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New Bioactive Flavonoid Derivative from the Leaves of *Caloncoba echinata*

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Abstract: Background: *Caloncoba echinata* is used in traditional medicine as vomiting, against lice and mange as well as in the treatment of dermal infection, leprosy, pustular eruption (small-pox) [1, 4]. It has been reported that the non-edible vegetable oil from *Caloncoba echinata* seeds possess potent antibacterial activity on *Escherichia coli* and *Staphylococcus aureus* [5]. Objective: This work addressed the phytochemical investigation of the methanolic extract of leaves of *Caloncoba echinate*. Both extracts and all the isolates were screened for the antibacterial activities. Method: All the compounds were characterized by spectroscopic and mass spectrometric methods, and by comparison with literature data. The antibacterial activity of both extract and some isolated compounds against bacteria was determined using broth microdilution method in 96-well microtitre sterile plates as previously described [11]. Results: From the methanolic crude extract of the leaves of *Caloncoba echinate*, a new derivative flavonol named Kaempferol-4',7-dimethoxy-3-*O*-(6"-*O*-acetyl)-*β-NULL*-glucopyranoside (*1*) together with nine known compounds namely ermanin-3-*O*-*β*-*D* glucopyranoside (*2*), Kaempferol-4′,7-dimethoxy-3-*O*-(3",4",6"-*O*-triacetyl)-*β-NULL* glucopyranoside (3), friedelan-3-one (4), 29-hydroxyfriedelan-3-one (*5*), mixture of *β*-sitosterol and stigmasterol (*6-7*), mixture of *β*-sitosterol and stigmasterol glucoside (*8-9*), Lupeol (10) were isolated. Furthermore, compounds (*2*) and (*5*) were reported here for the first time from the *Caloncoba* genus. Crude extract exhibited a significant activity against the five bacteria with the MIC = 62.5µg/mL for *Salmonella typhi*, *Escherishia coli*, *Shigella flexineri* and the MIC = 32.25µg/mL for *Salmonella typhimurium*, *S. enteritidis*. For the isolated compounds, the best activities were recorded by compound (*1*) showing a moderate activity against *Salmonella typhi* (MIC = 32.25µg/mL; MBC/MIC = 8), *Salmonella typhimurium* (62.5µg/mL; MBC/MIC = 4), *Salmonella enteritidis* (62.25 µg/mL; MBC/MIC = 2). Conclusion: These results showed that the antimicrobial activities could be mainly attributed to the constituents of flavonol glycoside *(1)*. In addition, the antibacterial bioactivities and determined constituents support the use of this specie by traditional healers to treat a certain number of bacterial diseases.

Keywords: Achariaceae, *Caloncoba echinate*, Flavonol, Antimicrobial Activities

1. Introduction

Caloncoba echinata belongs to the genus *Calonconba* (Achariaceae) which consists of about 20 species of trees and shrub located in Africa tropical climate zones [1-3]. In traditional medicine, *C. echinata* is used as vomiting, against lice and mange, in the treatment of dermal infection, leprosy, pustular eruption (small-pox) [1, 4]. Antimicrobial sensitivity

tests were carried out on the non-edible vegetable oil from seeds of *Caloncoba echinata* using bacterial and fungal isolates and it showed very potent antibacterial activity on *Escherichia coli* and *Staphylococcus aureus* [5]. Furthermore, the reported triterpenes from the leaves of the species inhibited *in vitro* growth of *Plasmodium falciparum* parasites [6]. Phytochemical reports on the genus *Caloncoba* have revealed the presence of triterpenes [6, 7], fatty acids [5, 8] (, Alkaloid [9], flavonoids and phytosterols [10]. In the present study, we report the isolation and characterization of one new flavonol, Kaempferol-4',7-dimethoxy-3-*O*-(6"-*O*-acetyl)-*β*-*NULL*-glucopyranoside (1) along with nine known compounds and their antimicrobial activities.

