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Research Article

NEW BIFLAVONOID FROM THE ROOTS OF *ALLEXIS CAULIFLORA* (VIOLACEAE) AND EVALUATION OF ANTIBACTERIAL ACTIVITIES

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A B S T R A C T

In the search for new compounds endowed with antibacterial, antioxidant and antiplasmodial properties in Cameroonian pharmacopoeia, a new compound was established using spectroscopic analysis techniques. The compound was isolated from the ethyl acetate fraction of *Allexis cauliflora*. The antibacterial, antioxidant and antiplasmodial activities of this compound isolated were assessed in this study. It showed good antibacterial activities, good antiplasmodial activity against the chloroquine-sensitive *Plasmodium falciparum* 3D7 strain but has presented a weak antioxidant activity.

INTRODUCTION

Poor people have always used plants for treatment. Today, because of the ever-increasing cost of available drugs associated with the emergence of multi-resistant microbes, there is renewed interest in the pharmacopoeia (Kaboré *et al*., 1997, Akoua *et al*., 2004). In view of these different financial and resistance barriers to the use of currently available antimicrobials, it is essential to look for new substances that are both effective and broad-spectrum. One of the strategies for this research is to explore the plants used in traditional medicine.Violaceae plants have been constantly used by traditional medicine to treat many diseases caused by these pathogens agents. Thus, the barks of *Allexis cauliflora* are used to treat fever and syphilis (Achoundong *et al*., 1998). Despite the availability of this ethnopharmacological information, there is very little phytochemical chemical report published on the *Allexis cauliflora* to date. However, previous phytochemical studies on some species of other Violaceae genera indicated the occurrence of potentially useful medicinal secondary metabolites such as flavonoids, triterpenoids, peptides (cyclotides). In the presentstudy, we report the isolation and structural elucidation of a new compound named 4,4''-dimethoxylophirone A (1) which was extracted from the roots of *Allexis cauliflora*. We also evaluated antibacterial and anti the antibacterial, antiplasmodial and antioxidant activities of this compound evaluated antibacterial activities.

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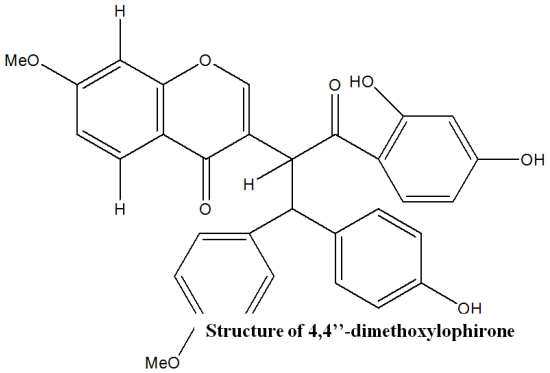
RESULTS AND DISCUSSIONS

The ethylacetate fraction of *Allexis cauliflora* roots was purified by column chromatography on silica gel. we obtained one new biflavonoid and a mixture of compounds. The new biflavonoid was obtained in the form of a white powder in the solvent system CH_2Cl_2 / MeOH (30/1). It showed the base peak of the molecular ion at m /z = 561.1356 $[M + Na]^+$, (calcd. 561.1360)in HRESI MS, corresponding to the formula$C_{32}H_{26}O_8Na$. the UV spectrum showed absorption bands at λmax 243, 247 and 277nm, suggesting an isoflavonoid nucleus (Abdullahi *et al*., 2014, A. N. Messi *et al*., 2016). The IR spectrum presented the vibration bands at vmax 3320 (hydroxyl groups) 1620 and 1570 cm-1 (aromatic chain). the ^{13}C NMR spectrum showed a total of 32 carbones, the DEPT showed 2 carbonyl groups (δ=206.4 and 173.2), 11 quaternary sp^2 carbons of which 5 were attached to oxygen, 15sp^2 tertiary carbons and 4sp^3 hybridized carbons highlighting the skeleton of a biflavonoid. The ^1H NMR spectrum has 14 aromatic protons, 1 vinyl proton, 2 methynic protons linked to SP^3 carbons and two methoxy proton groups according to the COSY spectrum, ^1H-H^1, we have 1,4-disubstituted 1,4-aromatic aromatic chains and two 1,2,4-trisubstituted aromatic rings. We also observed a singlet δH = 8.11, characteristic of the proton H-2 of isoflavones (Feng *et al*., 2012). The protons of the methoxy groups were observed at 3.67 and 3.71, the protons of the AB system at 5.98 and 4.65 (d, J = 12.5 Hz). all this information combined, in addition to those given by the presence of the 2 methynes at δ= 55.1 and 43.9, and the two carbonyls at 173.2 and 206.4, make us say

