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RESEARCH ARTICLE

NEW ISOFLAVONOID FROM THE ROOTS OF *ALLEXIS OBANENSIS* (VIOLACEAE) AND EVALUATION OF ANTIBACTERIAL, ANTIOXIDANT AND ANTIPLASMODIAL ACTIVITIES

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ABSTRACT

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 obanensis*. 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.

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INTRODUCTION

Allexis obanensis belonging to the family *Violaceae*, is commonly distributed from Congo to Cameroon (Hutchinson, 1954). *Violaceae* plants have been constantly used in traditional medicine to treat many diseases caused by pathogenic agents. The bark of *Allexis cauliflora* are used to treat fever and syphilis (Achoundong, 2010; Achoundong, 1998). *Allexis* genera have been reported as matrix metalloproteinase inhibitors (Nganso *et al*., 2011), antioxidants (Vukics *et al*., 2008), stomach aches and antiplasmodials (Moon, 2007), but experiments to isolate antiplasmodial and antioxidant agents from *Allexis obanensis* were not conducted. Despite the availability of this ethno pharmacological information, there is no phytochemical report published on *Allexis obanensis* to date. In the present study, we report the isolation and structural elucidation of a new compound named 7-hydroxy-3-(3-hydroxy-4- methoxyphenyl) -5-methoxy-4H-chromen-4-one (1) which was extracted from the roots of *Allexis obanensis*. We also evaluated the antibacterial, antiplasmodial and antioxidant activities of this compound.

RESULTS AND DISCUSSION

The ethyl acetate fraction of *Allexis obanensis* roots was purified by column chromatography on silica gel from which we obtained one new isoflavonoid. The new isoflavonoid was obtained in the form of a white powder in the CH_2Cl_2/MeOH (50:1) solvent system. It showed the base peak of the molecular ion at m/z = 314.1236 $(M + Na)^+$, (calcd. 312.10) in HRESI MS, corresponding to the formula $C_{17}H_{14}O_6Na$. the UV spectrum showed absorption bands at λmax 241, 248 and 277nm, suggesting an isoflavonoid ring (18). The IR spectrum presented vibration bands at vmax 3324 (hydroxyl groups) 1630 and 1580 cm^{-1} (aromatic chain).On its ^1H NMR spectrum, we observed a doublet of doublet of 1H integration at δ = 7.15 ppm (J = 8.2, 1.9 Hz) and an integration doublet 1H at δ = 6.93 ppm (J = 1.9 Hz) attributable to H-6'and H-2' respectively.

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An integration doublet 1H at δ = 6.84ppm (J = 8.2 Hz) attributable to H-5'.

These three signals indicate a disubstitution of ring B. Two doublets of integration 1H each, the first at δ = 6.28 ppm (J = 2 Hz) and the second at δ = 6.42 ppm (J = 2 Hz) attributable to H-8 and H-6 respectively indicating a disubstitution of ring A in positions 5 and 7. Two singlets of integration of 3H at δ = 3.91 and 3.9 ppm revealing the presence of two methoxyl groups in the molecule. The analysis of the spectrum relative to the HSQC experiment makes it possible to assign the signals of the protons to the corresponding carbon atoms thus: The H-6'allows the attribution of the signal at δ = 115.4 ppm to the carbon atom C-6'. H-2'allows the assignment of the signal at δ = 120.8 ppm to the carbon atom C-2'. H-5'allows the assignment of the signal at δ = 112.4 ppm to the C-5' carbon atom. The two families of protons of the methoxyl groups carried the carbons at δ = 55.6 ppm; δ = 48.01 ppm. According to the information collected from the HMBC spectrum, the H-2'and H-6' protons show: Two correlation spots with the same carbon atom at δ = 146.65 ppm which can only be attributed to carbon C-4. This same carbon atom shows a correlation spot with methoxyl at δH = 3.84 ppm (δ C = 55.6 ppm), this supposes that this methoxyl group is carried by the C-4'carbon. Two correlation spots with a carbon atom at δ = 148.8 ppm attributable to C-2. The H-5'proton shows two correlation spots: The first with the carbon atom at δ = 125.0 ppm attributable to C-1' and the second with the carbon atom at δ = 146.6 ppm attributable to C-3'. This attribution is confirmed by the correlation task of this ring (C-3') and H-2'. Oxygenated carbon C-7 at δ = 161.8 ppm shows three correlation spots: The first with proton H-8 at δ = 6.28 ppm, the second with proton H-6 at δ = 6.42 ppm and the third with protons of the methoxyl group δ = 3.9 ppm, which predicts the position of the methoxy group on the C-5 carbon. This is confirmed by the correlation between proton H-6 at δ = 7.9 ppm and oxygenated carbon C-5. Hence the structure:

Antimicrobial results: The results of antibacterial and antifungal activities of EA extract and compound 1 from *Allexisobanensis* are presented in tables 1, 2, 3 and 4.The activity of ethyl acetate was not very different from that of compound 1. Nine strains of Gram-negative bacteria including reference strains (ATCC) and multi-resistant clinical isolates were used. These strains belonging to different bacterial species are distributed 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 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. Antioxidant activities were achieved by two complementary tests, namely the DPPH assay for radical-scavenging activity and the Ferric reducing-antioxidant power (FRAP) assay for assessing reducing power. The scavenging activity of DPPH is considered as classic, simple, rapid and inexpensive method for evaluating antioxidant properties. This method is based on the ability of compounds to act as H donors to reduce free radicals. However, this method is unable to highlight the reducing power of an antioxidant compound. For this reason, the FRAP assay was added as a complementary method (Messi *et al*., 2016).

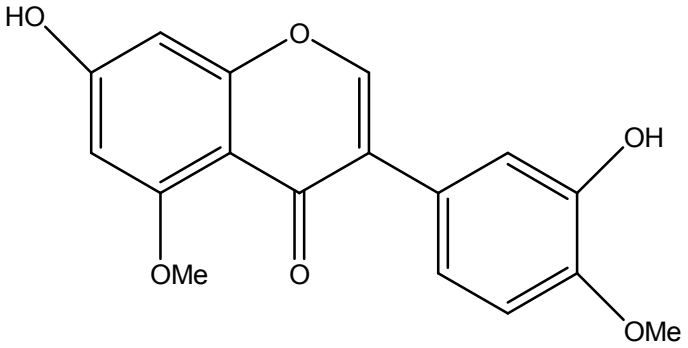
This table shows that compound 1(07.50±0.48) has presents a weakly DPPH radical scavenging activity than the standard reference ascorbic acid (SC_{50} = 4.50 mM). The reducing power assay is based on the reduction of Fe^{3+} in potassium ferricyanide to Fe^{2+} which forms a blue complex. The formation of this blue complex is monitored at ƛ= 593 nm. The greater the reducing power of the analyte, the greater the concentration of the complex formed, leading to higher absorbance values. The high antioxidant activity of phenolic substances is often attributed to their -OH moieties (18). These are potent H donors because they allow the delocalization of electrons across the molecule. This fact can explain why this isoflavonoid has a weak antioxidant activity, because some -OH groups are methoxylated. We evaluated antiplasmodial activity against the chloroquine-sensitive *Plasmodium falciparum* 3D7 strain for compound 1 and EA extract. In conclusion, one new compound was isolated from the ethyl acetate root extract of *Allexis obanensis*. The antibacterial, antioxidant and antiplasmodial activities of this compound isolated were assessed in this study. Compound 1 (IC_{50} =12.47±1.44 µM) showed good antiplasmodial activity against the chloroquine-sensitive *Plasmodium falciparum* 3D7 strain but has presented a weak antioxidant activity.It also presented good activities against many bacterial strains. The antiplasmodial properties of compound 1 support the ethno-medicinal use of *Allexis obanensis* roots in the treatment of malaria and stomach aches.

