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Bioactive Natural Products from Terrestrial and Marine Resources, Especially Terpenes, but not limited to… - Short Communication

Antibacterial and Antioxidant Activities of the Extract and Some Flavonoids From Aerial Parts of *Echinops Gracilis* O. Hoffm. (Asteraceae)

Fidèle Castro Weyepe Lah [1,2], Marius Balemaken Missi[1,3], Natasha October[3], Patrick Herve Betote Didoue[2], Nalova Hermia Nalova Ikome[2], Jean Pierre Abdou[4], Theodora Kopa Kowa[2], Gabriel Agbor[2], Alembert Tiabou Tchinda[2], and Etienne Dongo[2]

Abstract

*Mortality due to microbial diseases continues to be a major problem in many developing countries. The present study aims to evaluate the antibacterial and antioxidant activities of the ethyl acetate extract and some isolated compounds from aerial parts of Echinops gracilis. The phytochemical study resulted in the isolation of a new flavonoid derivative named apigenin-7-O-(4"-feruloyl)-β-D-glucoside (**1**), together with 2 known compounds: apigenin-7-O-(4"-trans-p-hydroxycinnamoyl)-β-D-glucoside (**2**), and apigenin-7-O-glucoside (**3**). Their chemical structures were determined using a combination of NMR and IR spectroscopic and MS techniques, as well as by comparison with literature data. **The extract and isolates were evaluated for their antibacterial and antioxydant properties**. The EtOAc extract and compounds **1** and **2** showed the ability to scavenge 2,2'-zino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS) with scavenging concentration (SC_{50}) values of 13.6 ± 0.8 µg/mL, 108.2 ± 4.3 µg/mL, and 28.5 ± 2.2 µg/mL, respectively. In addition, compound **1** displayed significant activity against Escherichia coli, Pseudomonas aeruginosa, and Klebsiella pneumonia, with minimum inhibition concentration (MIC) values of 31.2, 15.6, and 31.2 µg/mL respectively.*

Keywords

Echinops gracilis, asteraceae, flavonoids, apigenin-7-*O*-(4″-feruloyl)-β-D-glucoside, antioxidant, antibacterial activity

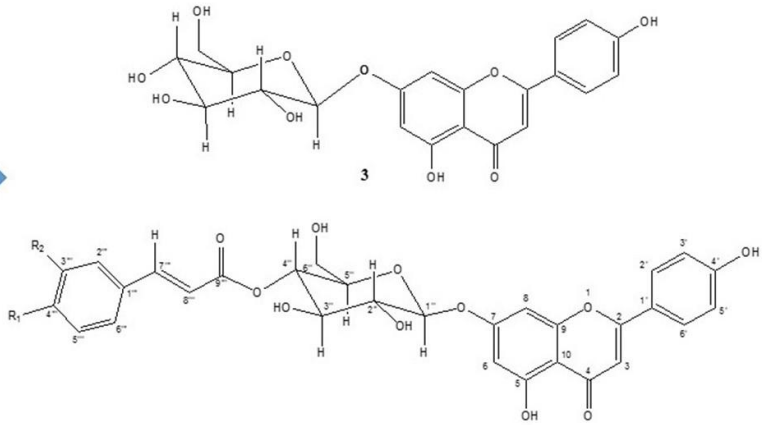
[1] Department of Organic Chemistry, Faculty of Science, University of Yaounde I, Yaounde, Cameroon
[2] Centre for Research on Medicinal Plants and Traditional Medicine, Institute of Medical Research and Medicinal Plants Studies, Yaounde, Cameroon
[3] Department of Chemistry, University of Pretoria, Pretoria, South Africa
[4] Department of Chemistry, Faculty of Science, University of Ngaoundere, Ngaoundere, Cameroon

Corresponding author(s):
Alembert Tiabou Tchinda, Centre for Research on Medicinal Plants and Traditional Medicine, Institute of Medical Research and Medicinal Plants Studies, Yaounde, Cameroon; talembert@gmail.com

Infectious diseases caused by microbes are responsible for more deaths worldwide than any other single cause. Many microbes are developing new properties to resist drug treatments that once effectively

destroyed them. Drug resistance has become a serious problem worldwide. In many regions affected by infectious diseases, local and indigenous plants are often the only available means of treating such infection. Plant natural products can also have antioxidant potential. These include phenolic compounds, alkaloids, terpenoids, and essential oils. Plant-based antioxidant compounds play a defensive role by preventing the generation of free radicals and hence are extremely beneficial to alleviate infectious diseases that generate free radicals as well as diseases caused by oxidative stress.

