Communication in Physical Sciences, 2024, 11(1):39-46

Isolation, Characterization and Antimycobacterial Potency of a Steroidal Derivative from the Chloroform Crude Extract of *Icacina trichantha Oliv* Tuber

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Received: 13 November 2023/Accepted: 28 February 2024 /Published: 02 March 2024

Abstract: *Literature has shown that the extract of Icacina trichantha is rich in various constituents, whose medicinal values have been confirmed. However, the major challenge is in the isolation of the component of interest. In this study, a steroidal derivative was isolated from the tubers of Icacina trichantha. The extraction was done using the cold maceration method, while further isolation and purification were carried out using column chromatography and thin-layer chromatography. Characterization was done using FT-IR, ^{1}HNMR, ^{13}CNMR, and COSY with literature which confirmed the isolated compound to be a steroidal derivative. The antimycobacterial activity was done on the isolated steroidal derivation, and the result obtained confirmed the speculation of its use for the treatment of "tough cough" by the locals. The findings from the study is remarkable in contributing information to phytochemistry.*

Keywords: *Icacina trichantha*, spectroscopy, antimycobacterial activity, functionality.

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1.0 Introduction

Natural products and their related moieties have historically been incredible as a source of therapeutic agents. Plants are a natural source of compounds; they have been used for the maintenance of human, health: and to improve quality of life. Medicinal plants have been known to have very high efficacy and that is why most people (about 78%) of the people in rural communities rely on them for effective methods for their primary health care because of the presence of diverse bioactive

components present in them, which have some pharmacological activities and nutritional values in the body of living organisms (Akoh and Mac-Kalunta 2021)

Some literature has reported leaf extract of *Icacina trichantha* as an active agent against bacterial parasitic micro-organisms of humans. *Icacina trichantha* Oliv. (*Icacinaceae*), characterized as being a drought-resistant plant originating from Central and West Africa, is a medicinal shrub utilized by the people, particularly the ethnic societies in Nigeria. It is called "Urumbia" or "Eriagbo" (denoting its emetic effect) among the Igbo tribe of Nigeria, or "Gbegbe" (connotating to purify) by the Yoruba tribe of western Nigeria.

Fig.1: Tuber of *Icacina trichantha*

A sequence of new marine-type diterpenes has been identified in the tubers of the *I. trichantha*. The tropical forest tree family *Icacinaceae* was initially acknowledged by Miers (Miers 1864) and it was successively reviewed by Karehed in 2001 on the background of deoxyribo nucleic acid sequence investigation (Karehed 2001). The genus currently consists of six recognized species and eight synonyms (Anon, 2015). *I. oliviformis* (false yam) is undoubtedly the most important which serves as a food plant of West tropical African communities among its species, (Anon, 2008). *Icacina trichantha* can be described as a shrub which grows up to two meters high (Burkill 1994). The nutrient and anti-nutritional properties (e.g. the bitter compounds such as oxalates alkaloids, hydrogen cyanide, tannins, and phytates) of the flour have been investigated. This showed the presence of carbohydrates (mostly starch), proteins and lipids as well as mineral elements such as sodium, potassium, and calcium (Umoh and Iweh, 2014; Umoh 2013; Udofia *et al*., 2014). The fruit of *I. trichantha* is a drupe with a soft sweet outer fleshy tissue which is palatable (Burkill 1994). *I. trichantha* is utilized as a popular household first-aid treatment for food poisoning and drugs for emergencies in Western Nigeria and neighbouring regions (Mbatchou and Dawda, 2012). The leaves and tubers of this plant are supposedly being used as aphrodisiacs (Burkill 1994; Quattrocchi, 2012). When the leaves and seeds are ground and macerated in local alcoholic beverages, they serve as medicine for the management of asthma and hypertension (Ajibesin *et a*l., 2008). Traditional medical healers make use of the tubers to treat several medical conditions including rheumatism, malaria, constipation, poisoning, and toothache as well as to induce abortion and emesis (Ariwaodo *et al.*, 2012). The juice extracted from the tuber can be used for curing mumps (Ubom 2010). The first pharmacological report on *I. trichantha* was reported by Asuzu *et.al* in 1990. Their report established diarrheal activities of aqueous extract of the tuber on mice, and the extract also potentiated pentobarbital-induced loss of righting reflex (Asuzu and Ugwueze, 1990). Extending pentobarbitone sleeping time in rats, prompting local anaesthetic outcomes in guinea pigs, and defending rats and mice from pentylenetetrazole poisoning proved the activity of the extracts on the central nervous system (Asuzu and Abubakir, 1995; Asuzu and Egwu, 1998).

