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Structural Elucidation of Two Unique Antimicrobial Cassane – Type Tricyclic Diterpenes from the Root of *Calliandra portoricensis (JACQ)-BENTH (Fabaceae)*

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Abstract

The burden of infectious diseases by bacteria and fungi had constituted a great concern to the entire human population. *Calliandra portoricensis (Fabaceae)* had been widely used over the years in ethnomedicine for the; treatment of various ailments such as swollen gum, tooth and throat inflammation often associated with microbial infections. At present, no active antimicrobial compound has been reported from this specie. The aim of this research was to identify, isolate and characterize the antimicrobial compounds from the root of *C. portoricensis.* The pulverized root sample (0.8 Kg) was extracted by successive cold maceration respectively for 72 hr.. The most bioactive ethyl acetate extract (4.61 gm) was subjected to chromatographic column fractionation (Silica Gel G, 200-400 mesh-stationary phase). Gradient mixtures of n-hexane: ethyl acetate: methanol (4:0:0; 3:1:0; 2:2:0; 1:3:0; 0:1:0; 0:3:1; 0:2:2; 0:1:3; 0:0:4; – v/v/v) were used for elution. Agar well diffusion method was adopted for the bioassays susceptibility tests and MIC determinations. Clinically viable human pathogens for the tests were; *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Klebsiella pneumonia*, *Streptococus fecalis*, *Candida albicans* and *Aspergillus niger*. Two major fractions (F_A and F_B) active against the test organisms were pooled The more active fraction F_B on further purification by preparative TLC (Silica Gel G, 0.5 mm thickness), yielded bioactive pure commands C_1 (9 mg) and C_2 (8 mg). Both compounds exhibited MIC values of 125.00±0.70 µg per ml against *Candida albicans* and *Aspergillus niger* These activities were found to be quite significant with respect to the reference controls (Ciprofloxacin and fluconazole) at $P \leq 0.05$. Characterization of C_1 and C_2 by spectroscopic analysis (UV, MS, FT – IR and NMR), identified two novel compounds as Cassane - type tricyclic diterpenoids. C_1 (Molecular Mass: 324, C_{20} H_{36} O_3) is (5,10- 8,9- 12,13)-seco- 4,4,10 – trimethyl, 14 – hydroxymethyl, 16 – keto, 13(15) – ene – cassane furanoditerpene and compound C_2 (Molecular Mass: 418, C_{24} H_{34} O_6) is (12,13) – seco - 12, 14 – epoxy, 12(16) -Oxo –,13(15), 16(17) – diene, 4, 10, 17 – trimethyl, 4, 7 – di – aceto cassanoate. Similar Cassane - type diterpenoids have been reported for promising antimicrobial properties.

Keywords: *Calliandra portoricensis (Fabaceae)*, antibacterial, antifungal, cassane-type diterpenoid derivatives.

INTRODUCTION

Over the years, humans have depended on natural products for basic needs such as food and medicines. Evidence abounds on how the ancient civilizations of Chinese, Indians and North Africans used plants for the treatment of various diseases [1].

There has been huge burden of the infectious diseases on the populace due to the newly emerging and re-emergent diseases as well as multiple drug– resistant microbial strains that have necessitated search for newer and better antimicrobial agents [2]. About 80 % of world inhabitants patronize herbal medicine [3], and this is most pronounced in the resource – limited countries of the globe [4].

Currently, plants are still rated as the most economical and effective alternative source of medicines and 'lead' for novel drug discovery [5, 6]. Studies are therefore needed to validate scientifically, the safety, efficacy, quality and dosage of medicinal plant used [7].

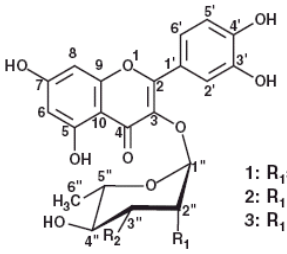
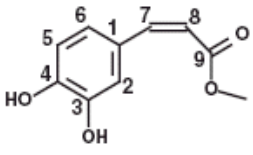
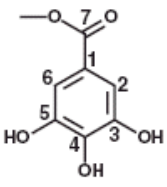
The plant *Calliandra portoricensisis* is a shrub distributed in tropical regions of America, India, West Indies and West African Nigeria [8]. Phytochemical constituents include; saponins, flavonoids, cardiac glycosides, steroids, triterpenoids, reducing compounds and alkalids [9].