The genus *Echinops*, **belongs to the** family of Asteraceae **which** comprises over 120 species, most of which are distributed in tropical Africa and in temperate areas of Europe and Central Asia.[1] *Echinops* species are traditionally used to treat different infectious diseases including trachomas, sepsis, typhoid, gonorrhea, and ulcerative lymphangitis. They are also used to treat different ailments that might be caused by bacterial or fungal infections including fever, respiratory diseases, toothache, leucorrhoea, and earache. Thus they have been investigated for their antimicrobial properties.[2] Previous chemical investigations of this genus established the presence of thiophenes,[3,4] acetylenic thiophenes,[5] sesquiterpene hydrocarbons,[6] triterpenes, flavonoids, alkaloids,[4] sesquiterpene lactones,[7] lignans,[8] and hydroxycinnamates.[9]



Antioxidant activity
Antibacterial activity

Previous phytochemical studies on the roots of *E. gracilis* revealed the presence of erythrinasinate, vogelate, ferulic acid, *p*-coumaric acid, ursolic acid, oleanolic acid, and quercetin. Moreover, the anti-inflammatory activity of *E. gracilis* extracts and some isolated compounds has been established.[10] We herein report the isolation and structural elucidation of one new flavonoid derivative; apigenin-7-*O*-(4”-feruloyl)-β-*D*-glucoside (**1**) and 2 known compounds apigenin-*7*-*O*-(4″-*trans*-*p*-hydroxycinnamoyl)-*β*-*D*-glucoside (**2**) and apigenin-*7*-*O*-glucoside (**3**) from the aerial parts of *E. gracilis*. Considering the traditional uses and the in vitro antimicrobial activities[2] of some *Echinops* species such as *E. amplexicaulis*, *E. giganteus,* and *E. keberiko,* and to contribute to the global fight against microbial diseases, antibacterial and antioxidant tests were performed on the ethyl acetate extract, as well as some isolated compounds of *E. gracilis*.

Results and Discussion

The ethyl acetate fraction of the methanol extract of *E. gracilis* aerial parts was subjected to open column chromatography (CC) over silica gel to give compound **1** and 2 known flavonoids, namely apigenin-*7-O*-(4″-*trans-p*-hydroxycinnamoyl)-*β-D*-glucoside] (**2**)[11] and apigenin-7-*O*-(β-D-glucoside) (**3**)[12] (Figure 1).