that the compound is a derivative of lophirone A (Ghogomu *et al*., 1987, Anuradha *et al*., 2006, LikhitWitayawuid *et al*., 2005) according to the information collected on the HMBC spectrum, we have a correlation between carbonyl at 173.2 (C-c_1) and protons at 7.94 (H-6), 8.14 (H-b_1) and 6.11 (H-a_2). the previous protons correlate with the carbon atoms at δc = 206.4 (C-c_2), 54.2 (C-b_2), 135.01 (C-1 " and C-1 '"). The carbonyl at 206.4 (C-c_2) correlates with the protons at 4.70 (H-b_2) and 6.11 (H-a_2), as well as with the aromatic protons H-6 (δH = 6.17). proton H-b_2 correlates with carbons (C-2'' and C-6'') and carbons (C-2'"and C-6'"). We observe in addition, a correlation between C-4"(158.9) and the protons of the aromatic ring at δH = 7.27 and 7.29, 6.63 and 6.62 (H-2", H-6", H-3" and H-5") respectively, as well as with the protons of the methoxy group at δH = 3.67.Another coupling is observed between the protons of MeO at 3.71 and C-4 (δC= 164.1). The structure of biflavonoid, in agreement with the preceding information is 4,4"- dimethoxylophirone (fig1)



Structure of 4,4''-dimethoxylophirone

Table 1 Spectroscopic data of 4,4''-dimethoxylophirone A

Position	δCmult	δH m, J(Hz)	HMBC
B_1-1	109.1	-	-
2	158.0	-	-
3	102.3	6.72 (d ; 2.5)	C-1 ; C-5
4	164.1	-	-
5	117.7	6.86 (dd ; 2.5 ; 9)	C-1 ; C-3
6	127.6	7.94 (d ; 9)	C-2 ; C-4 ; c_1
c_1	173.2	-	-
a_1	121.5	-	-
b_1	157.0	8.14(s)	C-2 ; c_1 ; a_1 ; a_2
B_2-1'	118.1	-	-
2'	165.5	-	-
3'	102.3	6,14 (d ; 2)	C-1' ; C-2' ; C-4' ; C-5'
4'	164.4	-	-
5'	103.2	6,38 (dd ; 2 ; 9)	C-1' ; C-3'
6'	134.0	6.17	
c_2	206.4	-	-
a_2	44.4	6.11 (d ; 12.2)	b_1 ; b_2 ; c_2 ; a_1 ; C-1'' ; C-1'''
b_2	53.4	4.70 (d ; 12.2)	C-1'' ; C-2'' ; C-1''' ; C-2''' ; c_2
A_1-1''	134.3	-	-
2''	129.0	7.27 (d ; 8.5)	C-3''' ; C-4'' ; b_2
3''	114.7	6.63 (d ; 8.5)	C-1'' ; C''-4''
4''	158.9	-	-
5''	114.6	6.62(d ; 8.5)	C-1'' ; C-3'' ; C'-4''
6''	128.0	7.29(d ; 8.5)	C-2'' ; C-4'' ; b_2
A_2-1'''	134.5	-	-
2'''	131.3	7.32 (d ; 8.5)	C-3''' ; C-4''' ; b_2
3'''	120.0	6.60 (d ; 8.5)	C-1''' ; C-4'''
4'''	155.6	-	-
5'''	117.9	6.60 (d ; 8.5)	C-1''' ; C-3''' ; C-4'''
6'''	130.0	6.65 (d ; 8.5)	C-2''' ; C-4''' ; b_2
4'''-OCH_3	53.6	3.67 (s)	-
4-OCH_3	53.9	3.71	-
OH	-	-	-
OH	-	-	-

Antimicrobial results

The results of antibacterial and antifungal activities of EA extract and compound 1 from *Allexis cauliflora* are presented in tables 2, 3, 4 and 5. The activity of ethyl acetate was not very different from that of compound 1.