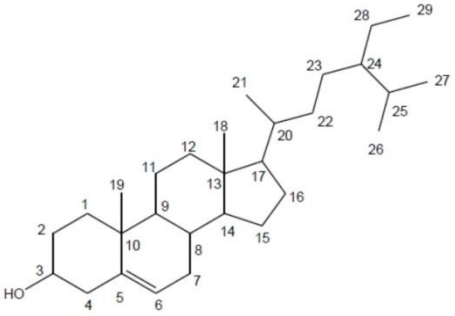
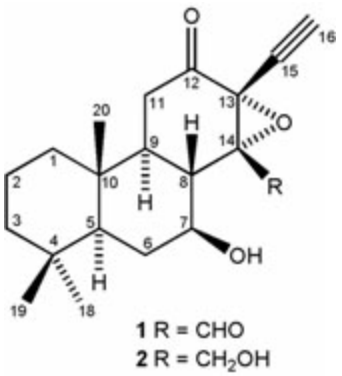
Fig-1: Photograph of Calliandra portoricensis showing, twigs, leaves and flowers

Ethnobotanically, the common names of *Calliandra portoricensis* include, "Sleeping plant" and "Corpse awakener," Tude (Yoruba of South Western Nigeria); [10]; "Ekweanahi" and "Avuvuagu" or "Eriagbo" among the Igbos of South Eastern Nigeria. In these regions, the plant has been used extensively in traditional medicine for the treatment of various ailments such as; throat and tooth inflammations, swollen tonsil, mouth ulcers. These medical conditions are usually caused by bacteria and fungi [11].

There were some previous scientific reports in the following domains; worm expeller, laxative, abortificient, antidote to viparean venon [12, 13]. Antidiarrhoea, anticonvulsant and antipyretic properties [14-16]. Also crude extracts of C. portoricensis, exhibited antimicrobial activity [17]. Antioxidant, antiangiogenic, and antiproliferative activities in human prostate cancer cells [18]. Antisickling properties [19]. Antioxidant and antihepatotoxic [20].

Table-1: Some chemical constituents previously isolated from the genus Calliandra

Structural formular	Name of Isolated compound	Morphological part	References
 1: R_1= caffeoyl, R_2 = OH 2: R_1 = OH, R_2 = galloyl 3: R_1 = R_2 = galloyl  *Z*-caffeoyl  galloyl	Quercitrin 2″-O-caffeate Quercitrin 3″-O-gallate Quercitrin 2″,3″-di-O-gallate	Leaves and stem of Calliandrahaematocephala	[21];)

	**β-sitosterol**	Leaves of C.haematocephala.	[22]
	**Cassane derivatives:** **Escobarine A (1)** **Escobarine B (2)**	Root of *Calliandra californica*	([23]

Aims and objective of the study

To identify, isolate and characterize the antimicrobial compounds from the root of C. portoricensis.

MATERIALS AND METHODS

Plant Material

The root sample of *Calliandra portoricensis* was collected in the month of June from Osisioma Local Government Area in Abia State of Nigeria. The plant was identified and authenticated in the Herbarium of the Department of Plant Science and Biotechnology, University of Port Harcourt, Rivers State, Nigeria by Dr. Chimezie Ekeke with the Voucher Number: UPH / V / 1240. The material was properly washed, air dried, pulverized and stored for subsequent use.