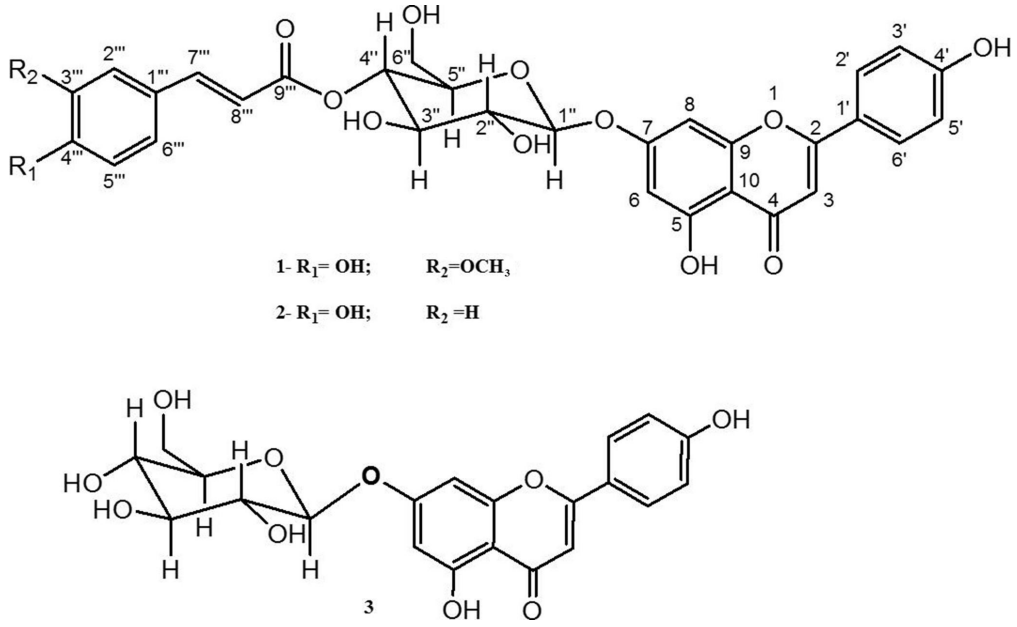


Figure 1 Structures of isolated compounds.

Compound **1** was obtained as yellow amorphous powder. It exhibited a molecular ion peak at *m/z* 607.1437 as $[M-H]^-$, corresponding to the molecular formula $C_{31}H_{28}O_{13}$ (calcd. 607.1452) in ESI-TOFMS (Supplemental Figure S3). The ^{13}C NMR spectrum confirmed the presence of 31 carbons consisting of 1 methyl, 1 methylene, 17 methines, and 12 non-protonated carbons, among which was a signal of carbonyl (C = O) at δ 166.0 ppm. The UV spectrum showed characteristic absorption bands of a flavone at λ_{max} 240 nm and 275 nm (log ε = 1.48)[13] (Supplemental Figure S5). IR absorptions suggested the presence of a hydroxyl group (3350 cm^{-1}), and an *α,β*-unsaturated carbonyl group (1638 cm^{-1})[13] (Supplemental Figure S2). In the ^1H NMR spectrum, we noticed the presence of AA'BB' signals at δ 7.95 (2H, d, *J* = 8.8 Hz, H-2′, H-6′) and δ 6.94 (2H, d, *J* = 8.8 Hz, H-3′, H-5′), which was characteristic for the B-ring in **1** (Figure 1). The coupling constant between the olefinic protons at δ 7.58 and 6.53 (*J* = 15.8 Hz) indicated that H-7‴ and H-8‴ are *trans*-oriented.[14] HMBC correlations between the olefinic proton at δ 7.58 (1H, d, *J* = 15.8 Hz, H-7‴) and the carbonyl C-9‴ at δ 166.0 ppm and between the same olefinic proton and carbons of the ABX system protons C-2‴ at δ 111.1 ppm, C-6‴ at δ 123.4 ppm and C-5‴ at δ 115.6 ppm indicated the presence of a disubstituted cinnamoyl moiety.

The ^1H NMR signals at δ 12.99 and 9.70 ppm, in addition to their HMBC correlations, indicated the presence of chelated C-5 and free C-4‴ hydroxyl groups in the cinnamoyl moiety. The HMBC correlations unambiguously confirmed a linkage between the anomeric proton, H-1″ of the glucose moiety and C-7 (δ 162.8 ppm) (Table 1, Figure 2). Furthermore, additional HMBC correlations between H-4″ (δ 4.79 ppm) and feruloyl carbonyl, C-9‴ at δ 166.0 ppm revealed that the feruloyl moiety is attached at position 4″ (Figure 2). These correlations are further supported by the NOESY spectrum, which showed correlations between H-4″ and the olefinic proton in the β position of carbonyl, C-9‴ (δ 166.0 ppm). All these NMR data are close to those of apigenin 7-*O*-(4″-*trans-p*-coumaroyl)-*β*-D-glucoside (**2**)[11] and chrysoeriol-7-*O*-(4''-*O*-(E)-coumaroyl)- β –glucopyranoside).[15] A signal, which resonated strongly at δ 55.7 ppm, was assigned to the methoxy group, which is linked to the cinnamoyl

