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Antimicrobial evaluation of Rhizome Extracts and Chemical Characterization of the hexane Extracts of *Costuslucanusianus J. Braun&Shum*

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ABSTRACT

Micro-organisms resistance to antimicrobial agents has prompted a quest for novel potent antimicrobial agents which are particularly derived from natural sources. *Costuslucanusianus*(*J.Braun&Shum*) is a plant generally utilized in traditional medicine for the treatment of innumerable infectious diseases including venereal diseases. However, there is limited information on the antimicrobial potency of its rhizome crude extracts and chemical compositions. This study is aimed at determining the chemical composition and antimicrobial potency of *C. lucanusianus* rhizome organ. The antimicrobial potency of various concentrations (12.5-100 µg/mL) of the ethyl acetate, hexane and methanol extracts of the rhizome was evaluated against six bacterial and four fungal strains using the Agar well diffusion techniques. Minimum inhibition concentration (MICs) of the most potent extracts was determined using Agar Microdilution method. The most potent plant extract was further subjected to chromatographic separation and then structural elucidation using NMR spectroscopy. The extracts displayed varying antimicrobial activity against the micro-organisms, and MICs for the most potent extracts ranged from 3.125 µg /ml to 6.25 µg/ml. Chromatographic studies of the hexane extract led to the isolation of a compound Z, a mixture of isonuatigenin and diosgenin whose structures were established with NMR and comparison with literature data. The *in vitro* antimicrobial activity displayed by the crude extracts from *C. lucanusianus* rhizomes highlights its potential for the treatment of infectious diseases in ethnomedicine.

KEYWORDS: *Costuslucanusianus*, Antimicrobial activity, Isonutiagenin, Diosgenin, Infectious diseases

1. INTRODUCTION

Infectious diseases continue to pose a significant world health concern, especially in low- and average-income regions where access to effective treatment remains limited[1]. The enormous burden of infectious diseases in developing countries is due to the emergence and spread of drug-resistant infections[2]2. A major challenge encountered in treating infectious diseases is the antimicrobial resistance. There is therefore an urgent requirement for new drugs that can counteract antimicrobial agent resistance mechanisms 3. Medicinal plants have traditionally served as a vital origin of bioactive compounds used to treat various diseases. These plants contain significant organic compounds such as carbohydrates, steroids, terpenes, alkaloids and flavonoids, many of which are liable for the antimicrobial properties displayed *in vitro* conditions [4].As a result, the interest in making use of compounds derived from plants as potential alternatives to conventional antimicrobial agents has increased.

Costuslucanusianus, commonly referred to as the Spiral ginger and African spiral flag, is a popular plant widely famous for its efficiency in the treatment of cough, urethral discharge, urinary tract infection, venereal diseases amongst others 5. The rhizome is frequently used in decoctions for therapeutic purposes in cases like leprosy and malaria 6. Various biological activities such like hepatoprotective, antimicrobial, anti-inflammatory and antihyperglycemic effects have been reported [7-9].

Despite this, limited information is available on the plant's chemical components 9. The *Costus* genus is rich in steroidal saponins, starches, oxalates, and sapogenins and other bioactive compounds [10], suggesting that C. lucanusianus may contain structurally relevant compounds with significant pharmacological potential. This study aims to evaluate the antimicrobial activity of rhizome extracts and characterize the chemical bioactive constituents accountable for these effects.

2. MATERIALS AND METHODS

2.1 Plant material

Fresh rhizomes of Costuslucanusianus italicize were collected from General gas area, Akobo Ibadan, Oyo state, Nigeria (at a geographical coordinate of 7 ° 25! 45.4" N 3 ° 56 ' 10.9" E; altitude 830 ft.). Voucher specimens (FH110048) of the plant were dropped at the FRIN herbarium.

2.2 Instrumentation

The NMR spectra were acquired by the use of BrukerTopSpin 600 and 150 MHz spectrometers for ^{1}H and ^{13}C, respectively, in $CDCl_3$. Silica gel 60-200 mesh size was utilized for column chromatographic procedures.

