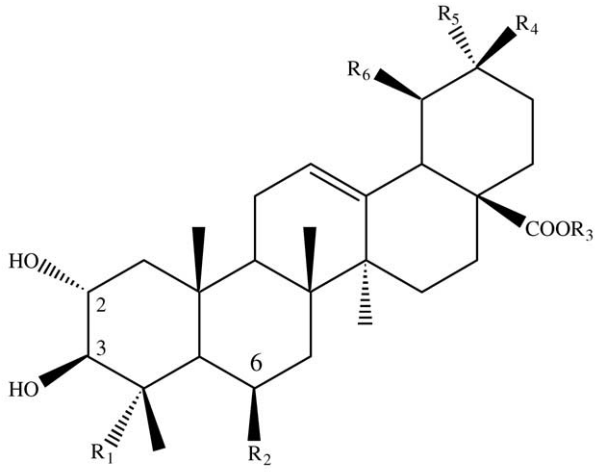
Table 1
MICs of compounds isolated from *Syzygium guineense* against gram-positive and -negative bacteria in (μg/spot)

Compound	*Escherichia coli*	*Bacillus subtilis*	*Shigella sonnei*
1	NA	NA	NT
2	NA	NA	NT
3a–3b	NA	NA	NT
4a	3	0.5	30
4b	5	0.75	30
5a–5b	6	3	50
6a–6b	NA	NA	NT
chloramphenicol	0.3	0.1	2

NA: no activity observed; NT: not tested.

of 2-hydroxylursolic acid **3a** and 2-hydroxyloleanolic acid **3b** with *m/z* 471 $(M–H)^-$ corresponding to $C_{30}H_{48}O_4$ (Chandan, 1990) and a mixture of isomers in fraction **4** with *m/z* 487 $(M–H)^-$relating to $C_{30}H_{48}O_5$. All fractions were submitted to the antibacterial assays and further purification was carried out on the active fractions. Only fraction **4** exhibits a significant antibacterial activity against *Escherichia coli* and *Bacillus subtilis* by thin-layer bioautography (Table 1). Compounds **4a** and **4b** precipitate as a white powder in $CHCl_3$:MeOH (9:1). ^1H NMR data were similar, with the main differences noted in the DEPT experiment, were **4a** and **4b** possessed ten and nine CH_2, six and eight CH, respectively, with the number of methyl groups for each compound being seven. The ^{13}C NMR spectra showed 30 carbon atoms for each. The chemical shift of the carbon atoms C_{12} and C_{13} at δ 122.4; 144.4 and δ 125.7; 138.8 for **4a** and **4b**, respectively, suggested the presence of two classes of triterpenes, the oleanane and ursane. Derivatisation of 100 mg of fraction **4** according to Lewis method (Lewis and Tucker, 1983) lead to 10 mg of arjunolic acid **4a** and 2 mg of asiatic acid **4b** (Tsutomu et al., 1987; Collins et al., 1992). For these compounds, the data obtained correlated very well with the literature.

The bioassay-guided fractionation of the *n*-butanol extract by column chromatography lead to fractions **5** with *m/z* 503 $(M–H)^-$ $C_{30}H_{48}O_6$ and *m/z* 649 $(M–H)^-C_{36}H_{58}O_{10}$. Each fraction was a mixture of two inseparable compounds. Fraction **5** was obtained as a white powder in $CHCl_3$:MeOH (8:2). The main differences compared to **4a** and **4b** being related to the nature of the R_2. The presence of one C_6H (δ_C 68.7, δ_H 5.11) in 5 and the absence of C_6H_2 (δ_C 18, δ_H 1.41) in the ^1H, ^{13}C and DEPT spectra led to the identification of **5a** as terminolic acid and **5b** as 6-hydroxy asiatic acid in the ration 3–2. While the constituents of fraction **6** were identified as arjunolic acid 28-β-glucopyranosyl ester **6a** (Adnyana et al., 2000) and a homologue to asiatic acid 28-β-glucopyranosyl ester **6b** (Tsutomu et al., 1987) following the ratio 1/1. The