moiety, with regard to an ABX system. NOESY correlations between the *β*-olefinic proton at δ 7.58 ppm and the methyl proton at δ 3.83 ppm and the proton at δ 7.35 ppm linked to C-2‴, showed that the methoxy group is in position 3‴ (Figure 2). Therefore, compound **1** was identified as a new flavonoid derivative with a semi systematic name of apigenin-7-*O*-(4”-feruloyl)-β-D-glucoside (Figure 1).

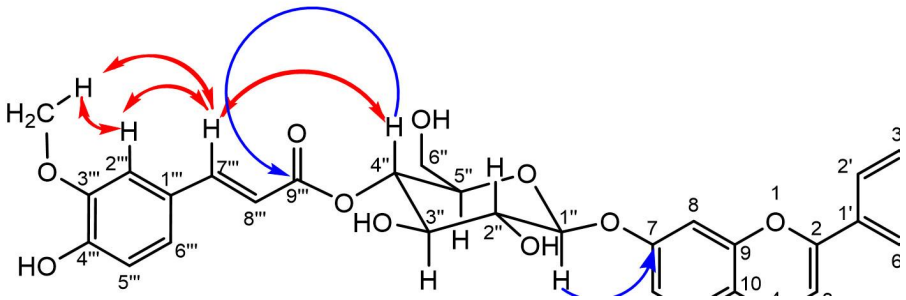


Figure 2 Some HMBC and NOESY correlations in compound 1.

Table 1. ^{13}C NMR (100 MHz, DMSO-D_6) and ^{1}H NMR (400 MHz, DMSO-D_6) Spectroscopic Data of Compound 1 (δ in ppm; *J* in Hz).

N°	δ_C	δ_H
2	164.4	
3	103.2	6.88 (s, 1 H)
4	182.1	
5	161.4	
6	99.5	6.50 (d, *J* = 2.2, 1H)
7	162.8	
8	95.0	6.86 (d, *J* = 2.2, 1H)
9	157.0	
10	105.5	
1'	121.0	
2'/6'	128.7	7.95 (dd, *J* = 2, 8.8, 2H)
3'/5'	116.1	6.94 (dd, *J* = 2, 8.8, 2H)
4'	161.2	
1‴	125.6	
2‴	111.1	7.35 (d, *J* = 2.0, 1H)

3'''	148.0	
4'''	149.5	
5'''	115.6	6.81 (d, J = 8.1, 1H)
6'''	123.4	7.13 (dd, J = 8.1, 2.0, 1H)
7'''	145.5	7.58 (d, J = 15.8, 1H)
8'''	114.4	6.53 (d, J = 15.8, 1H)
9'''	166.0	
1''	99.7	5.23 (d, J = 7.7, 1H)
2''	73.3	3.49 (m, 1H)
3''	74.9	3.80 (dd, J = 8.1, 2.0, 1H)
4''	70.7	4.79 (t, J = 9.7, 1H)
5''	73.9	3.62 (m, 1H)
6''	60.4	3.46 (m, 2H)
5-OH		12.99
4'-OH		9.70
OCH_3	55.7	3.83 (s, 3H)

The antioxidant activities of the EtOAc extract and isolated compounds (**1** and **2**) were evaluated and compared with gallic acid for a positive control. The EtOAc extract and compounds **1** and **2** showed ABTS radical scavenging abilities with SC_{50} of 13.6 ± 0.8 µg/mL, 28.5 ± 2.2 µg/mL, and 108.2 ± 4.3 µg/mL, respectively (Supplemental Table S1). In addition, compounds **1** and **2** and the ethyl acetate extract of *E. gracilis* displayed reduction potential (RP) abilities. From these results, we noticed that compound **1** exhibited better radical scavenging activity than compound **2**.