Experimental

General experimental procedures: Melting points were determined on an Electro thermal 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, ^{13}C, 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 ^{13}C NMR) spectrometer. ESIMS were obtained using an MSQ Thermofinnigan instrument. Chemical shifts are stated in parts per million (ppm) from 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 $KMnO_4$ reagents, followed by heating.

Plant material: *Allexis obanensis* was collected on 7th June 2014 at Bidou II, 20 km from the town of Kribi (South Cameroon) under the leadership of Mr. NANA (Botanist).

Extraction and isolation: Dried and powdered root of *Allexis obanensis* (1 kg) was extracted with MeOH (3L) at room temperature and evaporated under vacuum to yield a crude



Compound 1. 7-hydroxy-3-(3-hydroxy-4-méthoxyphenyl)-5-méthoxy-4H-chromen-4-one

Table 1. 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 2. 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

Table 3. 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

Table 4. Antioxidant Activities

	Radical scavenging activity		Reducing power
	SC_{50}(µM/L)	EC_{50}(µM/L)	FRAPµg EAA/mgdw
1	07.50±0.48	192.50	129.45±3.5421
AA	04.50±0.28	112.50	206.48±12.33

Table 5. Antiplasmodial activities

Compounds	**IC_{50} (3D7 strain *P. falciparum*)**
1	12.47±1.44 µM
EA	0.90±1.21 µM
Chloroquine	0.006±0.3 µM

The identification was carried out at the National Herbarium of Cameroon by Mr. NANA in comparison with specimen number 31839/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 15mm long (Achoundong and Onana, 1998).

100g of this extract was dissolved in MeOH-H_2O (8:2) and partitioned with *n*-hexane (3×150 mL) and ethyl acetate (3×200 mL). The ethyl acetate portion (35g) was subjected to column chromatography over silica gel eluting with gradients of CH_2Cl_2/MeOH to produce 79 fractions of 250 mL each. These fractions were combined based on their TLC profiles into 3 major fractions: A (5.2 g,1–40); B (3.6g, 41–55); C (3g, 56–79). Fractions A (CH_2Cl_2/MeOH50:1); B(CH_2Cl_2/MeOH 40:1); C (CH_2Cl_2/MeOH 30:1).

Fraction A (CH_2Cl_2/MeOH 50:1) was purified by silica gel column chromatography with a gradient of CH_2Cl_2/EtOAC (20:1) to yield compounds 1(18mg).

***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 burner.

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 inoculums: 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. 108CFU/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 antioxidant activity and antiplasmodial activity

DPPH radical-scavenging activity: 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).4 mL aliquot 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 then measured 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 SC_{50} against DPPH by linear regression. The activity of ascorbic acid, a recognised 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:

$$\%\ \text{inhibition} = \frac{(\text{absorbance of control} - \text{absorbance of sample})}{\text{absorbance of control}} - 100$$

Evaluation of reducing power: The reducing powers of compounds 1 was evaluated according to the method of Benzie and Strain (1999). 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 IC_{50} of Fe^{3+} reduction by linear regression. The activity of an ascorbic acid solution was used for normalisation.

***In vitro* antiplasmodial assays:** The culture of *P. falciparum* and antiplasmodial test were carried out as previously described (Frédérich, 2001).The asexual erythrocytic stage of *P. falciparum* chloroquine-sensitive 3D7 strain (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% CO_2, 5% O_2 and 90% N_2 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 days with initial conditions of 0.5% parasitaemia and 1% hematocrit. The EtOAC extract and pure compound were evaluated *in vitro*for their activity against *P. falciparum*(3D7). Artemisinin (98%, Sigma-Aldrich) was used as standard (IC_{50} 4.12 ng/mL). First, stock solutions of extract and pure compound 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. All tests were performed in triplicate. The results are expressed as the mean IC_{50} (the concentration of a drug that reduced the level of parasitaemiato 50 %).

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