Compound	R_1	R_2	R_3	R_4	R_5	R_6
3a 2-hydroxyoleanolic acid	CH_3	H	H	CH_3	CH_3	H
3b 2-hydroxyursolic acid	CH_3	H	H	H	CH_3	CH_3
4a arjulonic acid	CH_2OH	H	H	CH_3	CH_3	H
4b asiatic acid	CH_2OH	H	H	CH_3	H	CH_3
5a terminolic acid	CH_2OH	OH	H	CH_3	CH_3	H
5b 6-hydroxyasiatic acid	CH_2OH	OH	H	CH_3	H	CH_3
6a arjunolic acid 28- β-glucopyranosyl ester	CH_2OH	H	Glc	CH_3	CH_3	H
6b asiatic acid 28-β-glucopyranosyl ester	CH_2OH	H	Glc	CH_3	H	CH_3

Fig. 1. Chemical structures of isolated triterpenes.

^{1}H NMR spectrum of **6b** showed one anomeric proton signal at δ_H 5.37 (H-1′, $^3J_{1',2'}$ = 8.1 Hz) and carbon atom (δ_C 95.7) indicating the presence of one monosaccharide bonded as a glucosyl ester. The sugar moiety was identified as 28-β-glucopyranosyl ester based on the coupling constants of each proton and the ^{13}C NMR chemical shifts (δ_C 62.2, 71.2, 74.3, 78.8, 79.4, 95.7). The glucosidation of position C_{28} was indicated by the long-range correlation between the anomeric proton H-1′ and the carboxyl carbon (C-28, δ_C 178.06) in the HMBC spectrum. The ^{1}H and ^{13}C NMR data of the aglycone moiety were similar to those recorded for arjulonic acid **4a** and asiatic acid **4b**. Asiatic acid and asiaticoside are well known. This is the first time that compound **6b** (Fig. 1) is identified where only one glucose is found to be attached to the carboxylic acid group. While minimal antibacterial activity was noted in fraction **5** and no activity recorded in fraction **6**, no further purification was carried out due to lack of sufficient material.

4. Discussion and conclusions

Isolated compounds showed different activities according to the position of the different substituent groups. It appears from the biological assay results that the hydroxyl group at the position C_{23} makes an important contribution to the expression of activity in **4a** and **4b**. The presence of one hydroxyl group at the position C_6 reduces considerably the activity of metabolites **5a**–**5b**. Furthermore, when the proton of the acid function is substituted by a glucopyranosyl ester function or when the hydroxyl group at position C_{23} is absent, the metabolites loss all their activities.

Metabolites **4a** and **4b** showed significant antibacterial activity against *Escherichia coli*, *Bacillus subtilis* and interestingly against *Shigella sonnei* with MICs of 3, 0.5, and 30 μg for **4a** and 5, 0.75, and 30 μg for **4b**. The mixture **5a** and **5b** were less active with MICs of 6, 3, and 50 μg, whereas no activity was recorded on metabolites **1**, **2**, the mixture of **3a**–**3b**, and **6a**–**6b** on *Escherichia coli* and *Bacillus subtilis* (Table 1).

Asiatic acid found in *Centella asiatica* (Niranjan et al., 1989) has been traditionally used as a tonic in skin diseases and leprosy (Shukla et al., 1999) whereas arjunolic acid has been isolated from *Syzygium samarangense* (Srivastava et al., 1995) and *Syzygium cordatum* (Candy et al., 1968). Arjunolic and asiatic acid isolated from *Syzygium claviflorum* were identified as anti-HIV agents with IC_{50} of 36 and 8 μg/ml (Yoshiki et al., 1998), but this is the first time that their antibacterial activity specially on human pathogen bacteria is reported. Terminolic acid **5a** and 6-hydroxy asiatic acid **5b** are identified for the first time from the *Syzygium* genus. This might explain the use of this plant in folk medicine for treatment of various infectious diseases. Further investigations will focus on the in vivo antimicrobial activities.

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