Mycobacterium tuberculosis infections are transmitted through the breathing of infective bacilli. Bacteria are inhabited by alveolar

macrophages and develop infection centres in the lung tissue. These centres increase through bacterial development and the production of macrophages and lymphocytes that make up the granuloma that describes this infection. The granuloma appears to encourage partial bacterial development and inhibits metastasis of the infection. Nevertheless, the granuloma also shields the bacterium against the immune response and is possibly accountable for the persistent or hidden characteristics of the infection. Medical disease progresses when this immune-mediated constraint is repealed by immune compromise. Even in persons that have infection regulated at the granulomatous state or earlier stages, any future inequality of the host's immune system may support the reactivation of the sickness (Adams and Hamiliton, 1992).

2.0 Materials and Methods

2.1 Sample collection and identification

The plant was harvested at Umunakwukwu Chokoneze Mbaise, Imo state on 23rdAugust, 2017. The plant was identified by Mr. Ibeh K. Ndukwe of Plant taxonomy, Forestry Department of Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria and was assigned the herbarium number ICA DALZ 1094.

2.2 Sample preparation

The tubers were washed to remove sand, after which they were peeled and grated. The grated tuber was air-dried for four weeks and weighed. The weight was found to be 1.2 kg. Extraction was done by maceration with chloroform for 72 hours after which it was decanted, filtered using Whatman No.1 filter paper and concentrated under reduced pressure using a rotary evaporator to afford 9 g of the crude extract. The column used was 280 mm high and 35 mm in diameter. The chloroform crude already weighed 5.0 g . The slurry was formed with silica gel, crude mixtures (crude extract with 15 ml of chloroform) and celite in a ratio of 1:30:3. The slurry was left to evaporate for 48 hours in the laboratory. After which it was used in the column chromatography. The column chromatography was run according to the standard procedure using solvents of different polarities, from hexane to chloroform to ethyl acetate and then methanol. 49 eluents were collected and each of the eluents was spotted in a precoated TLC plate using a capillary tube. Fraction 33 which is an oil fraction being eluted from 50:50 (ethyl acetate: methanol) gave a singular spot at R_f value 0.69 in hexane: chloroform solvent mixture in the ratio of 2:1 (3ml), named OAU 4. OAU 4 was packaged in a sample bottle and sent to (ACEPRD) Jos.

2.3 Spectroscopic analysis

Spectroscopic studies were carried out to elucidate the structure of the isolated compound. The corresponding spectra of FT-IR, HMNR, 13CNMR, COSY were recorded with CDC as solvent.

2.4 Methodology for anti-tuberculosis assay

Mycobacterial strain Inoculum preparation was done as described by Molina-Salinas *et al*., (2006). *Mycobacterium tuberculosis* H37Rv, an American Type Culture Collection strain (ATCC 27294) and Multi-drug resistant strains of *M. tuberculosis* were used to investigate the antimycobacterial activity of the plant extracts. The Mycobacterial strains were first subcultured on Lowenstein-Jensen medium for 14 days, and pure colonies were then inoculated in sterile liquid medium (Middlebrook 7H9) supplemented with glycerol, Tween 80 and OADC (mixture of Oleic acid, Albumin, Dextrose and Catalase). The inoculum was incubated in a shaking incubator at 37^0C with 5% CO_2 for 14 5 days before adjusting the turbidity of the suspension to 0.5 MacFarland (1.5 x 108 CFU/ml). The adjusted suspension was used as inoculum to determine the inhibitory activity of the extracts.

