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Isolation and characterization of some flavonoids from the leaf of *Tapinanthus globiferus* growing on *Acacia nilotica*

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Tapinanthus globiferus is used ethnomedicinally for the treatment of bacterial infections, inflammation, stomach pain, ulcers among others. The aim of the study was to isolate bioactive compounds from the leaf of *T. globiferus* growing on *Acacia nilotica*. The powdered plant material was extracted with 90 % methanol using cold maceration and the resulting crude methanol leaf extract was partitioned into *n*-hexane, chloroform, ethylacetate and *n*-butanol fractions. The ethylacetate fraction was chromatographed on a silica gel, sephadex LH-20 column and preparative thin-layer chromatography. (-)-Epicatechin and Quercetin 3-O-β-D-glucopyranoside were isolated and characterized by means of physiochemical and spectroscopic (1D and 2D-NMR) analyses for the first time from *T. globiferus* growing on *A. nilotica.*

Keywords: *Tapinanthus globiferus*; Flavonoids; Isolation; NMR.

1. Introduction

The plant kingdom, with its remarkable diversity of natural compounds, has merited special interest (Lewinsohn and Gijzen, 2009). Among these compounds, flavonoids have received much research and attention (Harborne and Williams, 2000; Kesarkar *et al*., 2009; Buer *et al*., 2010). They not only function as stress protectants in plants (Hahlbrock and Scheel, 1989; Cespedes *et al.,* 2001), and UV protectants (Goto and Kondo, 1991; Li *et al*., 1993)), but also have multi-beneficial biological activities such as antioxidative (Ammar *et al*., 2010; Ghasemzadeh *et al*., 2010), anticarcinogenic (Seelinger *et al*., 2008), antimicrobial (Zhou *et al*., 2007; Pereira *et al*., 2007), antimutagenic (Liverio *et al*., 1994), anti-inflammatory (Ueda *et al*., 2002), antiallergic (Mastuda *et al*., 2002) and anti-obesity (Kamisoyama *et al*., 2008) properties.

Tapinanthus globiferus (A. Rich) belonging to the *Loranthaceae* family is a hemi-parasite with glabrous pendulous stems up to 1.2 m long with roots that mostly grows on the branches of a large number of tree species including *Acacia*, *Vitellaria*, *Kola*, *Citrus*, *Combretum*, *Aloe* and *Terminalia* as host trees (Waterberg *et al*., 1989; Polhill and Wiens, 1998). Ogunbolude *et al*. (2014) reported the identification and quantification of quercetin and some phenolic acids from *T. globiferus* growing on other host. Extensive literature search revealed that there is no report yet on the isolation and characterization of any compound from the plant *T. globiferus* growing on *Acacia nilotica*. We report herein, the isolation and characterization of (-)-epicatechin and Quercetin-3-O-β-D-glucopyranoside from the leaf of *T. globiferus* growing on *A. nilotica*.

2. Materials and Methods

2.1 General Procedures

The NMR experiments were conducted on a Bruker AVANCE spectrometer (500 MHz) with residual solvent (TMS) as internal standard. The melting points of the isolated compounds were determined on an electrothermal melting point apparatus. Thin layer chromatography (TLC) was carried out using silica gel 60 F_{254} pre-coated aluminium sheets (Sigma Aldrich, Germany). Column chromatography was conducted using LOBA Cheme silica gel (60-200) mesh in a sintered glass funnel. Gel filtration chromatography was performed using sephadex LH-20 Spots on TLC plates were visualized by spraying with 10 % H_2SO_4 followed by heating at 105 ^{O}C for 10 minutes.

2.2 Collection, Identification and Preparation of Plant Material

Tapinanthus globiferus growing on *Acacia nilotica* was collected at Dundaye village of Wamakko Local Government Area of Sokoto State, Nigeria in August 2019. It was identified and authenticated by Malam Abdul-azeez of the Herbarium unit, Department of Biological Sciences, Usmanu Danfodiyo University Sokoto, with a voucher specimen number (UDUH/ANS/0327). The plant material was shade dried, pulverized and stored in a polythene bag for further use.

