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A New Biflavonoid Glycoside from the Leaves of *Ziziphus mucronata* Willd. (Rhamnaceae)

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ABSTRACT

A new compound was isolated from the n-butanol fraction of the crude methanol extract of the leaves of *Ziziphus mucronata*, a plant that is widely used in ethnomedicine to treat inflammation, diarrhoea, tumour, cough, sores, asthma, measles, fever, infections and urinary problems, amongst numerous indications. The isolation of the compound was carried out by a combination of silica gel column and sephadex gel filtration chromatography and the structure was elucidated with the help of UV, IR, and 1D, 2D - ^{1}H and ^{13}C NMR spectroscopic analysis. The compound, a glycoside biflavonoid, was established as *bis* (quercetin 3-*O*-*β*-D-glucopyranoside). The compound might be responsible for some of the observed biological activities of the plant *Ziziphus mucronata*.

Keywords: *Ziziphus mucronata*, Isolation, Structure elucidation, *bis* (quercetin 3-*O*- *β*-D-glucopyranoside).

Introduction

The use of natural products especially plants for healing is as ancient and universal as medicine itself because they have been an integral part of the ancient traditional medicine systems such as the Chinese, Ayurvedic and Egyptian systems of medicine.[1] Natural products are known to possess several phytochemicals responsible for their pharmacological activities or acts as the precursor for the synthesis of novel drugs.[2] Even now, continuous traditions of natural product therapy exist throughout the third world, especially in the occident, where numerous minerals, animal substances and plants are still in common use. One of such plants is *Ziziphus mucronata* of the rhamnaceae family. It is commonly known as buffalo thorn and is a spiny shrub or small to medium-sized tree that grows up to 20 m high with a spreading canopy.[3] The leaves are used in African traditional medicine for the treatment of diarrhoea, tumour, cough, sores, ear inflammation, asthma, syphilis, gonorrhoea, measles, fever and psychiatric disorder.[3] Phytochemical screening of the methanol extract of the leaves had revealed the presence of saponins, flavonoids, tannins, triterpenes and steroids; catechins[4] and triterpenoids were previously isolated from the leaves of the plant. The ethnomedicinal use of the leaves in the treatment of pain and inflammation was recently validated scientifically.[5] Despite its varied medicinal potentials, there is paucity of information on the isolation and characterization of bioactive compounds from this plant; only the isolation of cyclopeptides have been reported in the scientific database.[6] In continuation of our work aimed at isolating the principles responsible for the observed diverse biological activities, we present herein, the isolation and structure elucidation of *bis* (quercetin 3-*O*-*β*-D-glucopyranoside) from the n-butanol fraction of the crude methanol leaf extract of *Z. mucronata*.

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Materials and Methods

General experimental procedures

The solvents used were of high quality (analytical grade) and include: methanol, n -hexane, chloroform, ethyl acetate and n-butanol purchased from Sigma Co. USA; silica gel 60-120 µm (Qualikems, India) was used for column chromatography, sephadex LH-20 (GE Healthcare) was used for purification of isolated compound, thin layer chromatography (TLC) was carried out on aluminum-backed Kieselgel 60 F_{254} TLC plate (Merck no. 5554, Darmstadt, Germany) and a Gallenkhamp electro thermal melting point apparatus was used to determine the melting point of isolated compound.

The UV was recorded on Thermo scientific biomate 6 UV-Visible spectrophotometer; the IR was recorded on Agilent Technologies Cary 6030 Fourier Transform Infrared Spectrophotometer; ^{1}H–NMR (500 MHz, MeOD) and ^{13}C–NMR (125 MHz, MeOD) spectra were recorded on a Bruker AVANCE-500 spectrophotometer (Japan). The chemical shift values (δ) were reported in parts per million (ppm) relative to internal standard TMS and coupling constants (*J* values) were given in Hertz.

Collection and identification of the plant material

The leaves of *Z. mucronata* were collected from Kudingi village, Giwa Local Government Area of Kaduna State, Nigeria in 2015. It was authenticated by taxonomist Musa Muhammad in the Herbarium Unit, Department of Biological Sciences, Ahmadu Bello University, Zaria by comparison with the existing herbarium specimen, voucher number 900328.

