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Original Research Article

Isolation and evaluation of the antibacterial constituents of *Synsepalum brevipes*

Fomene Tsapy Raphaelle[1], Ache Roland Ndifor[2,3]*, Ngnintedo Dominique[1], Ambassa Pantaléon[1], Céline Henoumont[4], Njinga Ngaitad Stanislaus[5], Sophie Laurent[4,6], Fotso Wabo Ghislain[1], Ngameni Bathelemy[7], Bonaventure Tchaleu Ngadjui[1,8]

[1]Department of Organic Chemistry, Faculty of Science, University of Yaoundé 1, Yaoundé, Cameroon, [2]Higher Technical Teachers Training College, University of Bamenda, Cameroon, [3]Department of Chemistry, Faculty of Science, University of Bamenda, Cameroon, [4]General, Organic and Biomedical Chemistry, NMR and Molecular Imaging Laboratory, University of Mons, Place du Parc 20, 7000 Mons, Belgium, [5]Department of Pharmaceutical and Medicinal Chemistry, University of Ilorin, Nigeria, [6]Center for Microscopy and Molecular Imaging (CMMI), Rue Adrienne Bolland 8, 6041 Gosseilies, Belgium, [7]Department of Pharmacognosy and Pharmaceutical Chemistry, Faculty of Medicine and Biomedical Sciences, University of Yaoundé, Cameroon, [8]University Institute of Sciences, Technologies and Ethics Yaoundé, Cameroon

For correspondence:* ***Email:* *rolyndifor@gmail.com;* ***Tel:*** *+237-676 444 329*

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Abstract

Purpose: *To isolate and evaluate the antibacterial constituents of Synsepalum brevipes.*
Methods: *The compounds were isolated using varying chromatographic separation methods. The structures were established using 1D and 2D nuclear magnetic resonance (NMR) spectroscopic methods, mass spectroscopy (MS), and by comparison with existing data. Extracts, fractions, and selected compounds were assessed against six bacterial strains using the broth microdilution method.*
Results: *Chemical constituents isolated were identified as taraxeryl acetate, taraxerol, ursolic acid, oleanolic acid, betulinic acid, lupeol, herannone, spinasterol, spinasterol-3-O-β-D-glucopyranoside, eicosanoic acid, asperphernamate, hyperoside, spinasterone, arganin C, and butyroside B. The crude extracts and fractions were inactive against all the bacterial strains. The three compounds, which include oleanolic acid, ursolic acid, and hyperoside, exhibited moderate inhibitory activity with minimum inhibitory concentration (MIC) ranging from 12.5 to 50 µg/mL against the tested bacteria. Oleanolic acid revealed moderate bactericidal effects on Escherichia coli, and methicillin-resistant Staphylococcus (minimum bactericidal concentration (MBC) = 100 µg/mL, while ursolic acid showed moderate bactericidal effects on methicillin-resistant Staphylococcus aureus (MBC = 50 µg/mL).*
Conclusion: *These results show that two triterpenoids and one phenolic exhibit moderate antibacterial effects against Escherichia coli, methicillin-resistant Staphylococcus, and Staphylococcus aureus.*

Keywords: *Synsepalum brevipes, Phytochemicals, Antibacterial activity, Sapotaceae*

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INTRODUCTION

Pathogenic bacteria and the diseases they cause remain a major health challenge, contributing significantly to morbidity and mortality. The emergence of antibiotic-resistant strains has further complicated treatment efforts, leading to frequent therapeutic failures [1]. Notwithstanding,

natural products, especially those derived from medicinal plants, are increasingly explored as alternative solutions to combat multidrug resistance [2]. Many metabolites from plants, such as alkaloids, flavonoids, isoflavonoids, phenolics, and triterpenoids, have demonstrated broad-spectrum antimicrobial activity [3]. Furthermore, extracts from various plants tested on many bacterial strains *in vitro* exhibited extensive activities against bacterial pathogens [4].

In the family Sapotaceae, extracts of *Tieghemella heckelii* were reported to demonstrate significant potency against methicillin-resistant *Staphylococcus aureus* [5]. The extracts of the fruits of *Synsepalum dulcificum* demonstrated antibacterial activity against different pathogenic bacteria with minimum inhibitory concentration (MIC) ranging from 0.156 to 2.500 mg/mL [6].