The methodology adopted the techniques as described earlier [24], for; extraction by successive cold maceration using n-hexane, ethyl acetate and 70 % aq. Methanol for 72 hr respectively, Anti-microbial in vitro susceptibility evaluation and Minimum Inhibitory Concentration (MIC) determinations by agar well diffusion method, preparation of test microorganisms which were; Staphylococcus aureus (Gram +ve cocci), Streptococcus fecalis (Gram +ve cocci), Escherichia coli (Gram –ve rod), Bacillus subtitlis (Gram +ve rod), Klebsiella pneumoneae (Gram -ve rod), Candida albican (fungi), Aspergillus niger (fungi), Also conducted was column Fractionation of bioactive ethyl acetate extracts (Silica Gel G, 200-400 mesh-stationary phase) with gradient mixtures of n-hexane: ethyl acetate: methanol (4:0:0; 3:1:0; 2:2:0; 1:3:0; 0:1:0; 0:3:1; 0:2:2; 0:1:3; 0:0:4; – v/v/v) used for the elution , The bioactive fraction (B) was subjected to further purification by preparative TLC, (Silica Gel G, 0.5 mm thickness), The resultant bioactive pure compounds C_1 and C_2 were subjected to spectroscopic analysis (UV, MS, FT – IR and NMR).

RESULTS AND DISCUSSION

Table-2: Result of antimicrobial susceptibility tests of TLC bands (C_1 and C_2) on selected human pathogens at concentration of 1 mg / ml

	TLC Band [C_1]		TLC Band [C_2]	
Microorganism	**C-$_1$**	**CTR**	**C-$_2$**	**CTR**
Staphylococcusaureus	*25.00±0.40	23.00±0.70	*31.00±0.12	24.00±0.70
Escherichia. Coli	*35.00±0.60	38.00±0.90	*20.00±0.60	25.00±0.70
Bacillus subtitis	*45.00±0.50	40.00±0.80	*22.00±0.50	35.00±0.40
Klebsiella pneumonae Streptococcus. fecalis	*35.00±0.70 *30.00±0.20	30.00±0.60 35.00±0.10	*18.00±0.40 *20.00±0.80	30.00±0.80 25.00±0.40
Candida albicans	*33.00±0.90	35.00±.00	*20.00±0.90	25.00±0.70
Aspergillus n.	*25.00±0.50	14.00±0.80	*30.00±0.50	45.00±0.60

Values are Diameter Zone of Inhibition (mm) and expressed as mean ±SEM; n = 3; CTR. = Control - Ciproflaxacin (20 µg per ml for bacteria) and Fluconazole (1000 µg per ml for fungi); (-) = no inhibition; 10 % aqueous DMSO (negative control, no inhibition).

* Represent the significant values with respect to the control at $P \leq 0.05$.

Table-3: Minimum Inhibitory Concentrations (MIC) values of TLC bands (C_1 and C_2) in µg per ml against the selected human pathogens

S/N	Micro organisms	TLC Bands.	
		C_1	C_2
1.	*Staphylococcus. Aureus*	125.00±0.80	125.00±0.10
2.	*Escherichia. Coli*	25000±0.30	125.00±0.70
3.	*Bacillus. Subtitis*	125.00±0.20	250.00±0.30
4.	*Klebsiella pneumoneae*	250.00±0.60	125.00±0.80
5.	*Streptococcus fecalis*	12500.±0.50	125.00±0.10
6.	*Candida albicans*	125.00±0.70	125.00±0.40
7.	*Aspergillus niger*	125.00±0.20.5	125.00±0.60

Values are expressed as mean ± SEM; n = 3.

Table-4: Interpretation of ^{1}H and ^{13}C NMR Spectral data (Deuterated Chloroform _$CDCl_3$ as solvent) for compounds C_1 and C_2