Preparation of the extract

The leaves of the plant were dried under shade at room temperature until a constant weight was obtained and size-reduced manually using mortar and pestle. About 1 kg of the pulverized leaf material was extracted with methanol by cold maceration for 72 hours with occasional shaking and concentrated *in vacuo* using rotary evaporator at 40ºC. This yielded a dark green gummy (134 g) crude extract; 100 g of the extract was suspended in 500 mL of distilled water and filtered using Whatman No. 1 filter paper.

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The water-soluble portion was partitioned successively with 400 mL each of ethyl acetate and n–butanol to obtain ethyl acetate fraction (EAF) and n-butanol fraction (nBF), respectively.

Column Chromatography of n-butanol fraction

About 4 g of the n-butanol soluble fraction of the extract was adsorbed onto about 6 g of silica gel and mounted over a glass column (75 cm x 3.5 cm) packed with 200 g silica gel and chromatographed. The column was eluted continuously with ethyl acetate 100 % followed by gradient mixtures of ethyl acetate and methanol; the progress of separation was monitored using TLC with solvent system: ethyl acetate/chloroform/methanol/water: 15:8:4:1; a total of 68 collections of 50 mL aliquots each, was made and similar collections were pooled together based on their TLC profile to obtain thirteen major fractions coded A-M.

Fraction F (12.2 mg) obtained from the solvent gradient mixture, ethyl acetate/methanol 90:10, containing three major spots was subjected to further purification using sephadex-LH20, eluting with methanol. The progress of separation was monitored on TLC plate. Repeated gel filtration afforded 4.8 mg yellow crystalline solid compound that was subsequently coded as 'S_1'.

The compound gave a single homogenous spot with two solvent systems of different polarity, ethyl acetate/chloroform/methanol/water; 15:8:4:1 and 15:4:4:1 indicating its purity. The isolated compound, S_1, was subjected to physical and some chemical tests as well as spectral analysis to elucidate its chemical structure.

Results and Discussion

The pulverized leaf material (1 kg) yielded 13.4 % w/w crude methanol extract; 7.5 % ethyl acetate fraction (EAF) and 12 % n-butanol fraction (nBF) were respectively obtained from the water-soluble portion of the extract.

The compound S_1 revealed an uncorrected melting point range of 243-245°C and was found to be soluble in methanol and insoluble in n-hexane. It gave a Prussian blue colour with freshly prepared ferric chloride solution indicating the presence of phenolic nucleus[7]; the compound also produced a red colour with concentrated hydrochloric acid in the presence of magnesium chips (Shinoda test). This is indicative of a flavonoid nucleus.[7]

Spectral Analysis

The UV spectrum of figure 1 showed two absorption bands within the UV region (λ_{max} 226 nm and 257 nm) and two bands within the visible region (λ_{max} 346 nm and 363 nm) in methanol. These shoulder bands are characteristic of compounds with a highly conjugated system.[8]

The Infrared spectrophotometer spectrum of figure 1 showed two important peaks at 1697 cm^{-1} and 3447 cm^{-1} on a KBr disc due to carbonyl and hydroxyl functional groups, respectively.

The ^{1}H–NMR spectra of figure 1 exhibited overlapping signals of two sets of metacoupled protons in a tetra-substituted benzene ring system: [δ_H 6.19 (2H, d, J = 2.0 Hz, H-6, 6'''), 6.38 (2H, d, J = 2.0 Hz, H-8, 8''')] and two sets of a tri-substituted benzene ring (ABX spin pattern): [δ_H 6.88 (2H, dd, J = 2.0 Hz, 8.5 Hz, H-5', 5''), 7.59 (2H, dd, J = 2.0 Hz, 8.5 Hz, H-6', 6''), 7.84 (1H, d, J = 2.0 Hz, H-2') and 7.71 (1H, d, J = 2.5 Hz, H-2'')]. The chemical shift values and the integrated protons in rings A and B of figure 1 suggest that the compound, S_1, has a biflavonoid nucleus. Ten of the methine signals clustered around δ_H (3.22 - 3.85) corresponding to carbon signals δ_C (62.0 - 78.5) suggest the presence of two sugar residues. The two anomeric proton signals at [δ_H 5.15 (1H, d, J = 8.0 Hz, H-1*) and 5.24 (1H, d, J = 7.5 Hz, H-1**)] are typical of glucose.[9] The high coupling constants observed (8.0 Hz and 7.5 Hz) are due to diaxial coupling with H-2 proton of each of the residue and confirmed the configuration of both sugars as β-D glucosyl.[9]

