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Cytotoxic Activities of Fractionated Ethanol Extract of the Root Bark of *Terminalia Catappa* and Isolation of Eriodictyol-7-*O*-β-D-glucopyranoside from the Ethyl acetate Soluble Fraction

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ABSTRACT

The crushed root bark of *Terminalia catappa* Linn was extracted using aqueous ethanol. The crude extract was suspended in water and successively partition into n-hexane, dichloromethane, ethyl acetate and n-butanol soluble fractions. The fractions i.e.; n-hexane, dichloromethane, ethyl acetate, n-butanol and water were subjected to cytotoxic activity using brine shrimp lethality bioassay and proton nuclear magnetic resonance (^{1}H NMR) analyses. The cytotoxicity of the isolates were evaluated in terms of lethality concentration (LC_{50}). Comparing the result obtained to vincristine sulphate with LC_{50} of 0.61 µg/ml, ethyl acetate and n-hexane fractions demonstrated a significant cytotoxic activity having LC_{50} value of 0.82 µg/ml and 1.21 µg/ml. The LC_{50} values of the water, dichloromethane and n-butanol fractions were 11.90 µg/ml, 13.25 µg/ml and 17.10 µg/ml respectively. The ethyl acetate fraction with significant activity in in-vitro cytotoxic activity and a good proton NMR profiles, was further fractionated and purified using column chromatography, preparative thin layer chromatography (PTLC) and the isolate obtained were characterized using spectroscopic techniques (MS, IR, ^{1}H and ^{13}C NMR, ^{1}H-^{1}H COSY and ^{1}H-^{13}C HSQC). We concluded that the compound isolated was eriodictyol-7-*O*-β-D-glucopyranoside.

Keywords: Crude extract, Cytotoxic activity, Spectroscopic methods, *Terminalia catappa*

INTRODUCTION

Terminalia catappa Linn. (Combretaceae) is a herbal medicinal plant commonly called 'Indian almond' in English, 'baushe' and 'fruutu' among the Hausa and Tiv speaking people of Nigeria. It is a fast growing tree that is easily propagated from its seeds, and can grow up to 35 m high. It also grows wild at low altitude or at about 800 m above the sea level, and is cultivated for its fruits. The plant is domicile in both the tropical and subtropical regions of Asia, Africa, and Australia (Ichôron *et al.*, 2018; Venkatalakshmi *et al.*, 2014; Lakshminarasimhaiah and Agarwal, 2014; Osunsina *et al.*, 2009). Different parts of this plant have been used by different traditional practitioner to treat different diseases. In Indian traditional medicine, its leaves are used to treat leprosy, scabies, bleeding, cough and asthma while the stem bark is used for bilious fever, diarrhoea, sore throat and abscess (Sangavi *et al.*, 2015; Gupta, 1993). In Nigeria, the aqueous extract of the leaves is used in the treatment of typhoid fever, dysmenorrhea and tonsillitis (Ichôron *et al.*, 2018; Aimola *et al.*, 2014). In Togo and Benin republic, the decocted root and stem bark is used for treating dermatitis, while in Philippines, Malaysia and Indonesia the leaves and root bark are used for antipyretic and haemostatic purposes (Poongulali and Sundararaman, 2016). Previous phytochemical examination of stem and root barks of the plant has resulted in the isolation of catechin, epicatechin, arjunolic acid, betulinic acid, daucosterol, β-sitosterol, tannin, leucocyanidin, acutissimin A and eugenigrandin A (Ichôron *et al.*, 2018; Lin and Hsu, 1997; Fan *et al.*, 2004; Chyau *et al.*, 2006). While chebulagic-acid, corilagin, gentisic-acid, geraniin, garanatin-b, kaempferol, quercetin, tercatin, tannin, squalene, rutin, gallic acid, ursolic acid, vitexin and isovitexin has been reported in the leaves (Ko *et al.*, 2002; Chen and Li., 2006; Yun-Lian *et al.*, 2000).