Synsepalum brevipes (Baker) T. D. Penn, a plant widely distributed in West, Central, and East Africa, is traditionally used to treat ailments such as coughs, stomach disorders, malaria, oedema, hernia, jaundice, vermifuges, liver problems, and nausea many of which may be linked to bacterial infections [7]. However, the antibacterial potential of its isolated compounds and extracts against resistant microbial strains remains largely unexplored. This study, therefore, was aimed at isolating and evaluating the antibacterial constituents of *Synsepalum brevipes* against selected drug-resistant pathogenic bacteria.

EXPERIMENTAL

Plant material

The leaves, stem bark, and roots of *Synsepalum brevipes* were collected from Bambui in Tubah subdivision and Mount Kala, Yaoundé, Cameroon, in November 2016. Identification was done by Mr. Eric Tchatchonang, a botanist at the Cameroon National Herbarium, Yaoundé. The specimen matched the herbarium specimen of Letouzey (reference no. 4891/SFRK) of the Cameroon National Herbarium.

Equipment and apparatus

All reagents were purchased from Merck, Darmstadt, Germany. Thin layer chromatography (TLC) was conducted on silica gel 60 F254. The spots were detected using fluorescence 254 nm or 366 nm on a UV-85/L basis, followed by spraying with 15 % dilute sulfuric acid (H_2SO_4) and heating at 75 °C. Chromatographic separations were done on Sephadex LH_{20}, C18 reversed-phase columns, and silica gel (063 nm; 230 - 400 mesh, ASTM; Merck, Germany). Nuclear magnetic resonance (NMR) spectra were recorded on an AVANCE-NEO Bruker spectrometer equipped with a magnetic field of 14T operating at a proton frequency of 600 MHz. Tetramethylsilane (TMS) was used as internal standard. The mass spectra were registered on a Waters ZQ-200 in electrospray ionization mode. A Quadruple Time of flight compact spectrometer equipped with an electrospray ionization (ESI) source was used to record the high-resolution mass.

Extraction and isolation of compounds

The extracts were prepared by maceration at room temperature. The powdered leaves (1.986 kg), stem bark (1.150 kg), and roots (2.161 kg) were extracted twice with 10 L of 50 % methylene chloride/methanol (CH_2Cl_2-MeOH) at room temperature. The filtrates were concentrated under pressure using a rotary evaporator. The process was performed twice. The extracts were air-dried to remove the remaining solvent and weighed to yield 327 g of black paste from the leaves, 153 g of red paste from the stem bark, and 143 g of brown paste from the roots. Thereafter, four (4 g) of each crude extract was kept at 4 ^oC until further investigation of bioassay. The stem bark extract (145 g) was suspended in 200 mL distilled water (H_2O) and partition sequentially with n-Hexane (n-Hex), methylene chloride (CH_2Cl_2), and n-butanol (n-BuOH) to obtain fractions of n-Hexane (26 g), CH_2Cl_2 (22 g), and n-BuOH (12 g), respectively. Of these, eleven grams (11g) of the CH_2Cl_2 fraction was removed, fixed on coarse silica, subjected to silica gel column chromatography, and eluted with different polarities of n-Hex, ethyl acetate (EtOAc), and MeOH to yield 180 subfractions. Identical subfractions were assembled according to thin layer chromatography (TLC) profiles. Compound **1** (4.5 mg) precipitated from subfraction SF23-28 (Hex-EtOAc 5 %), **2** (6.0 mg) from SF42-48 (Hex-EtOAc 20 %) and **11** (3.6 mg) from SF82-86 (Hex-EtOAc 70 %).