Assigned position/ identity of atoms	(δ) -^{13}C (ppm)		δ_H (ppm)		^{1}H-H COSY		HMBC $^{2,3,4}J_{HC}$		DEPT-135	
	C_1	C_2	C_1	C_2	C_1	C_2	C_1	C_2	C_1	C_2
C-1	22.0	29.85	-0.88	1.2-1.3					CH_2	CH_2
C-2	18.2	29.51	0.9,1.2	1.2-1.3					CH_2	CH_2
C-3	22.8	33.97	1.32	1.2-1.3					CH_2	CH_2
C-4	45.8	54.66		-						-
C-5	29.8	22.85	1.33	0.9					CH_2	CH
C-6	21.1	33.39	1.3	1.2-1.3					CH_2	CH_2
C-7	21.7	44.49	0.9	3.7		H_8			CH_2	CH
C-8	25.0	41.85	1.68	3.3		H_7			C H_2	CH
C-9	23.0	22.24	1.45	0.8					C H_2	CH
C-10	38.0	52.96	2.4	-					CH	-
C-11	30.2	32.08	1.4,1.75	1.2	H12				CH_2	CH_2
C-12	65.7	95.21	4.3	5.98	H11				CH_2OR	OCHO
C-13	127.9	111	7.6	5.40	H15				=CH	=CH
C-14	28.7	82	2.04	3.60				C_{21}	CH	OCH
C-15	129.9	109	7.4	6.05	H13				=CH	=CH
C-16	170ca	140	-	-					RO-C=O	-
C-17	13.1	95.09	0.99	6.04					CH_3	=CH
C-18	11	14.23	0.95	0.95					CH_3	CH_3
C-19	14	14.53	0.9	0.75			C2, C4		CH_3	CH_3
C-20	67.2	170	4.23	-					CH_2OH	R-O-C= O
C-21		164		-						R-O-C= O
C-22		18.81		1.0						CH_3
C-23		56.71		3.80				C_{20}		OCH_3
C-24		55.89		3.70				C_{21}		OCH_3

Both compounds exhibited DZI of 33.00±0.90 and 25.00±0.50 respectively against Candida albicans and Aspergillus niger as well as MIC values of 125.00±0.70 µg per ml for each. These activities were found to be quite significant with respect to the reference controls (Ciprofloxacin and fluconazole) at $P < 0.05$.

The susceptibility tests result shown in (Table 1) was found to be consistent with the report which suggested that Diameter Zone of Inhibition of 10 mm and above despite the current ease of acquired microbial resistance should be considered to possess some antimicrobial activity; while those equal to or above 20 mm could be considered potent [25].. Further, the result shown on (Table 2), on MIC values was in line with report of an investigation which expressed that extracts having activity where MIC values were below 8 mg /ml were considered to possess some antimicrobial activity, whereas natural products with MIC values below 1 mg /ml should be considered as noteworthy [26].

Compound C_1 had an R_f value of 0.72 (Silica Gel, 0.25 mm, n-hexane: ethyl acetate: methanol – 12: 4: 1) and fluoresced light green under the UV lamp at

365 nm. C_1 was a semi solid, oily and dark brown compound. The Ultra Violet (UV) spectrum, exhibited absorption maximum at 280 nm. This was consistent with values reported on Cassane - type tricyclic diterpenoids [27], and supported by the presence of conjugated chromophoric group on ring C of compound C_1. The structure of this compound was elucidated by using FT - IR, NMR (1- D and 2 – D experiments) and MS spectroscopic data., The IR bands were in the region; 3402.54 cm^{-1} and 2959.47 cm^{-1} , representing the -0H stretching and –CH vibrations respectively. Also evident were the carbonyl stretch at 1727.30 cm^{-1} , α and β, unsaturation at 1415.52 cm^{-1}

The proton (^{1}H) – NMR contained five peaks of deshielded protons at δ_H (ppm); 7.6, 7.4, 4.26, 4.25 and 2.04.

The cosy spectrum revealed the correlation of the proton peaks and exhibited cross – peak corrilatins as in; proton H -12 with H – 11 and H – 13 with H – 15. Three methyl protons singlets were evident at δ_H: (ppm); H – 17 – Me (0.99); H – 18 – Me (0.95) and H – 19 – Me (0.90). Evident too were nine methylene (CH_2) protons corresponding to H – 1, H – 2, H – 3, H – 5, H – 6, H – 7, H – 8, H – 9 and H – 11 respectively. Also present were four methine (CH) protons at; H – 10 and H – 14 respectively. The olefinic (Sp^2) = CH - protons were evident at H – 13 and H – 15. The methoxy (–OCH_2 –) proton at H – 12 was evident too. Also present were secondary alcohol protons (- CH_2 - OH) at H – 20. The clear designations and identity of atoms was achieved by use of 2 – Dimensional proton to carbon correlation (HMBC and HSQC). The other proton chemical shift peaks were equally rationalized on Table 4.