The proton noise decoupled ^{13}C-NMR disclosed 42 significantly overlapping peaks. The presence of two carbonyl carbon peaks at δ_C 179.48 and 179.44 further confirmed the dimeric nature of S_1. The DEPT experiment revealed the presence of 20 methine carbon resonances; the two methylene carbon signals at δ_C 62.6 (C-6*) and δ_C 62.0 (C-6**) also indicated the two sugar moieties as glucose. Moreover, the C-3 resonances observed in the downfield region (δ_C 135.7 and δ_C 135.8) relative to C-10 (δ_C 105.0) (the same chemical environment) further confirmed that the glycosylation is at C-3 position.[10] The ^{13}C–NMR also showed ten oxygen bearing quaternary carbons. Six carbons have hydroxyl groups attached and two are linked to pyranone oxygen. This suggested that the remaining two should be involved in interflavonoid ether linkage. Thus, S_1 must be a biflavonoid with either 4'-4'' or 3'-4'' ether linkage.[11] The downfield resonance of C-4'' compared to C-4' suggested a 4'-4'' linkage. The linkage was further confirmed by analyzing the heteronuclear multiple bond correlation (HMBC) spectral data (see below).

The ^{1}H - ^{1}H COSY spectra of S_1 also showed important cross peaks between δ_H 3.82 and δ_H 3.47 and the two sugar moieties at δ_H 5.15 and δ_H 5.24, in addition to the ortho and meta correlations observed in the benzene rings. Other important COSY correlations occurred between carbinol protons attached to adjacent carbons in the two sugar residues (Figure 2).

The heteronuclear multiple quantum coherence experiment (HMQC) facilitated the attachment of all protons to their respective carbons (Table 1).

The unambiguous assignment of the carbons and the placement of the sugar moieties were facilitated using the long-range correlation experiments (HMBC) (Table 2). In the spectrum, a common J_3 correlation between protons at δ_H 6.19 and 6.38 to carbons at δ_C 95.1 and 100.4, respectively confirmed their assignment to rings A of both flavonoid residues. Also, a J_2 correlation between protons at δ_H 6.88 to carbons at δ_C 123.0 and 123.3 and a J_3 correlation to carbon at δ_C 146.0 confirmed their assignments to ring B. A J_3 correlation between anomeric protons at δ_H 5.15 and 5.24 to the quaternary carbons at δ_C 135.7 and 135.8 confirmed that each of the sugar moiety is linked to the individual flavonoid aglycone through C-3 carbon, thereby confirming the biflavonoid diglycoside to be derived from a flavonol nucleus. Other correlations confirmed by the HMBC are those of the sugar residue. Based on the foregoing spectral analysis, S_1 was confirmed to be a biflavonoid and with the aid of 2D NMR correlations, the structure of S_1 was proposed to be *bis* (quercetin 3-*O*- β-D-glucopyranoside) (Figure 1).

C-O-C linked biflavonoids are those in which two monomeric units may be of the same or different structural types joined to each other through an ether linkage.[12] Unlike C-C linked biflavonoids, C-O-C linked biflavonoids have restricted distribution in the plant kingdom.[11] They are classified into various groups due to the nature of the interflavonyl linkage between them and have been used as chemotaxonomic markers for different families of plant e.g. Ochna flavones first isolated from the Ochnaceae family.[13]

Some of the reported pharmacological activities of biflavonoids include: inhibition of histamine release from mast cells and inhibition of lymphocyte proliferation, suggesting the anti-inflammatory/anti-allergic potential of biflavonoids.[12]

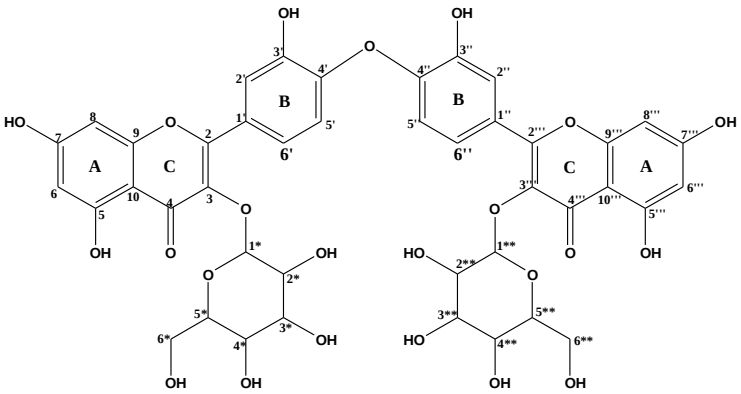

Figure 1: *bis* (quercetin 3-*O*- β-D glucopyranoside)