A total of 300 g of *Synsepalum brevipes* leaf extract was subjected to solid-liquid partitioning using n-Hex, EtOAc, and n-BuOH, with 600 mL of each solvent applied thrice. After concentration, the respective yields were: n-Hex (40 g, black syrup), EtOAc (40 g, brown syrup), and n-BuOH (107 g, red syrup). The ethyl acetate fraction (36 g) was fixed onto silica gel and chromatographed with n-Hex, EtOAc, and MeOH, under gradient conditions to obtain 164 subfractions. Thin layer chromatography (TLC)

guided pooling and purification using solvent systems of varying polarity yielded compounds **8** (15.5 mg, SF33-36, n-Hex-EtOAc 15 %), **3** (20 mg, SF53-55, n-Hex-EtOAc 25 %), **4** (18 mg, SF60-64, n-Hex-EtOAc 25 %), **5** (12.5 mg, SF65-69, n-Hex-EtOAc 30 %), **11** (6.0 mg, SF85-89, n-Hex-EtOAc 70 %), **9** (8 mg, SF126-129, n-Hex-EtOAc 85 %), and **12** (11.7 mg, SF146-147, (EtOAc-MeOH 5 %). Separately, 140 g of the root extract was subjected to liquid-liquid partitioning. After being suspended in 300 mL of water (H_2O), separation was done using n-Hex, EtOAc, and n-BuOH (3 x 300 mL each). Concentration yielded F_1 (yellow syrup, 10 g), F_2 (brown syrup, 10 g), and F_3 (red syrup, 23 g), respectively. Fraction F3 (10 g) was chromatographed utilising an isocratic system of EtOAc-MeOH-H_2O (10:4:1), yielding 22 subfractions (100 mL each). Subfraction SF12-14, selected via TLC, was further separated on Sephadex, LH_{20} using 100 % MeOH, yielding 30 subfractions (10 mL each). Subfraction SFsr10-11 was eluted on C18 reversed–phase silica with MeOH–H_2O (7:3), producing 37 subfractions (5 mL, each). Pooling SFsr 24-37 yielded 20 mg of brown solid (two spots), which was purified using EtOAc-MeOH-H_2O (10:4:1), obtaining compound **14** (5.0 mg, cream-white solid) and compound **15** (12.5 mg, cream-white solid). Fraction F_2 (9g) was fixed on coarse silica, subjected to separation on a silica column using various proportions of n-Hex, EtOAc, and MeOH to yield 122 subfractions. Regrouping of similar subfractions (SF) and washing of precipitates with MeOH, n-Hex, and acetone (CH_3COCH_3) obtained compounds **13** (SF21-22, 5.5 mg, n-Hex-EtOAc 7.5 %), **10** (5.0 mg, SF24-28, n-Hex-EtOAc 10 %), **6** (5 mg, SF53-54, n-Hex-EtOAc 20 %), **7** (4.5 mg, SF64-66, n-Hex-EtOAc 30 %) and **9** (12 mg, SF100-104, EtOAc-MeOH 5 %). All isolated compounds were distinguished using NMR and MS data in International System of Units (SI).

NMR data for some of the screened compounds

Ursolic acid (3)

White powder (20 mg), m.p. 285-286 ^oC**,** ^1H NMR(DMSO-d6, 500 MHz); 3.09 (1H, m, H-3), 0.72 (1H, s, H-5), 1.45 (2H, m, H-6α, H-6β), 1.32 (2H, m, H-7), 1.26 (1H, s, H-9), 1.86 (2H, m, H-11), 5.13 (1H, m, H-12), 1.45 (2H, m, H-15), 1.52 (1H, m, H-16), 2.13 (1H, d, *J* = 6.0 Hz, H-18), 1.29 (1H, m, H-19), 1.56 (1H, m, H-20), 1.26 (2H, m, H-21), 1.83 (2H, m, H-22), 0.96 (2H, m, H-23), 0.76 (3H, s, H-24), 0.63 (3H, s, H-25), 0.87 (3H, s, H-3), 1.10 (3H, s, H-26), 0.90 (3H, d, *J* = 6.0 Hz, Me-19β), 0.83 (3H, d, *J* = 6.0 Hz, Me-20α). ^{13}C NMR (DMSO-d6, 125MHz); δ: 38.4 (C-1), 23.7(C-2),76.8 (C-3), 38.3 (C-4), 52.3(C-5), 20.9 (C-6), 27.5 (C-7), 39.0 (C-8), 45.3 (C-9), 36.5 (C-10), 22.8 (C-11), 124.5 (C-12), 138.3 (C-13), 41.6 (C-14), 28.2 (C-15), 17.9 (C-16), 46.8 (C-17), 57.5 (C-18), 38.3 (C-19), 39.5(C-20), 32.7 (C-21), 30.3 (C-22), 25.4 (C-23), 17.9 (C-24), 16.9 (C-25), 16.8 (C-26), 15.9 (C-27), 178.3 (C-28), 15.1 (C-29), 23.2 (C-30). Molecular mass (456 g/mol); molecular formula ($C_{30}H_{48}O_3$).