The aim of the present research was to investigate the chemical composition of the root bark of *T. catappa* as well as the assessment of the cytotoxic activities of the solvents extracts.

Materials and Methods

All solvents and reagents used were of analytical grade purchased from Sigma-Aldrich (United Kingdom). Silica gel 60 (70-230 mesh) purchased from E. Merck (Darmstadt, Germany) was used for column chromatography. Similarly,

thin-layer chromatography and preparative thin layer chromatography was also carried out with a precoated silica gel plate (60 F254; E. Merck, Darmstadt, Germany). Spots on TLC were examined with a hand held UV lamp (UVL-14 EL) from Fisher Scientific after spraying with phosphomolybdic acid and followed by heating. Bruker Avance 400 MHz NMR spectrometer, operating at 400 MHz was used for ^{1}H and 100 MHz for ^{13}C as well as 2D-NMR (proton-proton correlation spectroscopy (^{1}H-^{1}H COSY)) and heteronuclear single quantum coherence (^{1}H-^{13}C HSQC) experiments which were performed with the usual pulse-sequence and TMS as an internal standard. Chemical shifts are expressed in δ (parts per million) referring to the solvent peaks δ_H 3.31 and δ_C 49.0 for deuterated methanol (CD_3OD) from Eurisotop (Saint-Aubin, France) and coupling constant, J, is in Hertz. The data were processed by the MestReNova program (Mestrelab Research, Santiago de Compostela, Spain). Mass spectrum was recorded on an Agilent 1200 HPLC (Agilent Technologies, Santa Clara, CA, USA), coupled to an Esquire 3000+ ion trap mass spectrometer using an ESI source from Bruker Daltonics (Billerica, MA, USA). Infrared spectrum (KBr) was recorded on a Perkin Elmer 100 FT-IR spectrophotometer.

Collection, Authentication and Preparation of Plant Materials

The root bark of *T. catappa* was collected at different location within Keffi, Nasarawa state, Nigeria, in July 2017. The plant was identified and authenticated at the Department of Forestry and Wildlife, Nasarawa State University, Keffi, Nigeria, with a Voucher specimen (No FWF 17063) deposited at the Departmental herbarium. The freshly harvested root bark of *T. catappa* was washed under tap water and air dried at room temperature for three weeks. The dried sample was crushed using mortar and pestle, and then stored in air tight container until further analysis.

Extraction

The air dried and ground root bark of *T. catappa* (1 kg) was extracted with 85 % ethanol for 7 days by maceration. The extract was first filtered through Whatman No.1 filter paper and the filtrate was further filtered again two more times through Whatman No.1 filter paper. The filtrate was concentrated using rotary evaporator at 45 °C to produced 51.92g dark brown gummy extract. The extract (50 g) was suspended in water (200 ml) and successively partitioned with equal volume of *n*-hexane, dichloromethane, ethyl acetate and n-butanol to give the hexane (3.7 g), dichloromethane (0.7 g), ethyl acetate (2.2 g), n-butanol (5.6 g) and water (25.7 g) soluble fractions.

Purification and Isolation

The ethyl acetate fraction was selected for further purification and isolation based on its cytotoxic activities and good ^{1}H NMR profiles. A clean column (4 × 50 cm) was packed with 100g of silica gel 60A, using the wet packing method in hexane. The sample was prepared by adsorbing 2.1 g of the fraction onto silica gel, dried in rotary evaporator and subsequently transferred to the top of the adsorbent layer in the column. The column was eluted with n-hexane: ethyl acetate: methanol (100:0:0→0:100:0→0:0:100, v/v/v) gradient elution system to obtained fifty one (51) eluates, these were pooled into seven fractions (F1 – F7), based on their R_f values on TLC. Fraction F3 (23 mg) was further purified by preparative thin layer chromatography. The plate was developed using Hexane – EtOAc – MeOH (7:5:2, v/v). The main band with yellowish-brown colour visualized under UV lamp was scraped from the plate and eluted with the same developing solvents, yielding compound Z (4 mg).