2.3 Extraction and Isolation of Compounds

The pulverized leaf of *T. globiferus* (1.437 kg) was exhaustively extracted with 4.5 L of 90 % methanol for 9 days with occasional shaking. The extract was filtered and the filtrate was evaporated to dryness using rotary evaporator at 40 ℃ to afford crude methanol leaf extract (128.5 g). The crude extract (120 g) was suspended in 500 mL of distilled water, then filtered and partitioned successively with solvents of increasing polarity to afford *n*-hexane, chloroform, ethylacetate and *n*-butanol fractions. The ethylacetate fraction (3.7 g) was subjected to silica gel column chromatography with eluting solvents systems consisting of different ratio of mixtures of *n*-hexane: ethyl acetate, 100 % ethylacetate, different ratio of mixtures of ethyl acetate: methanol, and 100 % methanol. TLC was used to monitor the column. A total of 150 collections were made and combined based on their TLC profile to afford eleven (11) major fractions coded EA-EK. Fraction EC was purified using sephadex LH-20 column with dichloromethane (100 %), mixtures of dichloromethane: ethylacetate, mixtures of ethylacetate: methanol to methanol (100 %) as solvent systems. Two (2) mL each of a total of 88 collections were made and combined based on their TLC profile to afford six (6) major fractions coded EC1-EC6. Fractions EC3 and EC6 were merged and further purified using preparative TLC with a mixture of ethylacetate: chloroform: methanol: water in the ratio of 15: 4: 4: 1 as solvent system. After development, the plates were dried and bands of interest were scraped using spatula and then dissolved in sufficient quantity of methanol and ethyl acetate. It was filtered, the filtrates were allowed to dry. TLC analysis of the samples obtained using a mixture of ethylacetate: chloroform: methanol: water (15: 4: 4: 1) as solvent system gave a single homogenous spot which led to the isolation of compounds L1 and L2.

3. Results and Discussion

3.1 Results

Spectral data

The isolate L1 is a yellow crystalline solid compound which is soluble in methanol and ethylacetate with a melting point of 178 - 179 ℃. The ^{1}H –NMR spectrum (in CD_3OD, 500 MHz) of the compound L1 showed signals at δ_H 5.89 (1H,d, J = 2.0 Hz, H – 8), δ_H 5.71 (1H, d, J = 2.0 Hz, H – 6), δ_H 2.66 (1H, dd, J = 4.0, 16.3 Hz, H – 4b), δ_H 2.69 (1H, dd, J = 4.5, 16.3 Hz, H – 4a), δ_H 4.73 (1H, brs, H – 2), δ_H 4.65 (1H, d, H – 3), δ_H 6.65 (1H, d, 1.0 Hz, H - 2′), δ_H 6.66 (1H, d, J = 2.5 Hz, H – 6′) and δ_H 6.89 (1H, s, H – 5′). The ^{13}C – NMR (500 MHz, CD_3OD) and DEPT experiments of L1 showed the presence of 15 carbon atoms; signals at δc 155.7 (C – 5), 156.6 (C – 7), 156.2 (C – 9), 98.4(C – 10), 28.2 (C – 4), 144.5 (C – 3′), 78.0 (C – 2), 144.4 (C – 4′), 95 (C- 6), 94.1 (C – 8), 114.7 (C – 2′), 117.9 (C – 6′) and 114.9 (C – 5′). The DEPT further revealed the multiplicity of the carbons as one methylene (CH_2), seven methine (CH) and seven quaternary (C) carbon atoms.

The isolate L2 is also a yellow crystalline solid compound, soluble in methanol but insoluble in chloroform; m.p 223 - 224 ^{o}C. The ^{1}H-NMR (CD_3OD, 500 MHz) of the compound L2 revealed chemical shift values/integration as follows: δ_H 6.34 (1H, d, J = 2.0 Hz, H-8), δ_H 6.18 (1H, d, J = 1.5 Hz, H-6), δ_H 7.34 (1H, d, J = 2.0 Hz, H-2'), δ_H 6.89 (1H, d, J = 8.5, H-5'), δ_H 7.30 (1H, dd, J = 2.0, 8.3 Hz, H-6'), δ_H 5.35 (1H, d, J = 1.5 Hz, H-1'') and δ_H 3.66 - 3.76 (m, sugar protons). The ^{13}C-NMR and ^{13}C-DEPT experiments (500 MHz, CD_3OD) of L2 revealed the presence of 21 carbon atoms. Seven aromatic carbon peaks were observed for L2 at δc 123.0 (C-6'), 122.8 (C-1'), 95.0 (C-8), 105.5 (C-10), 122.8 (C-1'), 116.9 (C-5') and 116.4 (C-2'). Other peaks include 159.1 (C-2), 136.1 (C-3), 179.5 (C-4), 73.3 (C-3''), 71.9 (C-4''), 64.4 (C-6'') and 72.1 (C-5'').