Oleanolic acid (4)

White powder (18 mg), m.p. 301-302 ^oC**,** ^1H NMR(CD_3OD, 600 MHz); 3.15 (1H, m, H-3), 0.82 (1H, s, H-5), 1.41 (1H, m, H-6α), 1.18 (1H, m, H-6β), 1.33(2H, m, H-7), 1.20 (1H, s, H-9), 1.94 (2H, dd, J = 6.0, 3.0 Hz, H-11), 5.23 (1H, m, H-12), 1.38 (2H, m, H-15), 1.59 (1H, m, H-16), 2.22 (1H, d, J = 6.0 Hz, H-18), 1.39 (1H, m, H-19), 1.55 (1H, m, H-20), 1.24 (2H, m, H-21), 1.62 (2H, m, H-22), 0.90 (2H, m, H-23), 0.78 (3H, s, H-24), 1.16 (3H, s, H-25), 0.85 (3H, s, H-3), 1.12 (3H, s, H-26), 0.96 (3H, s, Hz, Me-20β), 0.98 (3H, s, Me-20α). ^{13}C NMR (CD_3OD, 150 MHz); δ: 38.8 (C-1), 28.2 (C-2),78.2 (C-3), 38.7 (C-4), 55.3 (C-5), 18.4 (C-6), 39.3 (C-7), 37.1 (C-8), 47.7 (C-9), 37.1(C-10), 17.2 (C-11), 122.2 (C-12), 142.8 (C-13), 42.0 (C-14), 28.2 (C-15), 27.3 (C-16), 23.3 (C-17), 21.5 (C-18), 41.4 (C-19), 30.9 (C-20), 33.9 (C-21), 33.2 (C-22), 30.8 (C-23), 15.6 (C-24), 15.4 (C-25), 21.2 (C-26), 28.2 (C-27), 181.4 (C-28), 32.8 (C-29), 23.5 (C-30). Molecular mass (456 g/mol); molecular formula ($C_{30}H_{48}O_3$).

Hyperoside (12)

Yellow powder (11.7 mg), m.p. 225-226 ^oC, ^1H NMR (DMSO-d6, 500 MHz); δ: 12.6 (1H, s, H-4), 6.20 (1H, d, J = 3.5 Hz, H-6), 6.40 (1H, d, J = 3.5 Hz, H-8), 7.52 (1H, d, J = 3.5 Hz, H-2'), 6.80 (1H, d, J = 3.5 Hz, H-4'), 7.66 (1H, d, J = 3.5 Hz, H-5'), 7.67 (1H, d, J = 3.5 Hz, H-6'), 5.37 (1H, d, J = 3.5 Hz, H-1''), 3.59 (1H, d, J = 3.5 Hz, H-2''), 3.35(1H, d, J = 3.5 Hz, H-3''), 3.66 (1H, d, J = 3.5 Hz, H-4''), 3.33 (1H, d, J = 3.5 Hz, H-5''), 3.53 (1H, d, J = 3.5 Hz, H-6''). ^{13}C NMR (DMSO-d6, 125 MHz); δ: 156.2 (C-2), 133.1 (C-3), 177.4 (C-4), 161.1 (C-5), 98.4 (C-6), 164.2 (C-7), 93.4 (C-8), 156.2 (C-9), 103.5 (C-10), 121.1 (C-1'), 115.1 (C-2'), 144.8 (C-3'), 148.2 (C-4'), 115.9 (C-5'), 121.8 (C-6'), 101.8 (C-1''), 70.7 (C-2''), 73.2 (C-3''), 67.9 (C-4''), 76.5(C-5''), 60.2 (C-6''). HRTOFESI–MS: 487.0830, matches the molecular formula ($C_{21}H_{20}O_{12}Na$).