2.3 Study method

2.3.1 Extraction of plant organ

Air-dried and ground rhizome (50 g) was deffated with hexane using the cold-maceration extraction method repeatedly. The extract filtered usingWhatman filter paper number 1 and a Buchner funnel. The filtrate was concentrated under reduced pressure with a rotary evaporator at 40°c to obtain the crude extract. The defatted plant material was then extracted with Ethyl acetate and methanol sequentially with the same cold maceration technique. Subsequently, more extracts were obtained from the rhizome organ (2.70kg) using a Soxhlet apparatus. The rhizome organ was defatted with hexane, extracted with ethyl acetate and methanol successively. The percentage yields (w/w) of the extracts were determined.

2.3.2 Purification of the hexane extract

Hexane extract (6.80 g) was purified on silica gel (60-200 mesh, 205 g, 32.5 mm x 66 mm) using various concentrations of ethyl acetate in hexane to give 125 fractions of 200 mL each. Fractions 64-73 (1.03 g) were subjected to repeated column chromatography using a gradient of ethyl acetate in hexane to give a mixture of diosgenin (3) bold and isonutiagenin (4) bold (12 mg).

2.3.3 Antimicrobial activity of crude extracts

A total of six clinical bacterial strains (*Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus, Salmonella typhi, Klebsiellapneumoniae and Escherichia coli*) and four fungal strains (*Candida albicans, Rhizopusstolonifer, Penicilliumnotatum and Aspergillusniger*) were used in this study. All test organisms were provided by the laboratory of pharmaceutical microbiology, University of Ibadan. The antibacterial and antifungal potency of methanol, hexane and ethyl acetate extracts from *C. lucanusianus* were determined using Agar well diffusion techniques on Mueller-Hinton Agar medium (MHA) and Sabouraud Dextrose Agar medium (SDA), respectively, against the above-mentioned microorganisms. Culture media were sterilized by autoclaving prior to use

2.3.4 Preparation of graded concentrations

An initial concentration of 100 µg /ml of each extract was prepared by dissolving,extracts (1.0 mg each) into 10 ml of methanol. Double-fold serial dilution method was used to obtainfour different concentration solutions ranging from 12.5 µg/ml to 100 µg/ml

(a). Antibacterial assay

A mixture of micro-organisms andsterile nutrient agar was cautiously poured into sterilized petri dishes and permitted to solidify for about 45 minutes. Extracts (30µl), methanol and gentamicin were poured into the wells (8 mm diameter hole pierced in the Agar at distance of 25 mm apart using a sterilized cork borer). For adequate diffusion of the extracts into the medium, the plates were left at room temperature, afterwards incubated at 37 °C for 24 hours.

The bacterial growth zones were observed after the duration and inhibition zones were measured in mm. Tests were carried out in triplicates [11].

(b). Antifungal assay

The prepared sterile SDA (62 g/L) was permitted to solidify for about 45 minutes in sterile plates. The SDA plate surfaces were covered with fungal strain suspension using sterile cotton swabs under aseptic conditions. Different concentrations of the extracts, along with methanol and tioconazole{please chech the name} 70% were transferred into the wells for 120 minutes to allow perfect diffusion into the agar. After that, they were incubated uprightly for 48 hours at 28 °C. Measurements of inhibition zone diameter (IZD) in mm were determined [12.]

2.3.5 Determination of Minimum Inhibitory Concentration (MIC)

The hexane and methanol extracts solutions in methanol were prepared. The MIC was determined by making use of the Agar dilution technique (micro/dilution technique) in a 96-well micro-titer plate (MTP). Prepared sterilized and cooled Agar (18 mL each) was properly mixed with various concentrations of the extracts (2 mL each). The spreading of the resulting mixtures was evenly in (MTP) which was maintained in an oven for 24 hours at 37 °C for bacteria and 48 hours for fungi. Then, unto the surfaces were applied microorganisms. For the fungi, the visualization of turbidity in the wells, which represented the least concentration at which no microorganism growth was seen, was used to determine the MIC 12. The experiment was carried out in triplicate. MIC of the extracts in millilitres was determined,