3.2 Discussion

Compound L1 was obtained as a yellow solid substance; the sharp melting point observed by the compound indicates its purity (John, 1964) and it tested positive to ferric chloride reagent suggesting the presence of phenolic nucleus (Silva *et al*., 1998). The 1D- and 2D-NMR data of compound L1 revealed chemical shift values typical of flavonoids (Yusuf *et al*., 2019). The presence of an AX system (1,2,3,5 – tetra-substituted benzene ring A) was assigned from the protons at δ_H 5.89 (1H, d, J = 2.0 Hz, H – 8) and δ_H 5.71 (1H,d, J = 2.0 Hz, H – 6), while an ABX system (1,3,4 – trisubstituted benzene ring B) was depicted via the protons at δ_H 6.65 (1H,

d, *J* = 1.0 , H – 2ˈ), δ_H 6.66 (1H, d, *J* = 2.5 Hz, H – 6ˈ) and δ_H 6.89 (1H, s, H – 5ˈ). The presence of an aliphatic ring was clearly discerned from the proton chemical shift values observed at δ_H 4.73 (1H, s, H–2) and δ_H 4.65 (1H, d, *J* = 4.5 Hz, H–3) representing an oxymethine and a carbinol proton respectively, consistent with a saturated ring C (Yusuf *et al.*, 2019). Two protons with signals at δ_H 2.69 (1H, dd, *J* = 4.5, 16.3 Hz, H–4a) and δ_H 2.66 (1H, dd, *J* = 4.0, 16.3 Hz, H – 4b). These protons assigned to C – 4 are characteristic of a flavonoid of 3-flavon basic nucleus (Yusuf *et al*., 2019). The chemical shift value for H-2 δ_H (4.73) which appeared as a singlet is an indication that compound L1 is an (-) – epicatechin rather than (+) – catechin (Yusuf *et al*., 2019; De mello *et al.,* 1996). ^{1}H–^{1}H COSY established the correlations between the protons at δ_H 4.65 (H- 3) δ_H 2.69 (H–4b), and δ_H 2.66 (H–4a) which confirmed the assignment of ring C while the cross peaks correlations observed between δ_H 6.65 (H–2ˈ) and δ_H 6.89(H – 5ˈ) further strengthened the assignment of ring B. The ^{13}C – NMR (500MHz, CD_3OD) and DEPT experiments indicated the presence of 15 carbon atoms. Compound L1 exhibited 7 aromatic carbon peaks at δc 95.0 (C – 6), 94.1(C – 8), 98.4(C – 10), 130.6 (C – 1ˈ), 114.7 (C – 2ˈ), 114.9 (C – 5ˈ) and 117.9 (C – 6ˈ), five oxygenated carbon atoms at 155.7 (C – 5), 156.6 (C – 7), 156.2 (C – 9), 144.4 (C – 4ˈ) and 144.5 (C – 3ˈ) and the three aliphatic carbons at δc 78.0 (C – 2), 64.9 (C – 3) and the methylene carbon at 28.2 (C – 4) suggested that the compound L1 to be an epicatechin (Abdullahi *et al.,* 2017; Yusuf *et al*., 2019). The absence of a down field signal around δ_C 82 (C–2) confirms compound L1 to be an (-) – epicatechin rather than (+) – catechin (De mello *et al.,* 1996; Yusuf *et al*., 2019). HSQC experiments established the attachment of various protons to their respective carbons, the protons at δ_H 4.73 correlated with δc 78.0 and δ_H 2.69, 2.66 correlated with δc 28.2 among others. Comparison with a reference NMR data (Yusuf *et al.,* 2019*)* showed a good match and based on the above, the structure of compound L1 was confirmed to be (2R, 3R) -3,4-dihydro-2-(3, 4-di hydroxyl phenyl)-2H-chromene 3, 5, 7 triol or (-)- epicatechin (Figure 1).