Assessment of antibacterial activity

Stock solutions were prepared at 100 mg/mL for fractions and extracts, and 1 mg/mL for

compounds. Thereafter, 100 mg of extracts and fractions and 1 mg of dried compound were weighed and dissolved in 1 mL of absolute DMSO. The reference drug ciprofloxacin was prepared in similar conditions at 1 mg/mL in distilled water. The stock solutions were kept at 4 ℃ till further analysis of bioassays. Prior to each experiment, the bacteria species were activated for 24 h at 37℃ in Mueller Hinton Agar (MHA), and Mueller Hinton Broth (MHB) was used for antibacterial-based microdilution.

Accordingly, 0.5 mL of McFarland standard was used to prepare the bacterial inoculum. The stock suspension was prepared at 0.5 McFarland turbidity (corresponding to an approximate concentration of 1.5 x 10^8 cells/mL) from 24 h old young cultures on MHA and subsequently diluted to 5 x 10^5 CFU/mL for the antibacterial study.

Minimum inhibitory concentration (MIC)

The MICs were determined using the microdilution-based assay following the protocol M07A9 of Clinical and Laboratory Standards Institute (CLSI) [8], paired with resazurin-based viable cells assay. The experiment was conducted in sterile flat-bottom 96-well microplate. The crude extract (2 µL) and fractions concentrated at 100 mg/mL (DMSO) and 4 µL of the compounds prepared at 1 mg/mL were added in the first corresponding wells, containing respectively 198 µL and 196 µL of MHB, followed by 7 two-fold serial dilutions.

Thereafter, 100 µL of bacteria suspension (1.0 × 10^6 CFU/mL) was added to all the wells except for sterility control. The final concentrations ranged from 500 - 3.91 µg/mL for extracts, fractions, 100 - 0.781 µg/mL for compounds, and 16 - 0.00625 µg/mL for ciprofloxacin, with the final volume of 200 µL and final concentration of DMSO < 0.5 %.

The negative control was made up of bacterial suspension and culture media while the positive control was made up of ciprofloxacin, bacteria suspension, and culture media. The sterile control was culture media alone. The sealed plates, preserved at 37 ℃ were incubated for 24 h. At the end of the incubation period, 20 µL (0.15 mg/mL) of fresh resazurin was put in the wells. The plates were re-incubated for 30 min in the dark under the same conditions. The smallest concentration of each sample (extracts, fractions, and compounds) where there is no change of resazurin blue colour to resorufin pink colour, highlighting no bacterial growth, was recorded as the MIC. The assay was done in triplicate.

Minimum bactericidal concentration (MBC)

The MBC was determined by the subculture method from the MIC plate. The sample (25 µL) from the MIC determination wells of the two previous wells was removed and added to plates of new wells containing 175 µL of MHB. The sterility, negative, and positive controls were designed as previously described. The sealed plates kept at 37 ℃ were incubated for 48 h. At the end, all the viable cells were assessed via resazurin-based assay. The smallest concentration of extract or compound where there was no change in resazurin colour was recorded as MBC. The bactericidal effect was determined based on the calculations of the MBC/MIC value.

RESULTS

Identification of isolated compounds

The findings on *S. brevipes* resulted in the identification fifteen compounds, namely taraxeryl acetate (**1**) [9]; taraxerol (**2**) [9]; ursolic acid (**3**) [9]; oleanolic acid (**4**) [9]; betulinic acid (**5**) [9]; lupeol (**6**) [9]; herannone (**7**) [3]; spinasterol (**8**) [10]; spinasterol-3-O-β-D-glucopyranoside (**9**) [10]; eicosanoic acid (**10**) [11]; asperphernamate (**11**) [12]; hyperoside (**13**) [13]; spinasterone (**13**) [14]; arganin C (**14**) [15]; and butyroside B (**15**) [15] (Figure 1).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The *in vitro* antibacterial activities of the extracts, fractions, and selected compounds were carried out using Gram-positive (*S. aureus, P. aeruginosa,* Methicillin-resistant *S. aureus*, and *E. coli*) and Gram-negative (*S. typhi,* and *S. typhimurium*) bacterial strains. Ciprofloxacin was used as the reference drug (Table 1).