3.9.1: *Determination of Minimum Inhibitory Concentration using Microplate Alamar Blue Assay*

The antimycobacterial activity of the plant extracts was determined using the microplate Alamar Blue Assay (MABA) as previously described by O'Neil *et al.,* (2014) and Molina-Salinas *et al.* (2006). Briefly, the plant extracts were dissolved in 5% dimethyl Sulphoxide (DMSO) and then sonicated for 30 minutes to ensure a total solubility concentration of 100 mg was used as working solutions. Dilutions of the test extracts and approved anti-tuberculosis drugs (Isoniazid and Moxifloxacin were used as standard control drugs) were made with varying concentrations. Hundred (100) micrograms of each concentration of the plant extracts and standard drugs were dispensed into corresponding sterile 96-well microtitre plates except the wells used for growth control (containing microorganisms and culture media only) and negative control (containing only culture media). *M.tuberculosis* H37Rv amd *M.tuberculosis* resistant strains were also added to the corresponding 96 well plates except of the wells used for negative control, the final volume in each well was 200 µl.

Plates were covered and sealed with parafilm and incubated at 37^{o}C for 7 days. At day 7, 32.5 µl. of Alamar blue dye was added to all 96 wells and then incubated for 19 hours at 37^{o}C in the dark. After 19 hours, plates were read, and validation of the test was conditioned by oxidation-reduction reaction of Alamar blue dye and bacterial cells indicated by control wells (growth control, media control, and standard drug control wells). For the tested samples, the blue colour is synonymous with a lack of bacterial growth and therefore indicates the anti-tuberculosis activity. A turn to pink means bacterial growth. The MIC was defined as the lowest concentration, which prevented a colour change from blue to pink.

3.0 Results and Discussion

Table 1 presents FT-IR chart for eluent OAU 4 whereas Table 2 indicates ^{1}H NMR and ^{13}C NMR chemical shift for eluent OAU 4 which was recorded in $CDCl_3$

Table 1: Infrared spectral data of isolate compound OAU4

Vibration frequency (cm^{-1})	Assignment
3300.00	OH/NH_2
3006.86	Olefinic asymmetric stretch =C-H / Aromatic C-H Stretch
2922.60	Aliphatic C-H Stretch
2854.62	Aliphatic C-H Stretch
1740.21	C=O of ester
1712.09	C=O of ketone
1239.01	C-O Stretch
1375.39	C-H bending CH_3
1457.68	C-H bending of CH_2
1164.10	C-O

Table 2: ^{1}H NMR and ^{13}C NMR of isolate compound OAU4

POSITION	^{1}H signal ppm	Type of proton	^{13}C signal	Type of carbon
1	2.02m	2H	26.60	CH_2
2	3.21 m	2H	30.71	CH_2
3	5.50 p	1H/OH	86.99	CH – O
4	3.72 d	2H	33.89	CH_2
5	-	-	116.13	- C -
6	4.39 m	1H	103.07	= CH
7	2.8 m	2H	37.19	CH_2

8	2.25 m	1H	30.46	CH
9	2.25 m	1H	30.35	CH
10	2.28 m	1H	30.53	CH
11	2.14 m	2H	28.92	CH_2
12	1.30 m	1H	27.14	CH
13	3.72 d	1H	CH – O	123.37
14	3.01 d	1H	CH/CN	69.711
15	1.69 s	1H	-	114.50
16	-	-	C = O	169.93
$1^1 2^1 3^1 4^1 5^1$	1.11 d	11H	24.79	CH

The Infrared spectrum of OAU4 as represented in Table 1 revealed the presence of alcohol, O-H stretching at 3300.00 cm^{-1}, alkane, C-H stretching at 2922.60 cm^{-1} and 2854.62 cm^{-1}, absorption at 3006.86 cm^{-1} which indicates =C-H of alkene, absorption at 1740.21 cm^{-1} and 1712.09 cm^{-1} indicate the presence of carbonyl group, C = O stretching of a lactone ring. Absorption at 1239.01 cm-1 depicts C - N stretching, bands at 1457.68 cm^{-1} and 1375.39 cm^{-1} depict CH_3 and CH_2 respectively and bands at 1164.10 cm^{-1} indicate C - O stretching of alcohol.

^{1}H NMR spectrum of OAU 4 as represented in Table 2 revealed the presence of a methylene proton, at position - 1 with δ 2.02m, and another methylene proton at position - 2 with δ 3.21m. Position -3 revealed a methine proton with δ 5.50p, which is highly deshielded by the inductive effect of OH group attached to it. Position -4 showed another methylene proton at δ 3.72d, position -6 depicted an olefinic proton at δ 4.39m, position -7 also showed a methylene proton at δ 2.80m, H-8 showed a signal at δ 2.25m, H -9 also showed a signal at δ 2.25m. H -10 showed a signal at δ 2.28m, H-11 at δ 2.14m, H-12 with δ 1.30m, H -13 at δ 3.72d, H -14 at δ 3.01 d, H -15 at δ 1.69s, 1^1, 2^1, 3^1, 4^1 and 5^1 overlapped and appeared as a signal at δ 1.11d.