Table 2 MIC of EA extract and compound 1 on yeast strains (mg/mL)

			Reference	
Microorganism	**EA extract**	**1**	**Fluconazole**	**Ampicillin**
C. albicans	>0.5	>0.5	0.032	
C. krusei	>0.5	>0.5	0.032	
C. parapsilosis	>0.5	>0.5	0.032	
S. aureus NR46374	>0.5	>0.5		0.000488
K. pneumonia NR41916	>0.5	>0.5		0.000488
S. enterica NR13555	>0.5	>0.5		0.000488

Table 3 MFC of EA extract and compound 1 on yeast strains (mg/mL)

			Reference	
Microorganism	**EA extract**	**1**	**Fluconazole**	**Ampicillin**
C. albicans	>0.5	0.5	0.032	
C. krusei	>0.5	0.25	0.032	
C. parapsilosis	>0.5	>0.5	0.032	
S. aureus NR46374	>0.5	>0.5		0.000488
K.pneumonia NR41916	>0.5	>0.5		0.000488
S. enterica NR13555	>0.5	>0.5		0.000488

Nine strains of Gram-negative bacteria including reference strains (ATCC) and multi-resistant clinical isolates were used. These strains belonging to different bacterial species aredistributed as follows:

- A strain of *Escherichia coli* (*ATCC8739*).
- A strain and three clinical isolates of *Enterobacter aerogenes* (*ATCC13048; CM64; EA289; EA294*).
- Two clinical isolates of *Enterobacter cloacae* (*BM67, K2*).
- A clinical isolate from *Providencia stuartii* (*PS299645*).
- A strain of *Klebsiella pneumoniae* (*ATCC11296*).

The works of Berche (1993), Fauchère and Avril (2002), showed that when the CMB of an antibiotic on a given strain is

Table 4 Antibacterial activities

Compounds	***CM64***	***BM67***	***ATCC 8739***	***K2***	***PS299 645***	***ATCC 13048***	***EA289***	***ATCC 11296***	***EA294***
EA extract	2	ND	ND	1	ND	2	2	2	ND
1	2	2	2	ND	2	ND	2	2	2

close to the MIC (CMB/MIC = 1 or 2), the antibiotic is said to be bactericidal, in contrast, if these values are relatively distant (4 <CMB/CMI> 16), the antibiotic is said to be bacteriostatic. Finally, if CMB/CMI> 32, we speak of "tolerance" of the microbial strain.

Results indicated in Table 3 shows that the CMB/CMI ratio of the EA root extract varies from 1 to 2 for *CM64, K2, ATCC13048, EA289* and*ATCC11296* strains. While, the new isoflavonoid shows that the CMB/CMI ratio is 2 for *CM64, BM67, ATCC8739, EA289, PS299645,ATCC11296,* and *EA294* strains.

Table 5 Antiplasmodial activities

Compounds	IC_{50} (3D7 strain*P. falciparum*)
1	10.57±1.44 µM
EA	0.98±1.21 µM
Chloroquine	0.006±0.3 µM

The antiplasmodial activity of compound 1 could be explained by the presence of a methoxy group on C-4 and C-4'''. This group enhances the lipophilicity of the substance, improvingits incorporation intocells (Monks *et al*., 2002).

In conclusion, one new compound was isolated from the ethylacetate extract *Allexis cauliflora* roots. The antioxidant and antiplasmodial activities of this compound isolated was assessed in this study. Compound 1 (IC_{50} =10.57±1.44 µM) showed good antiplasmodial activity against the chloroquine-sensitive *Plasmodium falciparum* strain 3D7, but has presented a weak antioxydant activity. The antiplasmodial properties of compound 1 support the ethno medicinal use of *Allexis cauliflora* roots in the treatment of malaria.