The ethyl acetate extract, along with compounds **1** and **2**, were tested for their antibacterial effects against clinical isolates of *E. coli, P. aeruginosa, K. pneumoniae,* and *H. influenza,* with Levofloxacine as a reference drug. The antibacterial activity criteria were as follows: substances with a MIC <100 µg/mL were considered as significantly active; 100 < MIC ≤ 625 µg/mL moderately active and weakly active when the MIC >625 µg/mL.[16] Compounds **1** and **2** showed inhibition of clinical isolates of *E. coli, P. aeruginosa*, *K. pneumoniae,* and *H. influenza* with MIC values of 31.2, 15.6, 31.2, 12.5 µg/mL (compound **1**) and 62.5, 62.5, 31.2, 62.5 µg/mL (compound **2)** (Supplemental Table S2). These findings showed that the methoxy group influences antibacterial activity and increases the sensitivity of the bacterial strains to compound **1** compared to compound **2**. The results generated in this study are consistent with previous studies, confirming that substituted flavones exhibit pronounced antimicrobial[17,18] and antioxidant[19] activities.

Experimental

General Experimental Procedures

Electrospray ionization mass spectra (ESIMS) were recorded on a QSTARXL of AB Sciex Company, UV and visible spectra, recorded in MeOH at 25 °C, on a Kontron Uvikon spectrophotometer, IR spectra on a FT PerkinElmer 1750 FTIR spectrometer, and NMR spectra on a Bruker 400 MHz NMR Avance II spectrometer equipped with a cryoprobe, with TMS as internal reference. Chemical shifts were recorded in δ (ppm) and the coupling constants (*J*) are in Hertz. Silica gel 60 F254 (70-230; Merck; Darmstadt, Germany) was used for column chromatography. Precoated silica gel Kieselgel 60 F_{254} plates (0.25 mm thick) were used for TLC, and compounds were detected by spraying with 50% H_2SO_4 followed by heating at 100 °C. All solvents were distilled before use. Optical density values were determined on a Thermo-Fisher-Scientific: Evolution 300 UV-VIS.

Plant Material

Aerial parts of *Echinops gracilis* were collected from the Fongo-Tongo Leweh neighborhood in the West Region of Cameroon during February, 2014. A voucher specimen (No 66943/HNC Cam), authenticated by ethnobotanist Dr. Tsabang Nole, is located at the Cameroon National Herbarium, Yaoundé.

Extraction and Isolation

Dried plant powder (3 kg) was soaked in 12 L of methanol (MeOH) for 72 hours at room temperature to yield the crude extract (190 g), after evaporation under vacuum. This extract was subjected to liquid-liquid separation with a mixture of n-hexane (0.5 L × 4) and MeOH 80% (1L). The phase made up of 80% MeOH was treated with ethyl acetate (EtOAc) (0.5 L × 4) to afford 19.5 g of EtOAc extract. The extract was subjected to open column chromatography (CC) over silica gel, eluting with a DCM-MeOH mixture of increasing polarity (from 30:1 to 1:1). Eighty-eight fractions (250 ml each) were collected and grouped into 5 major fractions [**A** (650 mg), **B** (152 mg), **C** (254 mg), **D** (855 mg), and **E** (485 mg)] based on their TLC profiles. Compounds **1** (13.9 mg) and **2** (18.9 mg) were obtained directly from the main column in sub fractions **A** and **B**. Fraction **E** (485 mg) was successively purified by Merck silica gel column chromatography to give compound **3** (8 mg).