Brine shrimp lethality bioassay

The brine shrimp lethality bioassay was used to predict the cytotoxic activity of compound Z as well as *n*-hexane, dichloromethane, ethyl acetate, n-butanol and water soluble fractions using methods described by Meyer *et al.,* (1982) and Sarah *et al*., (2017). For the experiment, 1 mg of each of the extracts was dissolved in 1 ml of dimethylsulfoxide (DMSO) and solutions of varying concentrations (500, 250, 125, 62.5, 31.3, 15.6, 7.8 µg/ ml) were obtained by serial dilution technique using simulated seawater. Then, 1 ml each of these standard concentrations was added to test tubes containing 10 live brine shrimp nauplii in simulated brine water. After 24 hours, the tubes were inspected using a magnifying glass and the number of survived nauplii in each tube was counted. The median lethal concentration (LC_{50}) 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

Compound Z (2.5 mg) was dissolved in methanol (10 ml) and 10 ml of 5% H_2SO_4 was added and refluxed for 6 hours. On cooling, the reaction mixture was neutralized with saturated sodium carbonate and washed with EtOAc (2 × 5 ml) (Chaturvedula *et al*., 2013). The aqueous portion containing the sugar was concentrated and the EtOAc portions containing the aglycon were both subjected to ^{1}H NMR analyses.

RESULT AND DISCUSSION

Compound Z was isolated as a yellow needle which is soluble in methanol. It showed a quasi-molecular ion peak due to $[M - H]^-$ in the negative ESI-HRMS at *m/z* 449.0211. IR spectrum exhibits characteristic bands at 3354 (-OH), 1751

(C=O), 1644 (C=C), 1535 (C-H), 1084 (C-O) and 800 (C-H) cm^{-1} (Fig. 1).

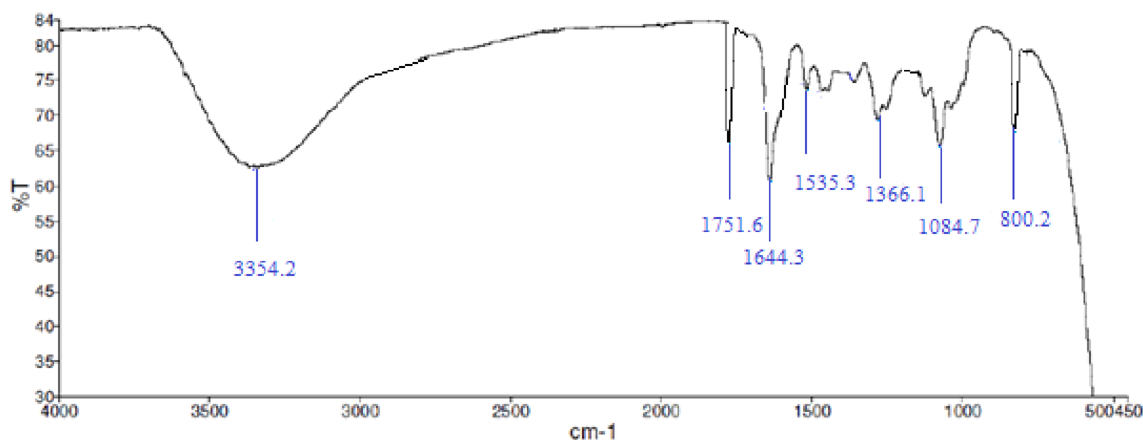

Fig. 1: The IR spectrum of eriodictyol 7-*O*-*β*-D-glucopyranoside

The ^{13}C NMR and DEPT spectra (Figs. 2 and 3) were measured in deuterated methanol (CD_3OD) on a Bruker Avance 400 MHz instrument, it showed twenty one carbon signals which comprised of two methylene, eleven methine and eight quaternary carbons. The resonance for the keto group bonded at C-4 appeared at 196.52. The anomeric carbon signal was sighted at δc 102.8 while other signals for the D-glucose appeared at δc 73.71 (C-2''), 76.35 (C-3''), 70.33 (C-4''), 77.25 (C-5'') and 61.27 (C-6'') which are typical signals for glucose (Dong-Joo and Young-Soo, 2011).