Experimental

General experimental procedures

Melting points were determined on a Electrothermal I A 9000 series digital melting point apparatus and were uncorrected. The UV spectra were recorded on UV-570/VIS/NIP and Shimadzu UV-24012A double-beam spectrophotometers. IR measurements were obtained on a PerkinElmer (model 1600) FTIR spectrometer. The 1D (1H, 13C, DEPT) and 2D (COSY, NOESY, HSQC and HMBC) NMR spectra were recorded in DMSO-d6 and MeOH-d4 using a Bruker 600 (600 MHz for 1H NMR, 150 MHz for 13C NMR) spec-trometer. ESIMS were obtained using a MSQ Thermofinnigan instrument. Chemical shifts are stated in parts per million (ppm) from the tetramethylsilane (TMS) internal standard. Flash column chromatography was performed using silica gel 60 (Merck, 0.040–0.063 mm). TLC was conducted on pre-coated Merck Kieselgel 60 F254 plates (20 _ 20 cm, 0.25 mm). Spots were checked on TLC plates under UV light (254 nm), and developed with vanillin or KMnO4 reagents, followed by heating.

Plant material

Allexis cauliflora was collected on 7th June 2014 at Bidou II, 20 km from the town of Kribi (South Cameroon) under the leadership of M. NANA (Botanist). The identification was carried out at the National Herbarium of Cameroon by M. NANA in comparison with specimen number 18374 / HNC. Identified in Gabon and southern Cameroon (Kribi) in the Kienke forest, it is a small Shrub up to 6 m tall, with a pale brown smooth stem and small leaves. The flowering is done on the stem. It has pedicel fifteen millimeters long (Achoundong and Onana, 1998).

Extraction and isolation

Dried and powdered root of *Allexis cauliflora* (1,5 kg) was extracted with MeOH (3L) at room temperature and evaporated under vacuum to yield a crude extract (124 g). 100g of this extract wasd issolved in MeOH-H_2O (8/2) and partitioned with n-hexane (3×150 mL) and ethylacetate (3×200 mL). The ethylacetate portion (22g) was subjected to column chromatography over silica gel eluting with gradients of CH_2Cl_2/MeOH to produce 95 fractions of 250 mLeach. These fractions were combined on the basis of their TLC profiles into 3 major fractions: A (1.68 g,1–67) ; B (1.46 g, 68–80); C (1.44 g, 80–95). Fractions A (CH_2Cl_2/MeOH50:1); B(CH_2Cl_2/MeOH 40:1); C (CH_2Cl_2/MeOH 30:1). Fraction C (CH_2Cl_2/MeOH30:1) was purified by silica gel columnchromatography with a gradient of CH_2Cl_2/EtOAC (20/1) to yield compounds 1(32 mg).

4,4''-dimethoxylophorone A

White powder; IR (KBr) ymax3320, 1620, 1656, 1570, 1514, 1456 and 1231 cm^{-1};^{1}H and ^{13}C NMR data (MeOH-d4, 600 and 150 MHz) see Table1; positive HRESIMS m/z 561.1356 $[M+ Na]^+$ (calculated for $C_{32}H_{26}O_9Na$).

In vitro evaluation of the antibacterial activity of the crude extracts

7.6 g of Mueller Hinton Agar (MHA) was dissolved in 200 mL of distilled water and then heated on autoclave at 121 ° C for 30 min. After cooling the mixture was poured into the petri dishes near the beak of Bunsen.

Liquid medium

13.65 g of Mueller Hinton Broth (MHB) were dissolved in 650 ml of distilled water. A part of this medium was distributed in tubes of 15mL (10.853mL per tube which will be used for inocula). Another part was distributed in the 2 mL tubes (1.7 mL per tube for the dilution of the extracts). These tubes and the rest of medium were heated in an autoclave at 121°C for 30 min.

Culture of bacterial strains

The different bacterial strains were subcultured by the method of the streaks on MHA agar medium poured into the Petri dishes. The petri dishes were introduced into the incubator at 37 ° C. for 18 hours in order to obtain a young culture and isolated colonies. The isolated colonies were used to prepare the inoculum.

Preparation of the inoculum

Using a sterile platinum loop, a few colonies of bacteria from each strain were taken from the activation medium and each introduced into a tube containing a sterile physiological solution (0.9% NaCl). The contents of each tube were homogenized using the vortex in order to obtain a turbidity comparable to the standard scale of Mc Farland (Table 1) corresponding to the concentration of 1.5. 108 CFU / mL. Subsequently, 147 µl of the resulting suspension was removed and introduced into 10.85 mL of MHB for a volume of 11000 mL of an inoculated medium at 2.10^6 CFU / mL.

