ISOLATION OF DIHYDROQUERCETIN GLYCOSIDE FROM THE ROOT BARK OF *CALOTROPIS PROCERA* AND ANTIOXIDANT AND CYTOTOXIC SCREENING OF THE CRUDE EXTRACTS.

A. Usman*¹, R.H. Mohammad², A.O. Abdullahi³, A.H. Zakari⁴ and N.L. Usman⁵

¹Department of Chemistry, Nasarawa State University, P.M.B. 1022 Keffi, Nigeria.

²General Science Department, Soran University, Iraq.

³Department of Chemistry, Gombe State University P.M.B 127, Gombe State, Nigeria.

⁴Department of SLT, Federal Polytechnic Nasarawa, Nasarawa State, Nigeria.

⁵Department of Science, Nasarawa State Polytechnic Lafia. Nigeria.

*Correspondent Author: ausman2015@yahoo.com, +23480100427276

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Abstract

The root bark of *Calotropis procera* (Family: *Asclepiadaceae*) was extracted with methanol. The methanolic extract was separated into hexane, chloroform, ethyl acetate, and water soluble fractions. The ethyl acetate fraction was subjected to bioassay-guided fractionation and final purification was achieved by column chromatography. The structure of the compound was elucidated by spectroscopic methods (ESI-MS, ¹H and ¹³C NMR, COSY, HSQC, and HMBC) and the comparison of the data obtained with that reported in the literature. It was concluded that the compounds isolated was taxifolin 4'-*O*- β -D-glucopyranoside, a dihydroquercetin glycoside. The crude extracts of hexane, chloroform, ethylacetate, and water soluble fractions of methanol extract and the isolated compound were subjected to antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and brine shrimp lethality bioassay. The result shows that the maximum inhibitions in the DPPH assays were isolated compound (51.7 %), methanol (80.7 %), and water (29.2 %) fractions while ascorbic acid standard was (84.4 %). However, the results for hexane, chloroform and ethyl acetate fractions in the DPPH assays were poor and hence discarded. The results for the in vitro cytotoxicity activity shows that ethyl acetate and hexane fractions showed significant cytotoxicity with LC50 value of 1.0 $\pm$ 0.2 and 2.4 $\pm$ 0.1 respectively.

Keywords: *Calotropis procera*, Column chromatography, Spectroscopic method, Cytotoxicity, Isolation.

Introduction

Calotropis procera, is a non-cultivated, xerophytic shrubs that are widely distributed in tropical and subtropical region of Africa, Asia, South America and North America, with only few species growing in temperate regions. It belongs to the family of *Asclepiadaceae* and it is commonly known as 'Milk weed or Swallow-wort' in English, 'Arka' in India and 'Tumpafiya' in Northern parts of Nigeria [1, 2, 3]. During the Vedic time in India, the leaves of this plant has great aesthetic and cultural value because of its use in Sun and Shiva worship [4], while the nomadic Fulani women of Northern Nigeria use this plant in the production of local soft cheese called 'warakasi' which is a cuddling agent [5].

This plant is highly regarded for its diverse medicinal properties in Sudanese, Unani, Arabic and Ayurveda traditional medicinal system, as well as its toxic potentials [6]. Different parts of *C. procera* has been used in the treatment of different diseases. The whole plant of *C. procera* is used

in the treatment of skin infections, the bark is used for spleen complaints, ringworms, protracted labour, leprosy, dysentery, and syphilis. Also, the root bark is used in the treatment of dyspepsia, constipation and jaundice [7]. The leaves are used as painkillers and for fever management, while the flowers is used for curing piles, asthma and cholera. And the latex has traditionally been used to treat and manage toothache, tumours, syphilis, and leprosy, as well as an antiseptic and also for poisoning arrows [8, 9, 10]. *C. procera* has also been regarded as very effective in the therapy and management of free radical related diseases [11].

C. procera has been reported to be poisonous or injurious to the body after prolonged usage or consumption of an overdose [12]. The latex if ingested between 4-5 ml may rupture the muscle of intestine and colon and this may lead to death [13]. It is also an irritant to the skin and mucous membrane and is highly toxic to human eyes and produces sudden painless dimness of vision with photophobia leading to blindness. And the latex

extracted from the stem has been used to make poison arrows [14]. The pharmacological studies of *C. procera* showed different parts to possess different biological activities. The whole plant has been reported to showed antimalarial activity [15], the root bark exhibit antifertility, antitumor and anticonvulsant [16, 17, 18], the latex displayed analgesic, antioxidant, antidiarrheal and inflammatory [14, 19, 20], while the flowers shows anthelmintic and hepatoprotective activities [21, 22].