ABTS Radical Cation Decolorization Assay

The method used to determine the antioxidant activities is based on following the discoloration kinetics of the $ABTS^{+\cdot}$ ion.[20] ABTS (2, 2'-azinobis-(3-ethylbenzothiazolin-6-sulfonic acid)) was prepared by mixing 0.0384 g of ABTS and 0.00662 g of potassium persulfate ($K_2S_2O_8$) with 10 ml of distilled water. The mixture was incubated for 16 hours at room temperature in a dark room. For the actual analysis, the ABTS solution was diluted with ethanol and the absorbance adjusted to 0.700 (±0.02) at 734 nm (initial optical density), which is stable at 30 °C. In a test tube, 3.0 ml of this diluted ABTS solution was added in 30 µL of the sample of different concentrations. The tubes were agitated to homogenize the mixture. Absorbance readings were taken at 734 nm immediately after incubation of 20 minutes. Gallic acid was used as the antioxidant reference at the same concentrations as the samples. The inhibition percentage was calculated according to the following formula:

$$I\ (\%) = \frac{\text{Abs control} - \text{Abs sample / gallic acid}}{\text{Abs control}} \times 100$$

where, $Abs_{control}$ is the absorbance of control and $Abs_{sample/gallic\ acid}$ is the absorbance of the sample or gallic acid.

Ferric Ions (Fe^{3+}) Reducing Antioxidant Power Assay

The ferric reducing antioxidant power assay (FRAP) is based on the reduction of the tripyridyltriazine ferric complex (Fe^{3+}-TPTZ) to the tripyridyltriazine ferrous complex (Fe^{2+}-TPTZ) in the presence of an antioxidant. To 2000 µL of FRAP in test tubes were added 75 µL of either extracts or gallic acid at different concentrations.[21] The tests were performed in triplicate, and the mixture was incubated for 30 minutes. The optical density was measured at 593 nm. The FRAP solution (pH 3.6) was prepared as follows: 14.1 mg of TPTZ was diluted in 9 ml 40 mM HCl, then ferric chloride ($FeCl_2$ 20 mM) and acetate buffer (300 mM, pH = 3.6) were mixed in the ratio of 1:1:10, respectively to form the FRAP solution.

In Vitro Antibacterial Activity

Clinical isolated **bacterial** strains of *Haemophilus influenza, Pseudomonas aeruginosa, Escherichia coli,* and *Klebsiella pneumonia* provided by the University Teaching Hospital in Yaounde, **were used to evaluate antibacterial activity**.

Solutions of the ethyl acetate extract and the isolated compounds were prepared in concentrations of 30 mg/mL and 1 mg/mL, respectively, in sterile distilled water to final volumes of 1 ml. Each solution was homogenized until clear solutions were obtained.

A microplate containing 96 wells was used for this study. 100 µL of Muller Hinton liquid medium supplemented with 10% glucose was introduced into each well. 100 µL of the stock solution of the ethyl acetate extract to be tested, prepared at 30 mg/mL, was introduced into the first term wells. An identical volume of the 1 mg/mL isolated compounds/levofloxacin solution was used. Final testing concentrations ranges were 7500 µg/mL to 7.32 µg/mL (for ethyl acetate extract) and 250 µg/mL to 0.488 µg/mL (for isolated compounds and levofloxacin). A volume of 100 µL of concentrated bacterial inoculum at 1.5×10^8 CFU/mL was introduced into each well to a final density of 7.5×10^7 CFU/mL. Thus, the final volume was 200 µL per well and all the tests were conducted in triplicate. In parallel, a third series of wells was used as a negative control containing only the culture medium and the sample, and other wells were used as a positive growth control and containing the culture medium and inoculum. The microplate was incubated at 37 °C for 18 to 24 hours. The color change was developed by adding a few drops of Alamar blue (Centre Pasteur; Cameroon) to each well and then incubating for 30 minutes. The change in coloration of the wells from blue to pink indicated bacterial growth. The MIC is defined as the lowest concentration of extract that inhibited bacterial growth visible to the naked eye.[22]

For MBC, 50 µL from each well with a concentration greater or equal to the MIC was taken and added to 150 µL of broth. The microplate was incubated for 18 to 24 hours and then developed with Alamar blue. The MBC was considered to be the cup with the lowest concentration of extract that did not change color.[22]

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Declaration of Conflicting Interests

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ORCID iD

Fidèle Castro Weyepe Lah https://orcid.org/0000-0003-4650-0847

Supplemental Material

Supplemental material for this article is available online.

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