DISCUSSION

This study has led to the isolation and identification of 15 compounds categorised as seven terpenoids (taraxeryl acetate, taraxerol, heranone, ursolic acid, lupeol, oleanolic acid, and betulinic acid), three steroids (spinasterol, spinasterone, and spinasterol-3-O-β-D-glucopyranoside), one fatty acid (eicosanoic acid), one polyphenol (hyperoside), one alkaloid (asperphernamate) and two saponins (arganin C and butyroside B). Except for asperphernamate, hyperoside, and eicosanoic acid, the other compounds were ubiquitous in the Sapotaceae family.

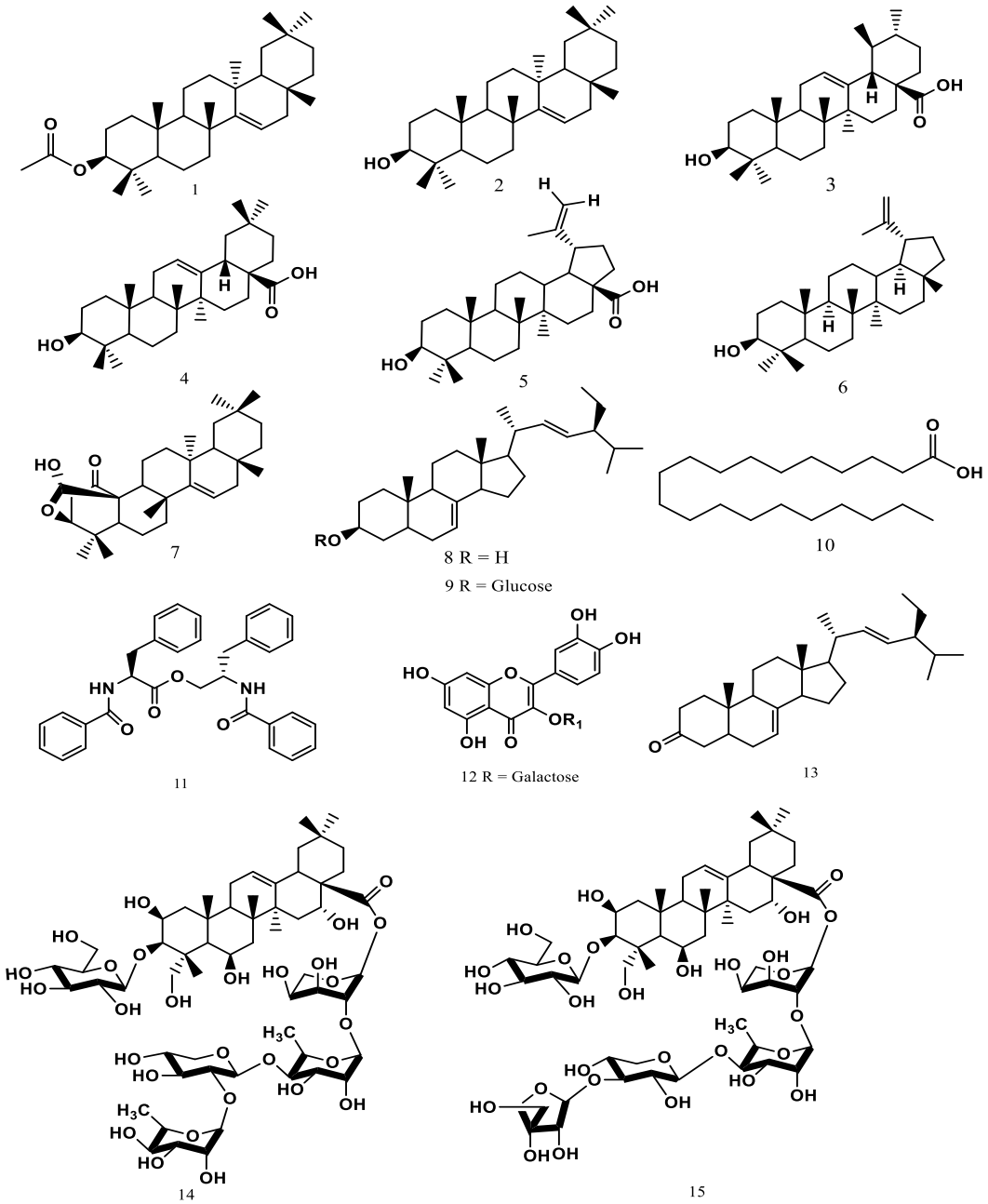

Figure 1: The structures of compounds **1** - **15** from *S. brevipes*

Table 1: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC; µg/mL)

Sample		PA NR 48982	EC ATCC 25922	ST CPC	STM CPC	SA ATCC 43300	MRSA ATCC 33591
Ursolic aicd	MIC	>100	25	>100	>100	>100	25
	MBC	>100	>100	100	>100	>100	100
Oleanolic acid	MIC	>100	25	>100	>100	50	12.5
	MBC	>100	>100	>100	>100	>100	50
Hyperoside	MIC	>100	25	>100	>100	>100	>100
	MBC	>100	>100	>100	>100	>100	>100
Ciprofloxacine	MIC	0.031	0.031	0.015	0.031	0.015	0.031

PA: *Pseudomonas aeruginosa*, EC: *Escherichia coli*; ST: *Salmonella typhi*, STM: *Salmonella typhimurium*; SA: *Staphylococcus aureus*, MRSA: Methicillin-resistant *Staphylococcus aureus*, >100: not active at 100 µg/mL, CPC: Centre Pasteur of Cameroon

According to the set standard, extracts are classified as moderate if 100 < MIC ≤ 625 µg/mL, weak if MIC > 625 µg/mL, and significant if MIC < 100 µg/mL. For pure compounds from plants, the activity is moderate if MIC ≤ 100 µg/mL, low if MIC > 100 µg/mL, and significant if MIC ≤ 10 µg/mL [15]. As presented in Table 1, among the tested compounds, **4** moderately impaired methicillin-resistant *S. aureus* (ATCC 33591; MIC = 12.5 µg/mL), **3** were moderately active against *E. coli* (ATCC 25922) and *S. aureus* (ATCC 43300; MIC = 25 µg/mL), and **12** were modestly active against *E. coli* (ATCC 25922; MIC = 25 µg/mL). Compounds **3** and **4** displayed moderate bactericidal effects against *E. coli* and methicillin-resistant *S. aureus*, with MBC values ranging from 50 - 100 µg/mL. The reference ciprofloxacin was active against all the bacterial strains.