The phytochemical studies of *C. procera* has resulted in the isolation of several useful compounds such as cardenolide, triterpenoids, alkaloids, resins, anthocyanins and proteolytic enzymes found in the latex [23, 24], the leaves contains α -amyrin, α -amyrin acetate, β -sitosterol, urosolic acid, cardenolides, calotropin 1, calotropagenin [25, 26]. The flower contains quercetin-3-rutinoside, sterol, calactin, calotoxin, calotropagenin, calotropin, polysaccharides with D-arabinose, glucose, glucosamine and L-rhamnose [27, 28]. While the bark contains triterpenes, calotropterpenyl ester, calotropursenyl acetate and calotropfriedelenyl acetate, akundarol isovalerate, mundarolisovalerate and quercetin-3-rutinoside [29, 30]. This study report for the first time, the isolation and characterization of dihydroquercetin glycoside, a flavonoid, from the ethyl acetate soluble fraction of the root bark of *C. procera*. The antioxidant capacity of the crude extracts and the isolated compound as well as their cytotoxic activity towards brine shrimps have been investigated.

Materials and methods

Thin layer chromatography (TLC) and Preparative thin layer chromatography (PTLC) were conducted on precoated E. Merck TLC silica gel 60 F254 glass plates. Column chromatography (CC) was performed on Fluorochem silica gel (60Å), and visualization of the compound was done using UV lamp,

UVL-14 EL hand held 220V 50Hz 4W 254nm white light by UVP. The ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance (400 MHz) spectrometer with internal references of δH 3.31 and δC 49.0 ppm for CD_3OD using TMS (Tetramethylsilane) as an internal standard. A Thermo Instruments HPLC system mass spectrometer with an electrospray ionization (ESI) source was used for recording of the mass and UV spectra. Optical rotation were measured in methanol solution on a ADP 440+ polarimeter. The melting point was determined by a Stuart instrument and is uncorrected.

Sample collection and Preparation

The root bark of *C. procera* were collected from mature trees at various locations within Lafia metropolis in Nasarawa State, Nigeria, West Africa. The sample was authenticated by the Botanist at the National Institute of Pharmaceutical Research and Development (NIPRD), Abuja where a voucher specimen NIPRD/H/6893 was deposited.

The sample was washed in clean water to remove dirt, air dried at room temperature for five weeks, pulverized with the aid of pestle and mortar into fine particles. It was stored in air tight container and kept away from moisture until required for the experiment.

Isolation

The powdered root bark of *C. procera* (1.5 kg) was soaked in 4 L of methanol for 7 days at room temperature. The extracts was filtered through fresh cotton bed and finally Whatman No. 1 filter paper. The filtrate was concentrated at 40 °C with a rotary evaporator to produced 110 g of total extract. Total extract (50 g) was dissolved in 500 ml of water and partitioned sequentially with hexane (4 × 500 ml), chloroform (4 × 500 ml) and ethyl acetate (4 × 500 ml) to give the hexane (7.1 g), chloroform (2.1 g), ethyl acetate (3.7 g) and water (18.9 g) soluble fractions. The proton NMR profiles of all the sample were assessed. The hexane, chloroform and water fractions showed poor proton NMR profiles and were discarded, while ethyl acetate fraction showed good profile and was investigated further. The ethyl acetate fraction (3.5 g) was subjected to silica gel column chromatography (100 g, 4 × 50 cm) using the wet packing method in methanol. Hexane and ethyl acetate were used as the eluents at the gradient mixture of (100:0→0:100 v/v) to yield 41 fractions and based on their TLC profiles were combined into six primary fractions A-F. Fraction C, (65 mg), obtained with 50 % hexane in EtOAc, were combined and evaporated to dryness under vacuum at 40°C. The combined extract was further purified by PTLC. The plate was developed using Hexane – EtOAc – MeOH (3:2:1, v/v). The main band with pink colour visualized under UV lamp was scraped from the plate and eluted with the same developing solvents, yielding the isolated compound (7 mg).

DPPH-free radical scavenging activity

The antioxidant activity of isolated compound and other crude extracts were determined by the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay, and was performed according to the method described by Lefahal *et al.*, [32] with slight modification. Fifty

microliters of the extracts and ascorbic acid standard at various concentrations (12.5–800 µg/mL) were added to 150 µL of a methanol solution of DPPH (0.4 M) in a sample bottles. The reaction mixtures were vigorously shaken for 40 seconds in a Vortex apparatus and then incubated at room temperature for 30 min in the dark. The absorbance of the mixture was measured at 517 nm with UV/VIS spectrophotometer. The ability of the sample to scavenge DPPH free radical was determined from the following equation:

$$\text{Percentage inhibition} = \frac{X_{\text{control}} - X_{\text{sample}}}{X_{\text{control}}} \times 100$$

Where, X control is the absorbance of the control reaction (containing all reagents except the test extract or standard), and X sample is the absorbance of the test extract or standard.