2. Experimental Section

2.1. General Experimental Procedures

The melting points were determined using capillary tubes on a Stuart Scientifique-type SMP11-like melting point apparatus. Infrared spectra were recorded on an Alpha spectrometer from the firm Bruker. Ultraviolet spectra were recorded on DAD in the range from spectrophotometer in MeOH. UPLC-HRMS analysis was performed on Dionex (Gemering, Germany) Ultimate 3000 RSLC system using a Waters (Eschborn, germany) BEH C18 column (50 x 2.1 mm, 1.7 µm) equipped with a Waters Van Guard BEH C18 1.7 µm Guard column. The LC flow is split to 75 µm/min before intering the Bruker Daltonics maxis 4G hrToF mass spectrometer (Bremen, Germany) equipped with Appolo II ESI source. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded using 700MHz and 175MHz Bruker Ascend 700 spectrometers for compound 1 and 2, also 125 MHz and 500 MHz Bruker NMR spectrometers for the rest respectively. Methyl, methylene and methine carbons were distinguished using the values of shift of carbons and proton, and the different integrations of proton signals. Homonuclear ^{1}H connectivities were determined by using the COSY experiment. One-bond ^{1}H-^{13}C connectivities were determined with HMQC gradient pulse factor selection. Two- and three-bond ^{1}H-^{13}C connectivities were determined by HMBC experiments. Chemical shifts are reported in δ (ppm) using TMS as internal standard and coupling constants (J) were measured in Hz. Column chromatography was carried out on silica gel (70–230 mesh, Merck). TLC was performed on Merck precoated silica gel 60 F254 aluminum foil, and spots were detected using diluted sulfuric acid spray reagent, UV lamp and iodine vapor.

2.2. Collection and Identification

Fresh leaves of *Caloncoba echinata* were collected from Nyete's locality located in the Ocean department in the South region, and identified by Victor Nana, a botanist at the National Herbarium, Yaoundé, Cameroon, where a voucher specimen was deposited under ref. 64634/HNC.

2.3. Extraction and Isolation

The dried leaves of *Caloncoba echinata* (1.58 kg) were extracted with MeOH for 48 h. After removal of the solvent by evaporation under reduced pressure, the residue (74.40 g) was submitted to silica gel column chromatography (CC, hexane, EtOAc, MeOH, v/v, gradient) and thirteen fractions (Fr 1-13.) were collected. Fr.2 was obtained by CC silica gel eluting with hexane-EtOAc (39:1 to 19:1) to yield friedelan-3-one (10.0 mg). Fr. 3 were obtained by CC on silica gel eluting with hexane-EtOAc (19:1) and hexane-EtOAc (200:15) and then subjected to repeated CC on silica gel eluting with hexane-EtOAc (39:1), hexane-EtOAc (19:1) and hexane-EtOAc (40:3) to afford of Lupeol (4.8 mg) and a mixture of *β*-sitosterol and stigmasterol (7.4mg). Fr.6 was obtained by CC on silica gel eluting with hexane-EtOAc (40:3 and 5:1) to give 29-hydroxy1friedelan-3-one (7.0 mg). Fr. 8 was obtained by CC on silica gel eluting with hexane-EtOAc (3:2) to give Kaempferol-4′,7-dimethoxy-3-*O*-(3",4",6"-*O*-triacetyl)-*β*-*NULL* glucopyranoside (30.7mg). Fr. 10 was obtained by CC on silica gel eluting with hexane-EtOAc (1:1) and hexane-EtOAc (1:3) to yield Kaempferol-4',7-dimethoxy-3-*O*-(6"-*O*-acetyl)-*β*-*NULL*-glucopyranoside (5.6mg). Fr.11 was obtained by CC on silica gel eluting with hexane-EtOAc (1:3) to afford a mixture of *β*-sitosterol and stigmasterol glucoside (13.9 mg). Fr. 12 was obtained by CC on silica gel eluting with EtOAc to give ermanin-3-*O*-*β*-*D* glucopyranoside (14.3mg).

2.4. Kaempferol-4',7-dimethoxy-3-O-(6"-O-acetyl)- β-NULL-glucopyranoside

Yellow crystals (DMSO); Mp = (222 ± 2)°C; Rf =0.75, silica gel 60 F254, Hex-AcOEt (3:7). UV (MeOH) max (log ε) = 222 nm. IR (KBr): ν = 1644.0 cm^{-1}, 1700.0 cm^{-1}, 3412.5 cm^{-1} and 1287.5cm^{-1}. ^{1}H NMR (DMSO, 500 MHz) and ^{13}C NMR (DMSO, 125 MHz) data (Table 1); HRMS ((+)-ESI): $[M+H]^{+}$ at m/z=519.1501 (calc. 519.1497) for $C_{25}H_{26}O_{12}$.