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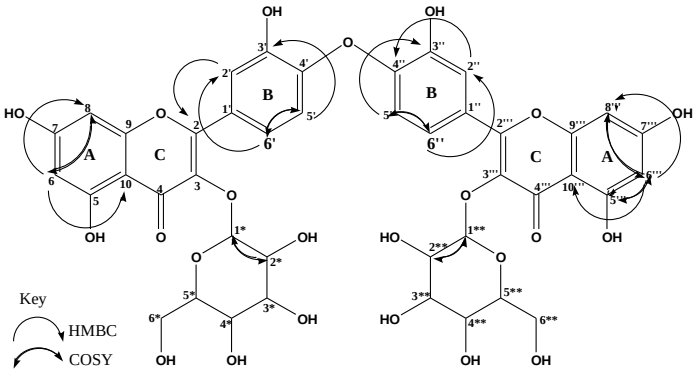

Figure 2: Important COSY and HMBC correlations of S_1

Conclusion

The isolation of this biflavonoid from *Z. mucronata* seems to support some folkloric use of the plant in African traditional medicine, in the management of inflammatory conditions. Anti-inflammatory studies and the possible mechanism of action of compound S_1 are in progress in our laboratory.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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Table 1: HMQC and DEPT spectral data of S_1

Position	δ_C	δ_H	DEPT
1			
2	158.98		C
3	135.66		C
4	179.44		C
5	163.06		C
6	100.42	6.19	CH
7	167.52		C
8	95.10	6.38	CH
9	158.98		C
10	105.34		C
1'	123.26		C
2'	117.83	7.84	CH
3'	146.04		C
4'	150.50		C
5'	116.09	6.88	CH
6'	123.25	7.59	CH
1''	123..26		C
2''	117.59	7.71	CH
3''	146.04		C
4''	158.50		C
5''	116.17	6.88	CH
6''	122.98	7.59	CH
1'''			
2'''	159.10		C
3'''	135.82		C
4'''	179.48		C
5'''	163.10		C
6'''	100.42	6.19	CH
7'''	167.52		C
8'''	95.10	6.38	CH
9'''	159.10		C
10'''	105.41		C
1^*	105.59	5.15	CH
2^*	75.83	3.54	CH
3^*	78.23	3.45	CH
4^*	70.11	3.85	CH
5^*	73.28	3.82	CH
6^*	62.46	3.72	CH_2
1^{**}	104.5	5.24	CH
2^{**}	75.21	3.57	CH
3^{**}	78.49	3.46	CH
4^{**}	71.30	3.84	CH
5^{**}	77.28	3.48	CH
6^{**}	62.02	3.72	CH_2

Table 2: HMBC correlations of S_1

δ_H	δ_C
6.19 (H-6,6''')	95.10(C-8,8'''),100.42(C-6,6'''),105.34(C-10),105.41(C-10'''),163.06(C-5),163.10(C 5'''),167.52(C-7,7''')
6.38 (H-8,8''')	95.10(C-8,8'''),100.42(C-6,6'''),105.34(C-10),105.41(C-10'''),158.98(C-9),159.10(C-9'''),167.52(C-7,7''')
6.88 (H-5',5'')	116.09(C-5'),116.17(C-5''),122.98(C-6''),123.25(C-6')123.26(C-1',1''),146.04(C-3',3''),150.50(C-4'),158.5(C-4'')
7.59 (H-6',6'')	117.59(C-2''),117.83(C-2'),122.98(C-6''),123.25(C-6')123.26(C-1',1''),150.50(C-4',),158.5(C-4''),158.98(C-9),159.10(C-9''')
7.71 (H-2'')	117.59(C-2''),122.98(C-6''),123.26(C-1''),158.50(C-4''),159.10(C-2')
7.84 (H-2')	117.83(C-2'),123.25(C-6'),123.26(C-1'),150.50(C-4'),158.98(C-2)

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