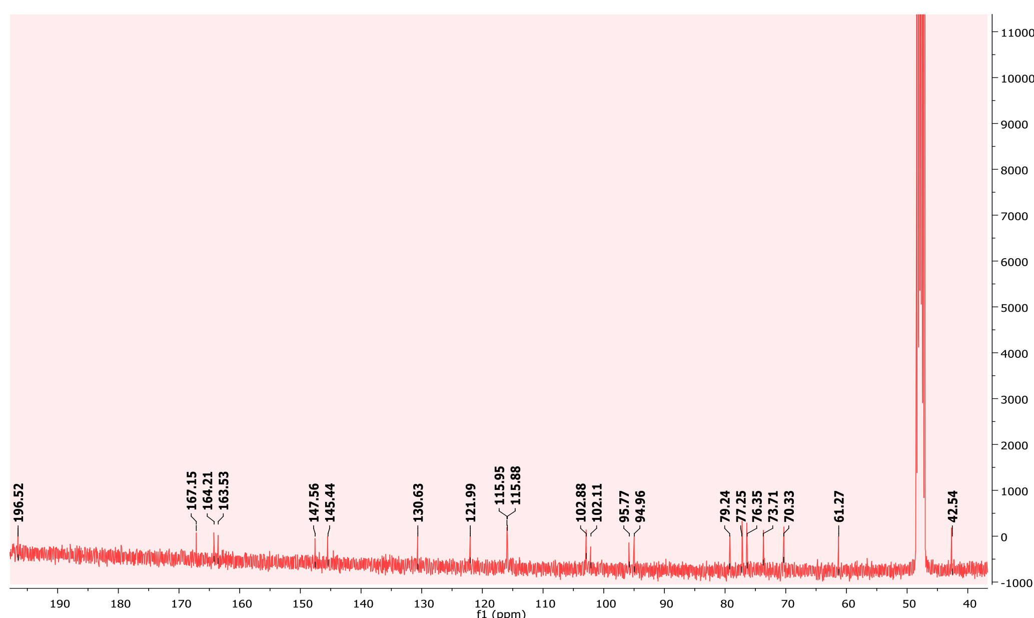

Fig. 2: ^{13}C NMR spectrum of eriodictyol 7-*O*-*β*-D-glucopyranoside

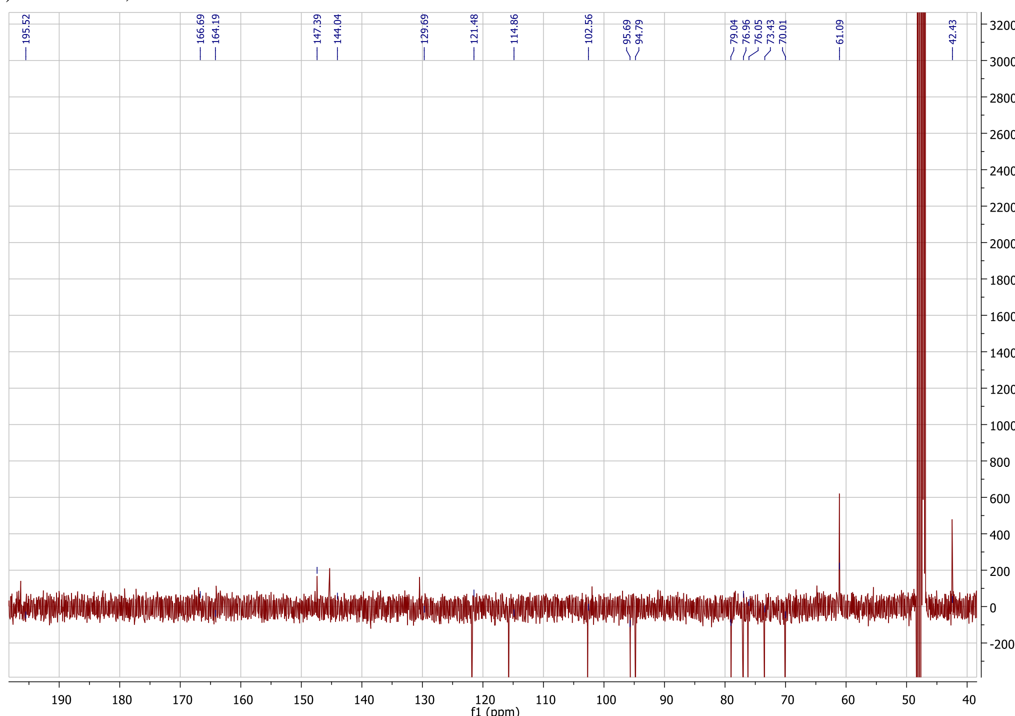

Fig. 3: The Depth spectrum of eriodictyol 7-*O*-*β*-D-glucopyranoside

In the ^{1}H NMR spectrum (Fig. 4), the signals that appeared at δ_H 3.04 (dd, 12.80 Hz, 17.02 Hz) and δ_H 2.63 (dd, 2.54 Hz, 17.02 Hz) belonged to the diastereotopic protons bonded at C-3 of ring M. The spectrum also showed two aromatic singlets at δ_H 5.79 and 5.81 allocated to ring L, and the three aromatic proton signals at δ_H 6.96 (d, 1.95 Hz, H-2'), 6.78 (d, 8.40 Hz, H-5') and 6.98 (d, 8.40 Hz, H-6') assigned to N-ring indicating a 1, 2, 4-trisubstituted aromatic ring.

In addition, one anomeric proton δ_H 4.70 (d, 7.18 Hz, H-1'') was also observed and based on this coupling constant, the configuration of the sugar moiety was determined to be β-oriented indicating a β-glycosyl moiety. The mutual coupling between proton signals can be observed in the COSY spectrum (Fig. 5), where signals δ_H 6.98 (H-6') and δ_H 6.77 (d, 8.6 Hz, H-5') were coupled.