2.5. Bioassays

The minimum inhibitory concentration (MIC) of the extracts of *Caloncoba echinata* were determined using broth microdilution method in 96-well microtitre sterile plates as previously described [11]. Briefly, two-fold serial dilutions of the extract, fractions and compound were seeded in a 96 well microtiter plate and inoculated with bacterial inoculum at 10^{6} CFU/mL (McFarland) in a final volume of 100 µL of Mueller Hinton broth medium (Oxoid, Thermo Scientific™). The final concentrations ranged from 500 to 0.244 µg/mL (for the plant extract) and 125 to 0.122 µg/mL (for isolated compound and ciprofloxacin used as the reference drug). The negative control made of broth medium and bacteria inoculum were treated with equivalent amount of DMSO at 0.5% (Loba chemie®, India). The sterile control wells containing broth medium was included in the experiment. The plates were incubated at 37°C for 24 h. The MICs were determined after addition of 20 µL of the yellow rezasurin (alamarblue TM Cell Viability Reagent) solution that viable bacteria reduce to pink color after 30 min of incubation at 37°C [12]. The MIC was considered as the lowest

concentration that gives no color change, indicating no microorganism growth. The Minimal Bactericidal Concentration (MBC) was determined by sub culturing 50 µL of culture media corresponding to wells without color changes (without rezasurin) into 150 µL of drug-free broth medium. After 24 h incubation at 37°C the MBCs were revealed by addition of rezasurin as above and define as the lowest concentration of with no color change. Tests were performed in triplicates at three different times. The classification criteria of the antimicrobial activity of extracts, fractions and compounds were based on the MIC threshold reported by the previous study [13]. The ratio MBC/MIC was calculated to determine the bactericidal (MBC/MIC ≤ 4) and bacteriostatic (MBC/MIC > 4) effects.

3. Results and Discussion

The methanolic extract of leaves of *Caloncoba echinata* was separated by repeated column chromatography on silica gel to afford one new flavonol and nine known compounds (Figure 1). The structures of known compounds were identified as ermanin-3-*O*-*β*-*D* glucopyranoside (2) which is new in the *Caloncoba* genus [14, 15], Kaempferol-4′,7-dimethoxy-3-*O*-(3",4″,6″-*O*-triacetyl)-*β*-*NULL* glucopyranoside (3) [10], friedelan-3-one (4) [16], 29-hydroxyfriedelan-3-one (5) wich is also new in the *Caloncoba* genus [17], mixtures of *β*-sitosterol and stigmasterol (6 and 7) [7], mixture of *β*-sitosterol and stigmasterol glucoside (8 and 9) [7], lupeol (10) [18]. The structures were confirmed by comparison of spectra data with authentic and published values.