A total of twenty spectral peaks were identified in ^{13}C – NMR experiment of compound C_1 . These were rationalized by the aid of DEPT – 135. Three methyl groups at δc (ppm); 13.10, 11.00 and 14.00 corresponding to C17 – Me, C18 – Me and C19 – Me respectively. Olefinic group (C = C) at δc (ppm): 127.90 (C – 13 and 129.90 (C – 15) respectively. Nine methylene (-CH_2) groups at δc (ppm); 22.00 (C-1), 18.20 (C – 2) 22.80 (C – 3), 29.80 (C -5), 21.10 (C -6), 25.00, (C – 7) 21.70, (C -8), 23.00 (C-9) and 30.20 (C-11). One dioxymethylene groups (O - CH_2 -0) at; 65.70 (C-12) was evident. Present also were two methine groups (-CH-) at; 38.00 (C – 10) and 28.70 (C – 14) respectively. Evident also were two querternary groups at; 45.80 (C – 4) and 170.00 (C – 16) respectively.

Other correlations were evident in HMBC as rationalized in Table 4. The number assignment of hydrogen, carbon and oxygen was further supported by the MS spectrum The Mass and NMR (1D AND 2D) spectral data suggested the presence of Cassane – type tricyclic diterpenoid. This skeleton is usually linked to certain sub group in Fabaceae family [28]. Again, a peak was shown at m/z 325 and corresponded to [M + 1] equivalent to molecular mass of 324 ($C_{20}H_{36}0_3$).

Compound C_1 is therefore a Cassane – type tricyclic diterpenoid derivative with (IUPAC) name as; (5, 10- 8,9- 12,13)-seco-4,4,10 – trimethyl, 14 – hydroxymethyl, 16 – keto, 13(15) – ene – cassane furanoditerpene (Figure 2).

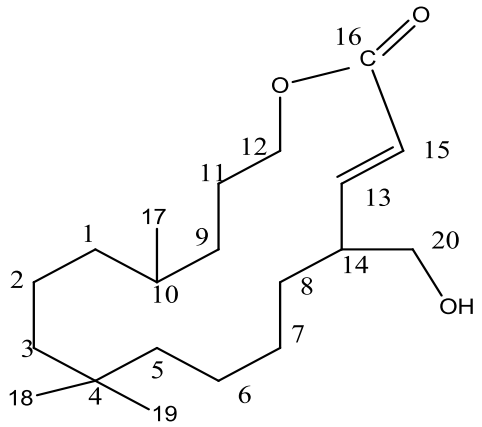


Fig-2: The structure of compound C_1. (5, 10- 8,9- 12,13)-Seco-4, 4, 10 – trimethyl, 14 – hydroxymethyl, 16 – keto, 13(15) – ene – cassane furanoditerpene

Compound C_2 had an R_f value of 0.59 on analytical TLC plate (Silica Gel, 0.25 mm, n-hexane: ethyl acetate: methanol – 12: 4: 1) and fluoresced deep purple under a UV lamp at 365 nm. It was also a semi-solid and oily compound with molecular formular of $C_{24}H_{34}O_6$ as could be deduced from the MS spectral data by a peak showing at m/z 419 corresponding to [M+1] equivalent to its molecular mass of 418.

The FT–IR of C_2 showed an carbomyl (C= 0) stretching in the band region of 1710.33 $cm^{-1.}$ Presence of –CH, CH_2, CH_3 vibrational frequencies were also evident.

The ^{1}H-NMR for compound C_2 exhibited signals of eight deshielded protons with chemical shifts at δ_H (ppm) 6.05, 6.04, 5.98, 5.40, 3.80, 3,70, 3.60 and 3.30, corresponding to; H-15, H-17, H-12, H-13, H-23, H-7/24, H-14 and H-8 respectively. Five quaternary carbons with no protons at; C-4, C-10, C- 16, C – 20, and C – 21, were evident.Also evident were the three methy protons singlets at chemical shifts δ_H (ppm); 0.95, 0.75 and 1.0, corresponding to; C-10-Me, C-4-Me, and C-17-Me respectively. Olefinic (Sp^2) protons at δ_H (ppm); H – 13 (5.40), H-15 (6.05) and H-17 (6.04). Present too were the oxymethine protons at δ_H (ppm); H-12 (5.98) and H-14 (3.60) respectively.