Evaluation of antioxidantactivity and antiplasmodialactivity

DPPH radical-scavengingactivity

The ability of compounds 1, and 3–7 to scavenge DPPH free radicals was evaluated according to the method of Brand-williams *et al*., 1995. A concentration series (2.0, 6.0, 10.5, 14.5 and 18.0 mg/ml in methanol) of each compound was prepared. A 4 mLaliquot of the sample solution was mixed with 1 mL of DPPH (0.04 mM in methanol). This mixture was vigorously shaken at room temperature for 30 min. The absorbance of the mixture was the nmeasured at 515 nm. A low absorbance value indicates effective free radical scavenging. Each solution was analysed in triplicate, and the average values were plotted to obtain the SC50 against DPPH

by linear regression. The activity of as corbic acid, a recognized antioxidant, was used as a standard over the same range of concentrations. The radical scavenging activity was evaluated as the percentage of inhibition according to the following equation: % inhibition = [((absorbance of control– absorbance of sample)/absorbance of control)] _ 100. 4.2. Evaluation of reducing power The reducing powers of compounds 1 and 3–7 were evaluated according to the method of Benzie and Strain (1999), withsome modifications. A concentration series (62.5, 125.0, 187.5, 250.0 and 312.5 mg/ml in ethanol) of each compound was prepared. A 25 mL test tube was loaded with 1.0 mL of sample solution, 2.5 mL of phosphate buffer (2 M, pH 6.6) and 2.5 mL of 1% (m/v) $K_3[Fe(CN)_6]$. The mixture was incubated at 45 _C for 20 min. Next, 2.5 mL of trichloroacetic acid (10% m/v) was added, and the solution was centrifuged at 4000 rpm for 15 min. A 2.5 mL aliquot of the supernatant was mixed with 2.5 mL of ultra-pure water and 0.5 mL of ferric chloride (0.1%). The absorbance of this mixture was measured at 700 nm. A greater absorbance value indicates greater reducing power. Each solution was analysed in triplicate, and the average values were plotted to obtain the IC50 of Fe3+ reduction by linear regression. The activity of an as corbic acid solution was used for normalisation. 4.3. In vitro antiplasmodial assays the culture of P. falciparum and antiplasmodial test werecarried out as previously described (Frédérich and Prosperi, 2001; Jonville *et al*., 2008). The asexualerythrocytic stage of P. falciparum chloroquine-sensitive strain 3D7 (from Prof. Grellier of Museum d'Histoire Naturelle, Paris-france) was cultivated continuously in vitro according to the procedure described by Trager and Jensen (1976) under an atmosphere of 5% CO2, 5% O2 and 90% N2 at 37 _C. The host cells were human red blood cells (A or Rh+). The culture medium was RPMI 1640 (Gibco) containing 32 mM $NaHCO_3$, 25 mM HEPES and 2.05 mM L-glutamine. The medium was supplemented with 1.76 g/l of glucose (Sigma-Aldrich), 44 mg/ml of hypoxanthin (Sigma-Aldrich), 100 mg/l of gentamycin (Gibco), and 10% human pooled serum (A or O Rh+). Parasites were subcultured every 3–4 dayswith initial conditions of 0.5% para-sitaemia and 1% hematocrit. The crudeextract and pure compounds were evaluated in vitro for the iractivity against P. falciparum (3D7). Artemisinin (98%, Sigma- Aldrich) was used as standard (IC50 4.12 ng/ml). First, stock solutions of extracts and pure compounds were prepared in DMSO at a concentration of 20 mg/ml. The solutions were further diluted in media to give 2 mg/ml stock solutions. The highest concentration of solvent to which the parasites were exposed was 1%, which was shown to have no measurable effect on parasite viability. Extracts were tested in eight serial twofold dilutions final concentrations: (200, 66.7, 22.2, 7.4, 2.5, 0.8, 0.3, 0.1 mg/ml) in 96-well microtitre plates. All tests were performed in triplicate. The results are expressed as the mean IC50 (the concentration of a drug that reduced the level of parasitaemia to 50%). 4.4. Statistical analysis The data are expressed as the means _ SD of three independentexperiments. The statistical analysis was performed with ANOVA. P-value lessthan 0.05 were considered to besignificant.

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