Cytotoxicity activities

The cytotoxicity screening of the isolated compound and other crude extracts against *Artemia salina* were carried out in an in vitro simplified assay, as described earlier with some modifications by Meyer *et al.*, [33]. Briefly, 0.5mL aliquot of various concentration of the sample extracts 500, 250, 125, 75, 37.5, 18.75 and 9.375 µg/ml were dissolved in 1 ml of DMSO. Then 1 ml each of these standard concentrations was added to test tubes containing 10 shrimps in simulated brine water. After 24 h, the median lethal concentration (LC50) of the test samples was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration. Vincristine sulphate was used as positive control in this assay to compare the cytotoxicity of the sample extracts.

Acid hydrolysis

Isolated compound (5 mg) was dissolved in methanol (20 ml) and 20 ml of 5% H₂SO₄ was added and refluxed for 6 hours. On cooling, the reaction mixture was neutralized with saturated sodium carbonate and washed with ethyl acetate (2 × 10 ml) [34]. The aqueous portion was concentrated and compared to standard sugars while the EtOAc portion was compared to aglycone using the TLC systems (CHCl₃/MeOH/H₂O, 7:3:1) [35].

Result and Discussion

Result

The isolated compound was a yellow needle; ESI-HRMS (negative ion mode): *m/z* 465.1012 [M-H]⁻, ¹H-NMR (CD₃OD) δ: 4.98 (1 H, d, *J* = 11.4 Hz, H-2), 4.57 (1 H, d, *J* = 11.4 Hz, H-3), 5.94 (1 H, s, H-6, 8), 7.40 (1 H, d, *J* = 1.4 Hz, H-2'), 6.91 (1 H, d, *J* = 8.4 Hz, H=5'), 7.11 (2 H, dd, *J* = 1.48,

8.4 Hz, H- 6'), 4.85 (1H, d, *J* = 7.58 Hz, H-1'), 3.26~3.51 (5H, m, H-2"-5"). 3.90 (2 H, dd, *J* = 1.50, 11.76 Hz, H- 6"); ¹³C-NMR (CD₃OD) δ: 84.9 (C-2), 73.5 (C-3), 198.4 (C-4), 168.9 (C-5), 97.4 (C-6), 165.3 (C-7), 96.3 (C-8), 164.4 (C-9), 101.8 (C-10), 130.0 (C-1'), 118.2(C-2'), 146.55 (C-3'), 149.0 (C-4'), 116.9 (C-5'), 124.6 (C-6'), 104.1 (C-1"), 74.9 (C-2"), 77.6 (C-3"), 71.5 (C-4"), 78.4(C-5"), 62.6 (C-6"). The ¹H-NMR and ¹³C-NMR data were in agreement with that of taxifolin 4'-O-β-D-glucopyranoside (Fig. 1).

Acid hydrolysis

The retention time of the isolated compound was 0.38 while that of standard sugars were as follows: D-galactose (0.32), D-glucose (0.38), D-mannose (0.42), D-xylose (0.58) and L-rhamnose (0.65). Comparing the retention time of the isolated compound with those of standard sugars, we concluded that the sugar was a D-glucose.

Discussion

The compound was isolated as a yellow needle, the ESI-HRMS showed *m/z* 465.1048 [M - H]⁻, suggesting a molecular formula of C₂₁H₂₂O₁₂. The IR spectrum showed peaks at 3368 cm⁻¹, 2927 cm⁻¹, 1727 cm⁻¹, 1640 cm⁻¹ and 1166 cm⁻¹ corresponding to O-H stretch, saturated C-H stretching, C=O, C=C and C-O stretching respectively. The ¹H NMR spectrum (Fig. 2) displayed a pair of meta coupling proton signals at 5.94 (s, H=6) and 5.91 (s, H=8) as shown in ring X. The proton signals of ring Y appeared at 4.98 (d, *J*=11.4 Hz, H=2) and 4.57 (d, *J*=11.4 Hz, H=3) while ring Z showed three aromatic protons signals at 7.40 (d, *J*=1.4 Hz, H=2'), 6.91 (d, *J*=8.4 Hz, H=5') and 7.11 (dd, *J*=1.4, 8.4 Hz, H=6') which is in the form of an ABD spin-system signifying a flavonol. The ¹³C spectrum (Fig. 3) showed twenty one carbon signals, which comprised of one methylene, twelve methine and eight quaternary carbons. On complete acid hydrolysis of the isolated compound, glucose was detected in the aqueous phase while ¹H and ¹³C NMR spectra of the organic phase showed signals that are similar to that of taxifolin [36] with the exception of signals for the glucose moiety and the downfield shift of the resonance at C-4' (δc 149.0), suggesting it to be the site of glycosylation. The spectrum also showed one anomeric proton at δ_H 4.85 (H-1'', d, *J*=7.5 Hz), and based on this coupling constant, the configuration of the sugar moiety was determined to be β-oriented indicating a β-glycosyl moiety. The anomeric carbons appeared at δc 104.1 and other signals for the glucose occurred at δc 73.50 (C-2''), 77.62 (C-