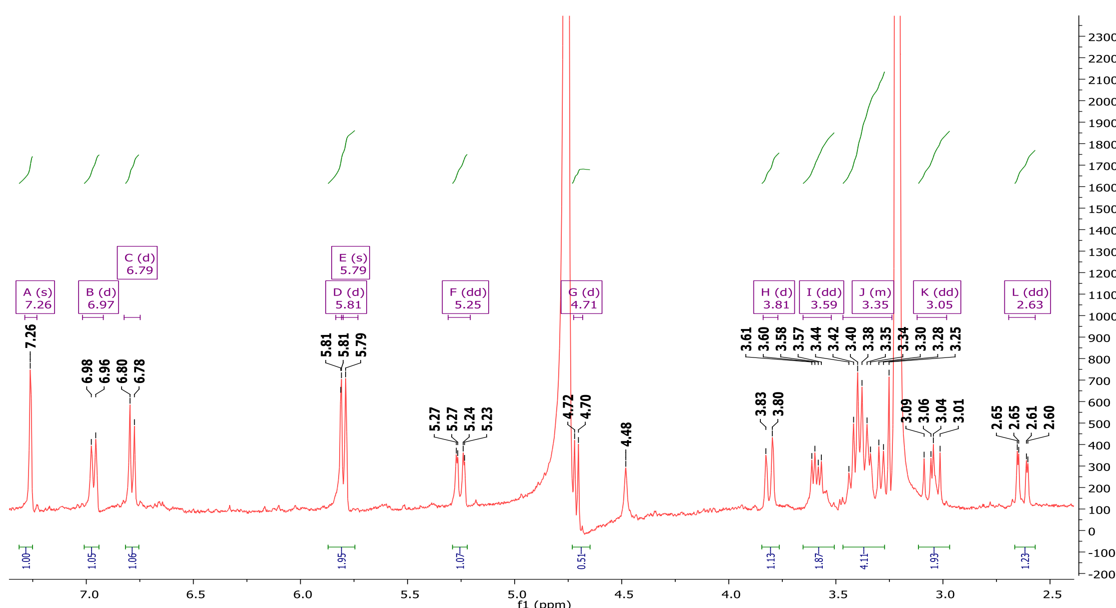

Fig. 4: ^{1}H NMR spectrum of eriodictyol 7-*O*-*β*-D-glucopyranoside

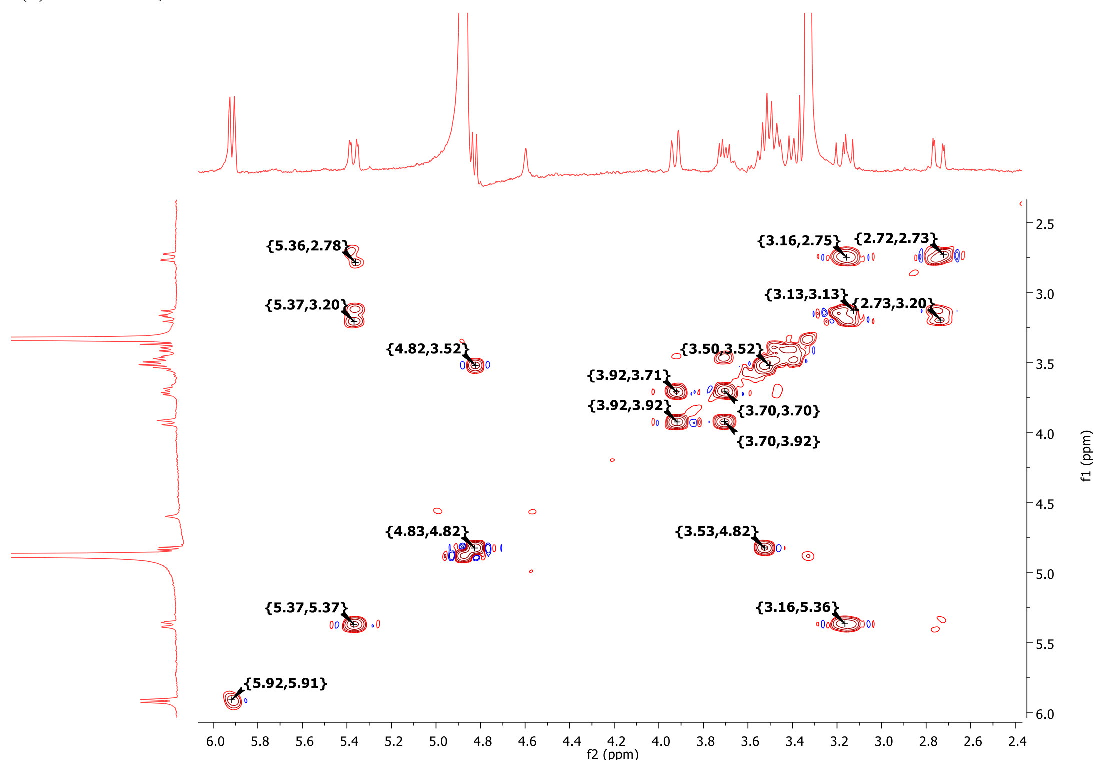

Fig. 5: ^{1}H-^{1}H-COSYspectrum of eriodictyol 7-*O*-*β*-D-glucopyranoside

On complete acid hydrolysis of the compound, the proton nmr spectrum of the aqueous phase shows signals that are typical of glucose moiety (Dong-Joo and Young-Soo, 2011) while the organic phase showed signals that are characteristic of eriodictyol (Guzel *et al.*, 2017), with the downfield shift of the resonance at C-7 (δc 167.15), suggesting it to be the site of glycosylation.