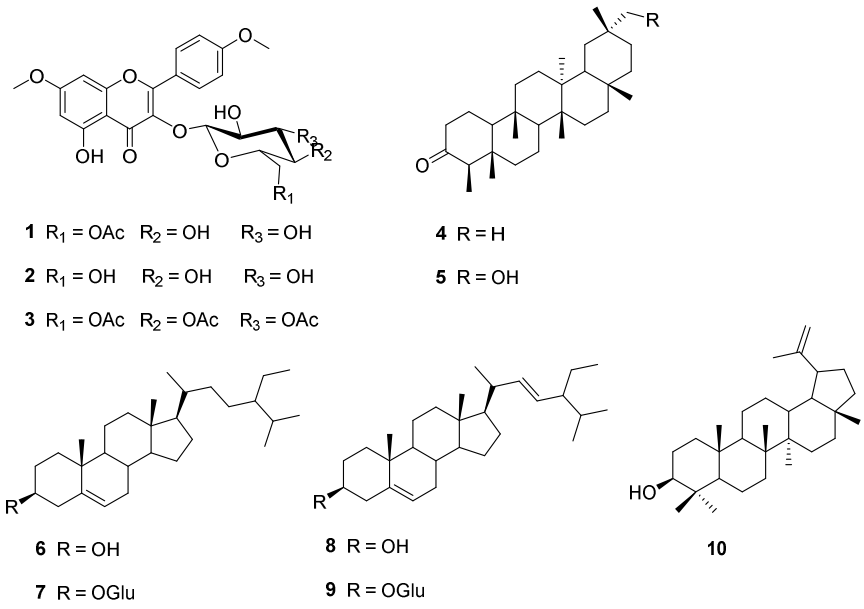

Figure 1. *Chemical structures of compounds 1-10.*

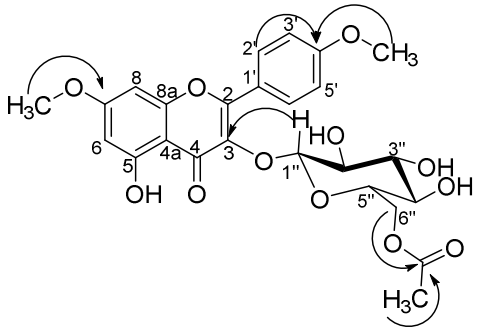

Figure 2. *Selected HMBC correlations of compound 1.*

Compound 1 was obtained as a yellow crystal and gave a positive reaction with ferric chloride indicating the presence of a phenolic hydroxyl group. The analysis of its mass spectrum FAB in positive mode showed peaks at m/z= 1037.2915 and 1059.2730 corresponding respectively to the pseudo-molecular ion $[2M+H]^+$ (1037.29) and $[2M+Na]^+$ (1059.27) to which was assigned the molecular formula $C_{25}H_{26}O_{12}$ corresponding to thirteen degrees of unsaturation. Its IR spectrum showed the presence of absorption bands of conjugated carbonyl for ketones (1644.0) cm^{-1}), carbonyl for ester function (1700.0 cm^{-1}), hydroxyl (3412.5 cm^{-1}) and ether (1287.5cm^{-1}) groups. Its UV spectrum exhibited absorption maxima at λ_{max}(MeOH) = 222 nm. The ^{1}H NMR spectrum combined to the COSY spectrum (Table 1), exhibited signal for one chelated hydroxyl proton at δ_H 12.49 (*s,* H-5) of a flavonol skeleton (Harborne et al., 1975), signals for two meta coupled aromatic protons at δ_H 6.37 (*d*, *J* = 2.1Hz, H-6) and δ_H 6.74 (*d*, *J* = 2.1Hz, H-8), characteristics of the ring A of flavonol skeleton [10]. Furthermore, it exhibited signal for an AA'BB' aromatic protons spin system at δ_H 8.10 (*d, J* = 8.9Hz, H-2', H-6') and δ_H 7.05 (*d*, *J* = 8.9Hz, H-3', H-5') attributable to a *para* ring B of flavonol and signals for protons of a glucose at δ_H 5.34 (*d*, *J* = 7.5Hz, H-1"), δ_H 3.22 (*m*, H-2", H-3"), δ_H 3.12 (*m*, H-4"), δ_H 3.30 (*m*, H-5") δ_H 3.93 (*dd*, *J* = 11.9; 6.1 Hz, H_α-6") and δ_H 4.08 (*dd*, *J* = 12.2; 2.2Hz, H_β-6") [19]. The coupling constant of the anomeric proton at δ_H 5.34 (*d*, *J* = 7.5Hz, H-1") indicated

a β-configuration of the glucoside unit [20]. In addition, proton signals of a methyl bearing acetyl group and two aromatic methoxy groups were observed at δ_H 1.69 (*s*), 3.84 (*s*) and δ_H 3.82 (*s*) respectively.