The structural configuration of this compound C_2 was further supported by a total of twenty four carbon signals in ^{13}C - NMR spectroscopy. These resonances were rationalized on the basis of DEPT-

135. (Table 4). Five quaternary carbons at δc (ppm); C-4 (54.66), C-10 (52.96), C – 16 (140.0), C-20 (170.0) and C – 21 (164.0). Three methy group carbons were evident at δc (ppm): Me - C-18 (14.23), Me - C-19 (14.53), and Me - C-22 (18.81). Five methylene groups (- CH_2 -) at δc (ppm) C-1 (29.85), C-2 (29.51), C-3 (33.97), C-6 (33.39) and C-11 (32.08). Present too were the two oxymethine groups (>CHO) at δc (ppm): C-12 (95.21) and C-14 (82.00). Four olefinic methine carbon atoms were evident at δc (ppm); C – 13 (111.00), C-15 (109.00) and C-17 (95.09).

The ^{1}H-H COSY spectral data indicated that proton H – 8 (δ_H = 3.30 ppm) exhibited cross – peak correlation with H -7(δ_H = 3.70 ppm). At the same time, the following correlations were observed with HMBC spectrum; proton H -14 (δ_H = 3.60 ppm) with C - 21(δ_C = 164.00 ppm), H – 23 (δ_H = 3.80 ppm) with C – 20 (δ_C = 170.00 ppm) and H – 24 (δ_H = 3.70 ppm) with C – 21 (δ_C = 164.00 ppm).

The absorption maximum in UV- VIS experiment was at 270 nm. This is consistent with earlier report on Cassane skeleton. This novel compound C_2 isolated from the root of *Calliandra portoricensis* was identified as (12, 13) – seco - 12, 14 – epoxy, 12(16) -Oxo –, 13(15), 16(17) – diene, 4, 10, 17 – trimethyl, 4, 7 – di – aceto cassanoate (Figure 3).

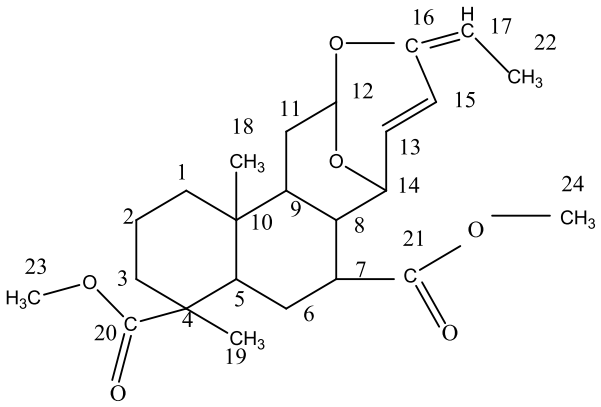


Fig-3: The structure of compound C_2: (12, 13) – seco - 12, 14 – epoxy, 12(16) -Oxo–, 13(15), 16(17) – diene, 4, 10, 17 – trimethyl, 4, 7 – di – aceto cassanoat.

The two compounds C_1 and C_2 being Cassane – type diterpenoid derivatives, could be linked to earlier reported activities exhibited by similar moieties against test bacteria, virus and candida albican – fungus [23, 29, 30]

CONCLUSION

This study had successfully isolated, identified and characterized two novel Cassane - type tricyclic diterpenoid derivatives. Compound C_1 as; (5,10- 8,9-12,13)-seco- 4,4,10 – trimethyl, 14 – hydroxymethyl, 16 – keto, 13(15) – ene – cassane furanoditerpene. (Molecular mass: 324 and molecular formular: C_{20} H_{36} O_3). and Compound C_2 as; (12,13) – seco - 12, 14 – epoxy, 12(16) -Oxo –,13(15), 16(17) – diene, 4, 10, 17 – trimethyl, 4, 7 – di – aceto cassanoate (Molecular mass: 418 and molecular formular: C_{24} H_{34} O_6). These Cassane - type diterpenoids have shown promising activities against human pathogenic bacteria and fungi (Candida albican).

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CONFLICT OF INTEREST

There was no conflict of interest involved in this research work.

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