The direct correlations between carbon and hydrogen of this compound is as shown in HSQC spectrum (Fig. 6), where the carbon resonances of the N-ring at δ_C 115.95 (C-2'), 115.88 (C-5') and 121.99 (C-6'') correlated with aromatic proton signals at δ_H 6.96 (H-2'), 6.78 (H-5') and 6.98 (H-6') respectively.

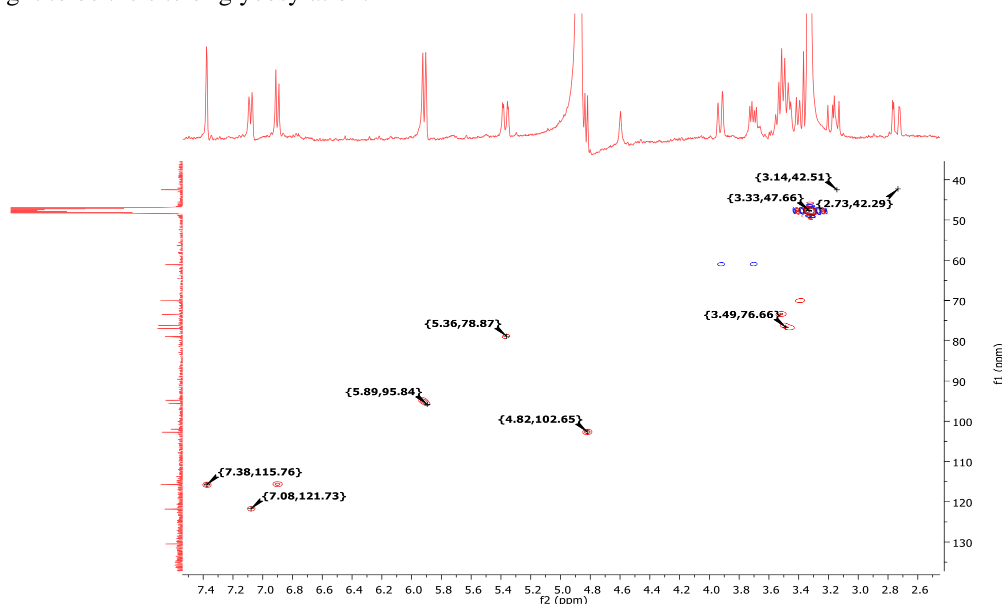

Fig. 6: HSQC spectrum of eriodictyol 7-*O*-*β*-D-glucopyranoside

The foregoing analyses of spectral data obtained for this sample correspond to the data of eriodictyol 7-*O*-β-D-glucopyranoside (Table 1 and Fig. 7) as reported by Lee and Shim, (2020).