2.3.6 Statistical Analysis

Statistical analysis of data obtained was determined by the use of one-way analysis of variance (ANOVA). A significant difference between obtained data was considered at $P \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Crude extract percentage yields

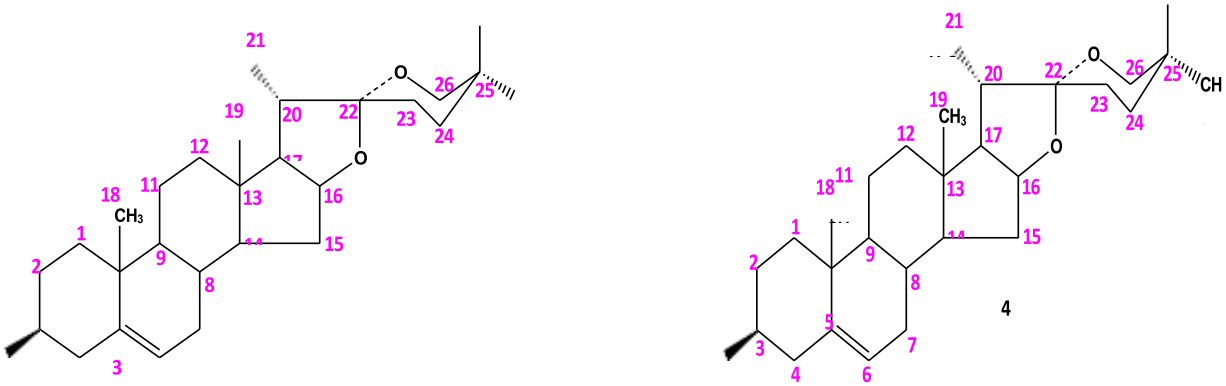
The percentage yields of the cold hexane, ethyl acetate and methanol extracts were 0.05 g, 0.01 g and 1.20 g, respectively. On the other hand, the hot methanol, hexane and ethyl acetate extracts yields were 0.26 g, 0.08 g and 1.41 g, respectively.

3.1.2 Isolation of bioactive constituents

In this study, we report the isolation of a mixture of isonutiagenin (3) and diosgenin (4) from the rhizomes of Costuslucanusianus. Fig.1 &2).

The known compounds (3 and 41) were identified by comparison of their NMR spectra data (Supplementary Figures A and (Tables 1-2) with the literature 13-15.

Fig.3 and 4.Chemicalcompoundsisolated fromtherhizomesof *Costuslucanusianus*J.Braun&K.Shum



The1H NMRand13CNMRdataofcompounds3&4 are reported inTables1 and2,respectively.

Compd.	H-3	H-6	H-18	H-19	H-21	H-26	H-27
3	3.35-3.37,m	5.36,d,1.9Hz	0.79,s,3H	1.02,s,3H	0.96, d, 7.0Hz	3.28,dd, 10.3,2.4, 3.6Hz	1.20,s,3H
4	3.45-3.47,m	5.73,d,1.9Hz	0.80,s,3H	1.02,s,3H	0.98,d,6.7 Hz	3.34, d,11.0, 3.44,d,11	0.82,s,3H

[1]H NMR(600Hz).3*,4*13-15

Table2:13CNMR dataofcompounds**3** and**4**

CarbonPosition	Compound**3***		Compound**4***	
	Experimental	Literature	Experimental	Literature
1	37.38	37.10	37.38	37.43
2	31.78	31.60	31.78	31.79
3	71.89	71.30	71.89	71.89
4	42.43	42.30	42.43	42.46
5	140.95	141.5	140.95	141.02
6	121.59	121.5	121.59	121.60
7	32.96	32.90	32.29	32.25
8	31.87	31.80	31.60	31.64
9	50.89	50.60	50.22	50.25
10	36.80	37.00	36.80	36.84
11	20.98	21.20	21.03	21.07
12	39.95	40.10	39.95	39.99
13	40.52	40.60	40.42	40.46
14	56.68	56.90	55.82	56.72
15	32.01	31.90	32.21	32.04
16	80.77	81.60	80.97	81.03
17	62.20	62.40	62.26	62.28
18	16.40	16.40	16.44	16.49
19	19.50	19.50	19.57	19.63
20	41.77	41.40	41.88	41.80
21	14.63	14.30	14.67	14.73
22	109.42	109.5	109.44	109.50
23	31.52	29.90	31.55	31.59
24	34.11	34.60	28.96	28.99
25	80.66	81.60	30.46	30.49
26	67.00	69.20	67.02	67.04
27	30.43	23.80	17.56	17.35