Compound L2 was- obtained as a yellow solid substance; it gave positive reaction with ferric chloride suggesting the presence of phenolic nucleus and Fehling's test indicating the presence of sugar moiety (Silva *et al*., 1998). The sharp melting point demonstrated by the compound indicated its purity (Tang *et al.,* 2000). The basic skeletal structure of L2 was confirmed from the ^{1}H-NMR data. The presence of two meta-coupled protons (ring A) at δ_H 6.18 (1H, d, *J* = 1.5 Hz) and δ_H 6.34 (1H, d, *J* = 2.0 Hz) assignable to H-6 and H-8 respectively was observed. An ABX system was clearly discerned from the protons signals at δ_H 6.89 (1H, d, *J* = 8.5 Hz, H-5'), δ_H 7.34 (1H, dd, *J* = 2.0 Hz, H-2') and δ_H 7.30 (1H, dd, *J* = 2.0, 8.3 Hz, H-6'). The sugar moiety was defined by the resonances around 3.65 – 3.76, the doublet at δ_H 3.66 (*J* = 5.0 Hz) typical of the $–CH_2$ group suggest the sugar to be a glupyranoside (Boots *et al*., 2008). The ^{1}H-^{1}H-COSY established the correlation between protons that are adjacent to each other, proton at δ_H 6.18 correlates with the proton at δ_H 6.34 ppm, while proton at δ_H 6.89 was found to be correlating with the proton at δ_H7.34 ppm. These correlations further confirmed the position of the protons in the ^{1}H-NMR of L2 (Sani *et al.,* 2015). The ^{13}C-NMR (500 MHz, CD_3OD) and DEPT experiments of compound L2 revealed the presence of 21- carbon atoms, seven aromatic carbon atoms signals at δc 105.5 (C-10), 100.3 (C-6), 95.0 (C-8), 122.8 (C-1'), 123.0 (C-6'), 116.9 (C-5') and δ 116.4 (C-2'). Eight quaternary oxygenated carbon atoms at δ 167.4 (C-7), 163.1 (C-5), 159.1 (C-9), 149.9 (C-4'), 146.5 (C-3'), 136.1 (C-3), 159.1 (C-2) and a down field signal due to carbonyl resonating at δc179.5 which further suggesting the compound to be Quercetin (Tang *et al*., 2000). The chemical shift value δc 103.5 was assigned to the anomeric carbon (C-1''). Other resonances such as 72.0 (C-2''), 73.3 (C-3''), 71.9 (C-4'') and 72.1 (C-5'') were characteristic of sugar absorptions and the $-CH_2$ absorption at δc 64.4 (C-6''). Suggest the sugar to be a glupyranoside which is consistent with ^{1}H-NMR data of quercetin glupyranoside (Boots *et al.,* 2008). The HSQC spectrum of L2 was used to attach each proton to their respective carbon atoms. Proton at δ_H 6.18 correlates with the carbon at δc 100.3, proton at δ_H 6.34 correlates with the carbon at δ_C 95.0, proton at δ_H 6.89 correlates with the carbon at δ_C 116.9, proton at δ_H 7.30 correlates with carbon at δ_C 123.0, proton at δ_H 7.34 correlates with carbon at δ_C 116.4, proton at δ_H 5.35 correlates with anomeric carbon at 103.5, δ_H 3.76 correlated with- δ_C 72, δ_H 3.74 correlated with δ_C 73.7 and δ_H 3.66 correlated with δ_C 64.4 among others. The connectivity between various fragments was established through Heteronuclear Multiple Bond Correlation spectroscopy (HMBC). It established the correlation between proton at δ_H 6.18 (H-6) with the carbons at δ_C 167.4 (C-7) and δ_C 105.5 (C-10) proton at δ_H 6.34 (H-8) showed a long range correlation with the carbons at δ_C 163.1 (C-5) and δ_C 105.5 (C-10) which further confirmed the 1,2,3,5- tetra-substituted benzene ring A. Correlation between proton at δ_H 6.84 (H-5') with the carbons at δ_C 122.8 (C-1'), δc 146.5 (C-3') and δ_C 149.9 (C-4'); proton at δ_H7.30 (H-6') is correlating with carbons at δ_C 116.4 (C-2') and δ_C 146.5 (C-3') and proton at δ_H 7.34 (H-2') is correlating with carbons at 123.0 (C-6'), δ_C 149.9

(C-4') and δ_C 159.1 (C-2) further confirmed the 1,3',4'-trisubstituted benzene ring B. Correlation between proton at δ_H 3.74 (H-3'') with carbon at δ_C 136.1 (C-3) and proton at δ_H 3.66 (H-4'') with carbon at δ_C 179.5 (C-4) also confirmed the glucose attachment at carbon position 3. Based on 1D and 2D-NMR data of L2 and comparison with the reported literature (Beck and Haberlein, 1999; Tang *et al.,* 2000), the structure of L2 was confirmed to be Quercetin -3 – O – ß – D – glupyranoside (Figure 2).