Table 1. ^{1}H and ^{13}C NMR data of compound Z

Atom No.	Lee and Shim, 2020 CD_3OD δc	Compound Z CD_3OD δc	Compound Z CD_3OD (δ_H)	Lee and Shim, 2020 CD_3OD (δ_H)
2	79.4	79.2	5.25 (1H, dd, J= 12.70, 2.61, H-2)	5.27 (1H, dd, J=13.20, 2.40 Hz, H-2)
3	42.8	42.5	3.04 (1H, dd, J=17.02, 12.80 Hz, H-3a)	3.10 (1H, dd, J=16.50, 13.10 Hz, H-3a)
3	42.8	42.5	2.63 (1H, dd, J=17.02, 2.54 Hz, H-3b)	2.66 (1H, dd, J=16.50, 2.50 Hz, H-3b)
4	197.2	196.5		
5	163.3	164.2		
6	96.7	95.8	5.81 (1H, s, H-6)	6.08 (1H, s, H-6)
7	163.6	163.5		
8	95.6	95.0	5.79 (1H, s, H-8)	6.10 (1H, s, H-8)
9	166.7	167.2		
10	131.4	130.6		
1’	103.4	102.6		
2’	113.4	115.9	6.96 (1H, d, J=1.95 Hz, H- 2’)	6.83 (1H, s, H- 2’)
3’	148.3	147.6		
4’	145.2	145.4		
5’	114.9	115.9	6.78 (1H, d, J=8.40 Hz, H-5’)	6.71 (1H, d, J= 8.30 Hz, H-5’)
6’	122.1	121.9	6.98 (1H, d, J= 8.40 Hz, H-6’)	6.68 (1H, d, J= 8.30 Hz, H-6’)
1’’	101.6	102.8	4.70 (1H, d, J=7.18 Hz, H-1’’)	4.91 (1H, d, J=6.50 Hz, H-1’’)
2’’	73.1	73.7	3.40 (m)	3.40 (m)
3’’	75.8	76.3	3.38 (m)	3.38 (m)
4’’	70.6	70.3	3.42 (m)	3.50 (m)
5’’	76.7	77.3	3.61 (m)	3.63 (m)
6’’	60.9	61.2	3.59 (1H, dd, J=2.05, 12.11 Hz, H-6’’) 3.91 (1H, dd, J=5.75, 12.11 Hz, H-6’’)	3.71 (1H, dd, J= 2.42, 11.70 Hz, H-6’’) 3.92 (1H, dd, J= 5.91, 11.70 Hz, H-6’’)

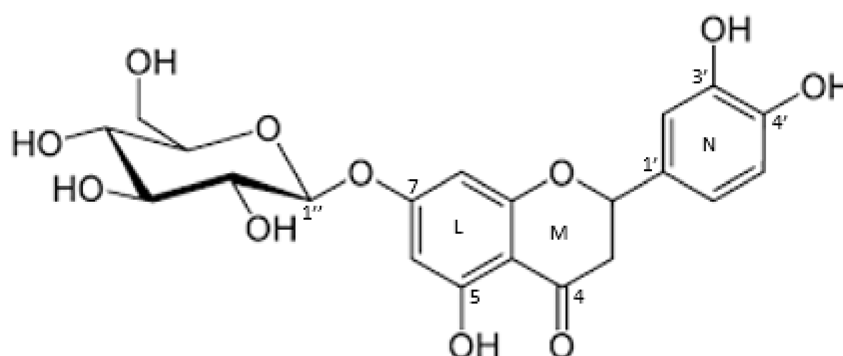

Fig. 7: Structure of eriodictyol 7-*O*-*β*-D-glucopyranoside

In the cytotoxic screening, the lethality of compound Z, hexane, dichloromethane, ethyl acetate, butanol and water extracts was determined using brine shrimp lethality bioassay for probable cytotoxic activity. The LC_{50} obtained from the best fit line slope for the compound Z, standard and the fractions are presented in Table 2. The result obtained for the ethyl acetate (0.82 µg/ml) and hexane (1.21 µg/ml) soluble fractions were comparable to that of vincristine sulphate (0.61 µg/ml). Therefore, the cytotoxicity exhibited by n-hexane and ethyl acetate extracts was significant as compared to dichloromethane, water and n-butanol fractions respectively.

Table 2. LC_{50} data of *T. catappa* root solvents extracts, compound Z and vincristine sulphate.

Samples	LC_{50} (µg/ml)
Vincristine sulphate (Standard)	0.61
Hexane	1.21
Dichloromethane	13.25
Ethyl acetate	0.82
Butanol	17.10
Water	11.90
Compound Z	12.16

CONCLUSION

T. catappa's ethyl acetate extract, has a potential cytotoxic activity against brine shrimp nauplii. To the best of our knowledge, this is the first time the presence of this flavonoid glycoside, eriodictyol 7-*O*-β-D-glucopyranoside is reported in this plant species (*T. catappa*) in Nigeria. In view of this study, further chemical and pharmacological investigations to identify other secondary metabolites present in this species, and to evaluate the cytotoxic potential *in vivo* is recommended.

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