13CSpectrum(CDL_3)recordedonBrukerAvanceIIIat150.0MHz.3*,4*(*3= 13,16*4=14-15.

3.2 Discussion

3.2.1 Extract yields

Low extraction yields were recorded for both the cold (0.01-1.20%) and hot (0.08-1.41%) extracts. This aligns with previous reports, which have attributed low recovery of crude extracts to the limited abundance of extractable secondary metabolites 17-18.

3.2.2 Isolation of bioactive constituents

This was the initial isolation of steroids from the hexane extract of the *C. lucanusianus* rhizome. *Costus* genus rhizomes are known as the major sourceof diosgenin and similar compounds have been identified from its other parts such as leaves, stems and flowers. In addition, steroidal saponins, steroids and sapogenins have also been reported to be characteristic constituents of the genus [10]. In this study, isonuatigenin was identified alongside diosgenin, marking its first reported occurrence within the Costus genus. The examination of

spectroscopic data of compound Z, in comparison with the published data for (25R)-isonuatigenin and diosgenin confirmed that it was a mixture of two identical steroidal sapogenins13-16

Few proton signals were easily recognizable due to extensive interproton coupling in the 1H NMR. A total of eight methyl signals at (18-CH_3, 19-CH_3) and (21-CH_3, 27-CH_3) were observed as the upfield signals. The overlapping multiplets in the methylene envelope (δ 2.23-1.6 ppm) were as a result of the remaining methylene and methine signals. Overlapping downfield signals greater than 3ppm were assigned to the olefinic signals at δ 5.3-5.7ppm and oxysubstitutedmethine resonance at δ 3.3-3.4 ppm. The signals for H-16 appeared as a doublet of doublet at δ 4.4 ppm and H-26 at δ 3.2-3.3 ppm. The overlapping of signals observed in the H NMR can be attributed to the presence of two similar compounds with similar chemical shifts. The signal corresponding to the H-27 is of particular diagnostic value in distinguishing between the two compounds. The chemical shift value of the C-27 protons at 30.43ppm further confirmed that the structure is isonuatigenin.

A total of fifty-four (54) carbon signals observed were assigned to compounds 3 and 4.

The characteristic signals of the steroidal sapogenin are those of C-16, C-26 and C-22, which are usually between (80-110 ppm). 16

The angular methyl groups, and C-21 chemical shifts were established to be similar to those reported for isonuatigenin and diosgenin. The signals of diosgenin and isonuatigenin were nearly identical except for the induction of the expected chemical shift of the carbon atoms (25-C and 27-C) of ring F by the addition of a -OH group at C-25 and a small downfield shift of the C-27 value. Some ^{1}H-^{1}H and ^{1}H-^{13}C couplings were also established with the 2D NMR

3.2.3 Antibacterial and antifungal activity

High inhibition zones were observed by the hexane extract against *S. typhi* (30 mm), followed by *S. aureus* and *K. pneumonia* (28 mm) while the methanol extract exhibited a high inhibition zone against *B. subtilis* (24 mm) and *P. aeruginosa* (23 mm). These findings are comparable to previous studies on the antibacterial potency of *C. igneus, C. pictus* and *C. speciosus* rhizome petroleum ether (non -polar) extracts against *E. coli*, *S. aureus, P. aeruginosa*and *B. subtilis* at (500-2000 µg/mL) 19. Also, *C. speciosus* rhizome hexane, ethyl acetate and methanol extracts inhibited the growth of *S. aureus* and *B. subtilis* (9 mm-15mm) at 2.5-5.0 mg/disc 17.