3''), 71.69 (C-4''), 78.37 (C-5'') and 62.55 (C-6'')

which are typical signals for the glucose.

Table 1. ¹H and ¹³C NMR data of taxifolin 4'-O-β-D-glucopyranoside in deuterated methanol (CD₃OD).

Atom no.	Oh, 2014 (δ _c)	Isolated Compound (δ _c)	Isolated Compound (δ _H)	Oh, 2014 (δ _H)
2	83.4	84.9	4.98, d (11.40)	5.24 d (9.0)
3	72.4	73.5	4.57, d (11.40)	4.88 d (9.0)
4	196.2	198.4		
5	169.1	168.9		
6	97.3	97.4	5.94 (s)	5.89 (s)
7	165.6	165.3		
8	95.4	96.3	5.91 (s)	5.89 (s)
9	164.1	164.4		
10	102.4	101.8		
1'	128.9	130.0		
2'	116.0	118.2	7.40, d (1.40)	6.96 (d, 1.8)
3'	146.0	146.5		
4'	147.0	149.0		
5'	116.3	116.9	6.91 d(8.40)	6.75 (d, 8.1)
6'	121.2	124.6	7.11 dd, (1.40, 8.40)	6.80 (dd, 1.8, 8.1)
1''	104.6	104.1	4.85 d (7.58)	4.67 (d, 7.8)
2''	75.4	74.9	3.51 (m)	3.10-3.21(m)
3''	77.7	77.6	3.49 (m)	3.10-3.21(m)
4''	71.5	71.5	3.26 (m)	3.10-3.21(m)
5''	78.0	78.4	3.37 (m)	3.10-3.21(m)
6''	62.9	62.6	3.90 dd (1.50, 11.76)	3.80 (dd, 1.2, 11.7)
			3.67 dd (5.91, 11.76)	3.57 (dd, 3.9, 11.7)

The correlation between anomeric proton and carbon (H1''/C1'', 4.86/103.85) and other correlations between carbons and hydrogens are displayed in HSQC spectrum (Fig. 4). The coupling of the anomeric proton signal H-1'' with H-2'' (δ_H 3.51) and H-2 with H-3 (δ_H 4.57) was observed in the COSY spectrum (Fig. 5) while in the the HMBC spectrum (Fig. 6), the H-1'' was linked to C-4' (δ_C 149.01), signifying that the β-D-glucose moiety was located at C-4' of the taxifolin skeleton. The ¹H and ¹³C NMR data (Table 1) of this compound are consistent with the reported literature values [37].

Plants phytochemicals such as phenolic, flavonoids, tannins, coumarins and stilbenes have antioxidant activity due to their redox properties and chemical structures. Recent studies has shown that only flavonoids with certain structure and hydroxyl group in a particular positions in the molecule can act as proton donors and show radical scavenging ability [38, 39]. Flavonoids suppress reactive oxygen formation, chelate trace

elements involved in free-radical production, scavenge reactive species and up-regulate and protect antioxidant defences [40]. Natural antioxidants have been reported to have less side effects as compared to their synthetic analogues like ascorbic acid, caffeic acid, gallic acid, p-coumaric acids and butylated hydroxytoluene (BHT) that causes oxidative stress related diseases [41, 42]. The DPPH radical scavenging potentials of different concentrations of the isolated compound, methanol, water, hexane, chloroform and ethyl acetate extracts of *C. procera* root bark and ascorbic acid standard were evaluated as displayed in Table 2. The results of the hexane, chloroform and ethyl acetate extracts were poor and was then discarded. And the maximum DPPH scavenging activity of the isolated compound, methanol and water extracts were 67.4%, 80.7% and 29.2% while the control, ascorbic acid was 85.3%. It was observed that the radical scavenging effect of methanol extract and the isolated compound were concentrations dependant. The high DPPH radical scavenging ability of the