Previous reports of 3 and 4 revealed significant/moderate activity against methicillin-sensitive *Staphylococcus aureus* with MIC values ranging from 8 - 64 μg/mL [16,17]. In the present study, compounds **1**, **2**, **8**, **9**, and **11 - 15** with MIC > 100 μg/mL were considered inactive. For the family Sapotaceae, the extract of *Synsepalum dulcificum* has been reported to exhibit significant and moderate antibacterial activities [6], however, in this study, extracts and fractions of *S. brevipes* were inactive on all bacterial strains. Concerning compounds **5 - 7** and **10**, they were not experimentally evaluated in this study due to other constraints.

CONCLUSION

This study isolated 15 components from *S. brevipes,* which include two terpenoids (oleanolic and ursolic acids) and one polyphenol (hyperoside) with moderate antibacterial activity against three bacterial pathogens. These results revealed the presence of antagonism and confirm previous findings on triterpenoids showing little or no antibacterial effect.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work

Data availability statement

The data that support the findings of this study are found in the supplementary material.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Conceptualization and methodology: Fomene Tsapy Raphaelle, Ache Roland Ndifor and Ngameni Bathelemy; collected and analysed the data: Céline Henoumont, Sophie Laurent, and Fotso Wabo Ghislain; writing-original draft preparation: Ache Roland Ndifor, Ngnintedo Dominique, Njinga Ngaitad Stanislaus, Ambassa Pantaléon; supervision and project administration: Bonaventure Tchaleu Ngadjui.

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