A similar trend was observed in antifungal assays where maximum antifungal activity was also observed at 100 µg/ml (Table 4). Hexane extract displayed a slightly higher antifungal activity than the methanol and ethyl acetate extracts. Antifungal activity of the extracts against tested fungal strains is in line with previous reports on the antifungal potency of the root and leaf ethanol extracts of *Costusafer*, a related African species, against *C. albicans* and *A. niger*20.

Antibacterial and antifungal activity resultsof the crude extracts revealed that the active components liable for the observed properties are predominantly present in the non-polar extract (hexane extract).

SolventExtracts	***S.aureus***				***E.coli***				***P.aeruginosa***				***K.pneumonia***				
(µg/mL)Concentration	**100**	**50**	**25**	**12.5**	**100**	**50**	**25**	**12.5**	**100**	**50**	**25**	**12.5**	**100**	**50**	**25**	**12.5**	**100**
Hexane(Rhizome)	- 28.0± 1.848	- 24.3± 1.067	- 20	- 18	- 27.3 ±1.06 7	- 25.3 ±1.067	- 22-3 ±0.53 3	- 19.0 ±1.60 0	- 26.7 ±1. 067	- 26	- 21.7 ±1.41 1	- 20	- 28	- 25.3 ±1.06 7	- 22.7 ±1.41 1	- 19.7± 1.411	- 25.3 ±1.067
Ethylacetate(Rhizome)	16.7± 1.067	14	12	10	18	16.7 ±1.067	14.7 ±1.06 7	12.7 ±1.06 7	18	16	14	12	17.3± 1.067	15.3 ±1.06 7	12.7 ±1.06 7	10	20
Methanol(Rhizome)	20 ±1.848 0.533	17.3 ±1.067	15.3± 1.067	13. 3± 1. 06	22.7 ±1.06 7	19.7± 1.411	17.7 ±1.41 1	15.0 ±1.60 0	23	20.7 ±0.53 3	17.7 ±0.53	15.7 ±0.5 3	22	18	16	14	24
+vestandardGentamicin	40						38										
-veStandardDMSO	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

S.auereus=Staphylococcusaureus,B.subtilis = Bacillus subtilis,E.coli=Escherichiacoli,P.aureginosa=Pseudomonasaureginosa,K.pneumonia=Klebsiellapneumonia,S.typhi =Salmonellatyphi

Table4:AntifungalActivityOfCrudeExtracts

Partsofplant used	SolventExtracts	*C.albicans*				*A.niger*				*P.notatu*		
	Concentration (µg/mL)	100	50	25	12.5	100	50	25	12.5	100	50	2
		-	-	-	-	-	-	-	-	-	-	-
Rhizome	Hexane	18.7± 1.067	16.7± 1.067	15	14	19.3±1.067	17.3± 1.067	15.3±1.067	13.3± 1.067	19.0± 0.924	17.3± 0.533	1 ±
	Ethylacetate	17.3± 1.067	14.7± 1.067	12	10	17.3±1.067	15.3± 1.067	12.7 ± 1.067	10	19.3± 1.067	17.3± 1.067	1
	Methanol	18.7± 1.067	17.3± 1.067	14.7± 1.067	12	17.3±1.067	14	12	10	16.7 ±1.067	14	1
	+ve Standard Tioconazole(70%)	28				26				28		
	-ve Standard DMSO	-				-				-		

C.albicans= Candidaalbicans, P. notatum= Penicilliumnotatum,A.niger=Aspergillusniger ,R.stolonifer