methanol extract as compared to ascorbic acid standard could be attributed to synergistic effects of different metabolites present in the extract. The findings of the solvent extracts of this study are in agreement with the report of Mako et al., [43]. The moderate scavenging potential of the isolated compound may be due to the presence of OH-group in position 3' and sugar moiety in position 4' of ring Y, which is view as a free radical target site because of the ease of hydrogen abstraction in the free radical scavenging process [44]. Similarly, the presence of a 3-OH group and a 4-oxo group (keto double bond at position 4) on the Y-ring also appear to increase scavenger activity because these confer stability to the phenoxy radicals produced [45]. Although, studies have shown that glycosylation of flavonoids weakens their activity

when compared with their corresponding aglycones [46]. Thus, the antioxidant potential of the isolated compound agreed well with this fact.

The brine shrimp lethality bioassay study of the isolated compound and other solvents extracts of *C. procera* root bark is as shown in Table 3. The results show that ethyl acetate and hexane extracts exhibited high activity on brine shrimp larvae of *Artemia salina* with LC50 values of 1.02 and 2.40 $\mu\text{g ml}^{-1}$ as compared to positive control, the vincristine sulphate with LC50 values of 0.4 $\mu\text{g/ml}$. The cytotoxicity exhibited by the isolated compound (10.1 $\mu\text{g ml}^{-1}$) against this organism was insignificant as compared to the positive control disagreeing with the report of Mona et al., [47] that flavonoids with free hydroxyl groups on the ring A or B have high cytotoxic activity.

Table 2: Result of LC50 data of *C. procerar* root bark extracts and vincristine sulphate standard.

Sample extract	LC50($\mu\text{g/ml}$).
Vincrisine sulphate standard	0.4 $\pm$ 0.0
Ethyl acetate	1.0 $\pm$ 0.2
Hexane extract	2.4 $\pm$ 0.1
Chloroform	11.9 $\pm$ 0.4
Methanol extract	15.2 $\pm$ 0.9
Water extract	17.3 $\pm$ 1.4
Isolated compound	10.1 $\pm$ 0.3

Values are presented as mean (SD of three replicates)

Table 3: Result of antioxidant activities of *C. procera* root bark extracts and ascorbic acid standard.

Concentration ($\mu\text{g/ml}$)	Isolated compound	Methanol extract	Water extract	Ascorbic acid
800	51.7 $\pm$ 0.9	80.7 $\pm$ 1.0	26.7 $\pm$ 0.3	84.4 $\pm$ 0.9
400	50.4 $\pm$ 0.5	80.2 $\pm$ 0.9	28.1 $\pm$ 0.5	83.9 $\pm$ 1.1
200	46.2 $\pm$ 0.8	78.3 $\pm$ 1.1	29.2 $\pm$ 0.2	85.3 $\pm$ 1.0
100	44.3 $\pm$ 0.6	70.6 $\pm$ 0.7	25.7 $\pm$ 0.4	84.2 $\pm$ 1.0
50	38.7 $\pm$ 0.9	55.1 $\pm$ 0.8	26.1 $\pm$ 0.3	83.8 $\pm$ 1.2
25	31.3 $\pm$ 0.4	50.9 $\pm$ 0.9	25.1 $\pm$ 0.2	81.7 $\pm$ 0.7
12.5	27.9 $\pm$ 0.5	50.3 $\pm$ 0.9	20.7 $\pm$ 0.4	80.9 $\pm$ 0.9

Values are presented as mean (SD of three replicates)

Conclusion

The phytochemical screening of the root bark of *C. procera* resulted in the isolation of taxifolin 4'-O- β -D-glucopyranoside, a dihydroquercetin glycoside. Several chromatographic separation techniques such as TLC, CC and spectroscopic methods (ESI-MS, ^1H and ^{13}C NMR, COSY,

HSQC, and HMBC) were utilized. The isolated compound and methanol extract of this plant sample display a moderate to a high antioxidant activity, while ethyl acetate and hexane soluble fractions displays a moderate cytotoxic activity in the brine shrimp lethality bioassay in vitro.

Figure 1: Structure of taxifolin 4'-*O*- β -D-glucopyranoside

Figure 2: ^1H NMR spectrum of taxifolin 4'-*O*- β -D-glucopyranoside

Figure 3: ^{13}C NMR spectrum of taxifolin 4'-O- β -D-glucopyranoside

Figure 4: HSQC spectrum of taxifolin 4'-O- β -D-glucopyranoside

Figure 5: ^1H - ^1H COSY spectrum of taxifolin 4'-O- β -D-glucopyranoside

Figure 6: HMBC spectrum of taxifolin 4'-O- β -D-glucopyranoside

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