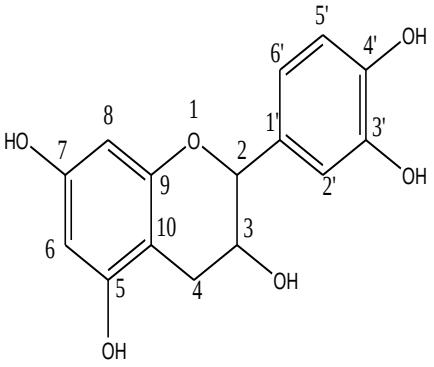

Figure 1: (-)-Epicatechin

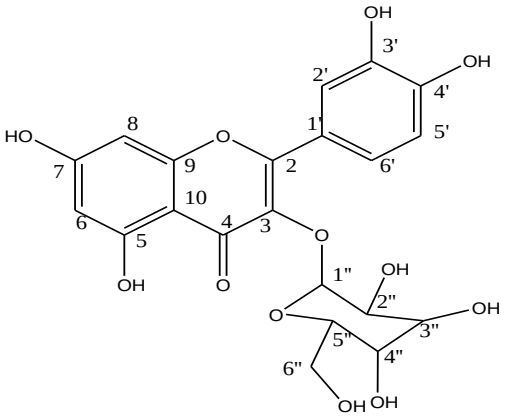

Figure 2: Quercetin -3 - O - ß - D - glupyranoside

Table 1: 1D and 2D spectral data summary for Compound L1 (CD_3OD, 500 MHz)

Position	δ ^{1}H (Hz)	δ ^{13}C	DEPT	COSY
1	-	-	-	-
2	4.73 (1H, brs)	78.0	CH	-
3	4.65 (1H, d, *J* = 4.5)	64.9	CH	-
4	2.69 (1H, dd, *J* = 4.5, 16.3)	28.2	CH2	H-4a
	2.66 (1H, dd, *J* = 4.0, 16.3)			H-4b
5	-	155.7	C	-
6	5.71 (1H, d, *J* = 2.0)	95.0	CH	H-8
7	-	156.6	C	-
8	5.89 (1H, d, *J* = 2.0)	94.1	CH	H-6
9	-	156.2	C	-
10	-	98.4	C	-
1'	-	130.6	C	-
2'	6.65 (1H, d, *J* = 1.0)	114.7	CH	-
3'	-	144.5	C	-
4'	-	144.4	C	-
5'	6.89 (1H, s)	114.9	CH	H-6'
6'	6.66 (1H, d, *J* = 2.5)	117.9	CH	H-5'

Table 2: 1D and 2D -spectral data summary for Compound L2 (CD_3OD, 500 MHz)

Position	δ ^{1}H (Hz)	δ ^{13}C	DEPT	COSY	HMBC
1	-	-	-	-	-
2	-	159.1	C	-	-
3	-	136.1	C	-	-
4	-	179.5	C	-	-
5	-	163.1	C	-	-
6	6.18 (1H, d, *J*= 1.5, H-6)	100.3	CH	H – 8	C-7; C-10
7	-	167.4	C	-	-
8	6.34 (1H, d, *J*= 2.0, H-8)	95.0	CH	H – 6	C-5; C10
9	-	159.1	C	-	-
10	-	105.5	C	-	-
1'	-	122.8	C	-	C-3'; C-4'
2'	7.34 (1H, d, *J*= 2.0, H-2')	116.4	CH	H-5'	-
3'	-	146.5	C	-	-
4'	-	149.9	C	-	-
5'	6.89 (1H, d, *J*= 8.5, H-5')	116.9	CH	H-2'	C-1'; C-3'; C-4'
6'	7.30 (1H, dd, *J*= 2.0, 8.3)	123.0	CH	-	-
1''	5.35 (1H, d, *J*= 1.5, H-1'')	103.5	CH	-	-
2''	3.76 (1H, d, *J*= 3.0, H-2'')	72.0	CH	-	-
3''	3.74(1H, dd, *J*=3.0, 9.5 H-3'')	73.3	CH	-	C-3
4''	3.68 (1H, H-4'')	71.9	CH	-	C-4
5''	3.67 (1H, d, *J*= 3.5)	72.1	CH	-	-
6''	3.66 (2H, d, *J*= 5.0)	64.4	$-CH_2$	-	-

4. Conclusion

Chromatographic studies of ethylacetate fraction of *T. globiferus* afforded two flavonoids (2R, 3R)-3, 4-di hydro-2- (3, 4-di hydroxyl phenyl)-2H-chromene-3, 5, 7 triol or (-)-epicatechin and Quercetin 3-O-β-D-glucopyranoside.

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Conflict of interest

The authors declare no conflict of interest.

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