3.3.4 Minimum Inhibitory Concentration (MIC) Results

The hexane extract exhibited MIC values at 3.125 µg /ml against tested microorganisms except *A. niger* and *P. notatum*, which displayed no prominent growth (Table 5) while the MIC of the methanol extract was at 6.25 µg /ml against all tested microorganisms (Table 6). C. speciosus rhizome hexane extract displayed a MIC value of 1.25 mg /ml against *B. subtilis*and *S. aureus*and 500 mg /ml against *A. niger*. Also, a MIC of 1.25 mg /ml was displayed by *C. speciosus* rhizome methanol and ethyl acetate extracts against *B. subtilis* and *S. aureus* respectively. The methanol extract also displayed a MIC of 0.625 mg/mL against *S. aureus* 17. *Costusigneus* rhizome petroleum ether extract also displayed a MIC value of 0.25mg /ml against *P. aeruginosa, S. aureus* and *B. subtilis*, while *CostusPictus* petroleum ether exhibited MIC of 0.25 mg /ml for *B. subtilis, S. aureus,* and 0.5 mg /ml for *P. aeruginosa and E. coli*[19].

4. CONCLUSION

The Chromatographic studies of the rhizome hexane extract led to the isolation of a mixture of diosgenin and isonuatigenin. The antimicrobial activity displayed by the rhizome extracts validates its application in the treatment of infectious diseases traditionally. Further work is ongoing to purify and separate the compounds for individual antimicrobial activities.

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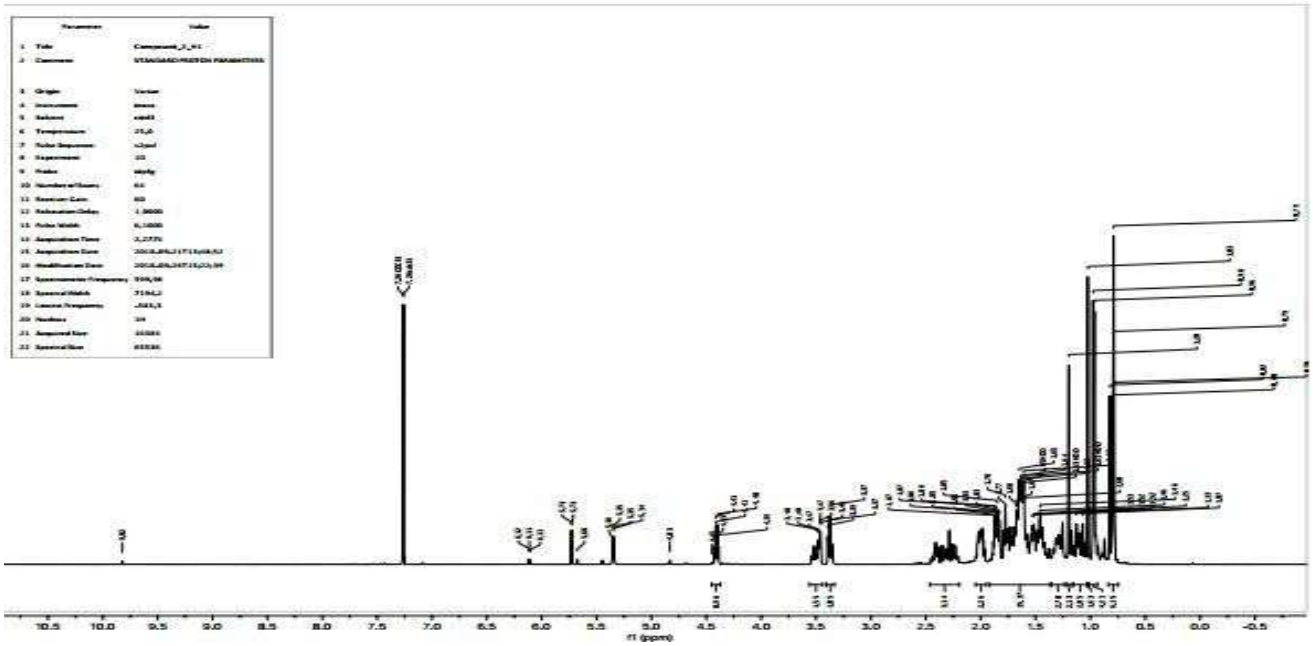

FigureB1:ProtonNMRofCompounds**3**and**4**

FigureB2:Carbon13NMRofCompounds**3**and**4**