Table 1. *NMR data of compound (1) (^{1}H: 500 MHz, ^{13}C: 125 MHz) measured in DMSO-d_6 (δ in ppm).*

Position	1		
	^{13}C	^{1}H (*m*, *J* in Hz)	HMBC
2	157.0	-	
3	133.9	-	
4	177.2	-	
4a	105.3	-	
5	161.2	-	
6	98.4	6.37 (*d*, 2.2)	C-4; C-5; C-7; C-8; C-4a
7	165.7	-	
8	92.7	6.74 (*d*, 2.2)	C-4; C-6; C-7; C-4a; C-8a
8a	156.7	-	
1'	122.6	-	
2'	131.2	8.10 (*d*, 8.9)	C-2; C-2'; C-3'; C-4'
3'	114.1	7.05 (*d*, 8.9)	C-1'; C-3'; C-4'
4'	161.8	-	4'-OMe
5'	114.1	7.05 (*d*, 8.9)	C-1'; C-3'; C-4'
6'	131.2	8.10 (*d*, 8.9)	C-2; C-2'; C-3'; C-4'
5-OH	-	12.49 (*s*)	C-4a; C-5; C-6; C-7
7-OMe	55.8	3.84 (*s*)	C-7;
4'-OMe	56.5	3.82 (*s*)	C-4';
1"	101.4	5.34 (*d*, 7.5)	C-3; C-1"; C-2"; C-3"; C-5"
2"	74.4	3.22 (*m*)	C-1"; C-2"; C-3"; C-4"
3"	76.4	3.22 (*m*)	C-1"; C-2"; C-3"; C-4"; C-5"
4"	70.1	3.12 (*m*)	C-2; C-3; C-4; C-5; C-6
5"	74.3	3.30 (*m*)	C-1"; C-3"; C-4"; C-5"; C-6"
6"	63.0	3.93 (*dd*, 11.9; 6.1) 4.08 (*dd*, 12.2; 2.2)	C-6"; C-4"; C-5"
2"-OH		5.53 (*d*, 4.4)	C-1"; C-2"; C-3"
3"-OH		5.27 (*d*, 4.7)	C-4"; C-2"; C-3"
4"-OH		5.34 (*d*, 6.2)	C-4"; C-5"; C-3"
6"-$COCH_3$	20.5	1.69 (*s*)	6"-$COCH_3$; $COCH_3$
6"-$COCH_3$	170.4		6"-$COCH_3$

The ^{13}C NMR spectrum of compound 1 supported the flavonol skeleton with the characteristic carbon signals at δ_C 177.4 (C-4), 157.0 (C-2), 133.9 (C-3), 161.2 (C-5), 165.7 (C-7), 156.7 (8a) [20, 21]. Further signals of the ^{13}C NMR spectrum of compound 1 at δ_C 122.6 (C-1'), 161.8 (C-4'), 105.3 (C-4a), 98.4 (C-6), 92.7 (C-8), 114.1 (C-3', C-5'), 131.2 (C-2', C-6') suggested that the flavonol skeleton is kaempferol [10, 22]. In addition, the ^{13}C NMR displayed signals for glucoside moiety [δ_C 101.4 (C-1"), δ_C 74.4 (C-2"), δ_C 76.4 (C-3"), δ_C 70.1 (C-4"), δ_C 74.3 (C-5"), δ_C 63.0 (C-6") [19], acetyl carbonyl group at δ_C 170.4, methyl bearing carbonyl group at δ_C 20.5 and two methoxyle groups at δ_C 55.8 and δ_C 56.5 respectively. The chemical shift of anomeric carbon at δ_C 101.4 (C-1") suggested an *O*-linkage [20]. The correlations observed in the HMBC spectrum between the methoxyle protons group at δ_H *3.82* and the carbon at δ_C 161.8 (C-4'), between the proton H-2' (δ_H 8.10) and the same carbon indicated that this methoxyle group was located in carbon C-4'. This spectrum also showed correlations between the methoxyle protons group at δ_H 3.84 with the carbon at δ_C 165.7 (C-7), between the protons H-6 and H-8 with the same carbon at δ_C 165.7 indicated that this methoxyle group is located at carbon C-7. The position of acetyl group in position C-6" of glycoside unit was supported by the ^3J correlations between the methylene protons H-6" (δ_H4.08, δ_H3.93) and the carbonyl acetyl group at δ_C 170.4. The linkage between the Kaempferol moiety and glucoside unit was established through the ^3J correlation between the anomeric proton H-1"(δ_H5.34) with the carbon C-3 (δ_C 133.9). From the above results, compound 1 was characterized as Kaempferol-4',7-dimethoxy-3-*O*-(6"-*O*-acetyl)- β- *NULL*-glucopyranoside.

Table 2. *Inhibition parameters (MIC, MBC) of compounds and extract against different tested microorganisms.*

Extracts/Compounds	Parameters	Microbial organisms				
		St 19430	Stm	Se 13076	Ec 25922	Sf NR 518
	MIC (µg/mL)	31.25	62.5	62.5	125	125
1	MBC (µg/mL)	250	250	125	250	250
	MBC/MIC	8	4	2	2	2
	MIC (µg/mL)	125	NA	125	NA	125
2	MBC (µg/mL)	250	ND	250	ND	250
	MBC/MIC	2	ND	2	ND	2
	MIC (µg/mL)	NA	NA	NA	NA	NA
3	MBC (µg/mL)	ND	ND	ND	ND	ND
	MBC/MIC	ND	ND	ND	ND	ND
	MIC (µg/mL)	62.5	NA	62.5	NA	125
5	MBC (µg/mL)	125	ND	125	ND	250
	MBC/MIC	2	ND	2	ND	2
	MIC (µg/mL)	62.5	31.25	31.25	62.5	62.5
Crude extract	MBC (µg/mL)	250	125	125	250	250
	MBC/MIC	4	4	4	4	4
	MIC (µg/mL)	0.5	1	0.5	0.5	0.5
Cipro	MBC (µg/mL)	2	2	2	2	2
	MBC/MIC	4	2	4	4	4

ST: *Salmonella typhi* (ATCC 19430); STm: *Salmonella typhimurium*; SE: *Salmonella enteritidis* (ATCC 13076); EC: *Escherishia coli* (ATCC 25922); SF: *Shigella flexineri* (NR 518); NA: not active; ND: not determined. MIC = Minimum inhibitory concentration; MBC = Minimum bactericidal concentration.

The antimicrobial activities of methanolic crude extract and compounds (1), (2), (3),(5) (Table 2) were evaluated

using the broth microdilution method against five Gram-negative bacteria strains including *Salmonella typhi*, *Salmonella typhimurium*, *Salmonella. enteritidis*, *Escherishia coli* and *Shigella flexineri* based on the MIC and the ratio MIC/MBC values [11]. The results revealed that the crude extract showed strong activity against the five bacteria tested with the minimum inhibitory concentration values of 62.5µg/mL (*S. typhi*, *E. coli* and *S. flexineri*), 32.25µg/mL (*S. typhimurium* and *S. enteritidis*) and possessed bactericidal effect according to their ratio MBC/MIC = 4. Among all the compounds tested, compounds 1 and 5 exhibited moderate activity according to the minimum inhibitory concentration values respectively on *S. typhi* (32.25µg/mL;), *S. typhimurium*, *S. enteritidis* (62.50µg/mL;) and on *S. typhi*, *S. enteritidis* (62.25µg/mL;), *S. enteritidis* (62.50µg/mL;) compared to ciprofloxacin on *S. typhi*, *S. enteritidis*, *E. coli*, *S. flexineri* (0.5 µg/mL) and *S. typhimurium* (1 µg/mL) as reference. Furthermore, compound (1) possessed bactericidal effect on *S. typhi* (MBC/MIC = 8), *S. typhimurium* and *S. enteritidis* (MBC/MIC = 4) while compound 5 revealed bacteriostatic activity on *S. typhi* and *S. enteritidis* (MBC/MIC = 2) [23]. The highest activity obtained from the methanolic crude extract suggested a synergistic effect due to the various metabolites in the plant. These results justified the use of the extract of this plant by traditional healers to treat bacterial diseases.

4. Conclusion

This paper addressed the phytochemical investigation of the methanolic extract of leaves of *Caloncoba echinate*. This research led to the isolation and structural elucidation of one new flavonol named Kaempferol-4',7-dimethoxy-3-*O*-(6"-*O*-acetyl)-*β*-*NULL*-glucopyranoside (1) together with nine known compounds by the means of usual spectroscopic methods. The antimicrobial activities of some isolated compounds and crude extract were evaluated. This work probably explains the use of extracts from this plant by traditional healers against a certain number of bacterial diseases.

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