13C NMR spectrum of OAU 4 as represented in Table 2 revealed the presence of methylene carbon, C -1 at δ 26.60, C -2 at 30.71, C -3 at δ 86.99, is a quaternary carbon that is deshielded by the inductive effect of an oxygen atom, C -4 at δ 33.89, C -5 at δ 116.13, is an olefenic quaternary carbon, C -6 at δ 103.07 is also an olefenic quaternary carbon which is deshielded, C -7 at δ 37.19 is a methylenic carbon, C -8 at δ 30.46, C -9 at δ 30.35 and C -10 at δ 30.53 are methine carbon atoms. C -11 at δ 28.92 is a methylene carbon, C -12 at δ 27.14 is a methine carbon, C -13 at δ 123.37 is a methine carbon that is deshielded as a result of an oxygen attachment to it. C -14 at δ 69.71 is a methine carbon that is deshielded as a result of its direct bonding to a nitrogen atom. C -16 at δ 169.93 is a carbonyl carbon. Carbon atoms at positions 1^1, 2^1, 3^1, 4^1 and 5^1 overlapped and showed a signal at δ 24.79.

In 2 - D NMR, 1H-1H COSY, there are three cross peaks labeled X, Y and Z which reveal coupled protons, that is , protons that split each other's signals. The cross peak X indicates that H - 13 and H - 14 at δ 4.24d and δ 5.20d are coupled, cross peak Y depicts that H - 3 and H - 4 at δ 5.50p and δ 2.8m and the cross peak at Z indicates that H - 3 and H - 2 at δ 5.50p and 2.02m are also coupled. Again, the spectrum also reveals a diagonal sword-like structure which depicts that the structure proposed is a steroid. Steroids are anti-inflammatory medicines used to treat a range of conditions.

3- hydroxyl – 12 – (3 – methyl but – 2- yl) – 17 – oxa – 15 – aza -cyclopenta [*a*] dodeca-hydro phenanthrene-16-one.

In Table 3, the MIC result of OAU 4 is presented. The result indicated the MIC for plant isolates and for the control.

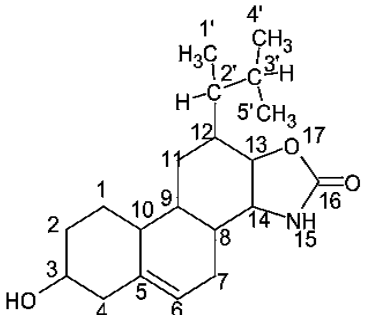

Fig.2:Proposed structure of OAU4

Table 3: Minimum inhibitory concentration result

Plant isolates	MIC (mg/ml) DR-TB Strain	MIC (mg/ml) H37RV Strain
OAU 4	6.33±0.14[a]	6.33±0.14[a]
Control 1 – Moxifloxacin (for MDR-TB Strain)	4.25±1.95[a]	
Control 2- Isoniazid (for H37RV Strain)		1.31±0.46[a]

****Values are presented as mean ± standard deviation (n = 3); and values with a different letter superscript are significantly ($p < 0.05$) different from the paired mean with the column.**

From the TB investigation, isolate OAU4 revealed the same degree of activity against the MDR-TB strain and H37RV strain as compared to the actual controls, Moxifloxacin and Isoniazid, which indicates a high level of potency against MDR-TB strain more than the sister organism H37RV. This further confirms the folk story of its use in the treatment of "cough.

4.0 Conclusion

From the ^{1}HNMR spectrum, the clusters down field depicts signals peculiar to steroidal moiety. The ^{13}CNMR spectrum also reveals the presence of carbonyl functionality down field. The anti-tuberculosis potency of this compound makes it even more interesting and has confirmed and contributed to the folk stories of its use in the treatment of cough.

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Compliance with Ethical Standards Declarations

TThe authors declare that they have no conflict of interest.

Data availability

All data used in this study will be readily available to the public.

Consent for publication

Not Applicable

Availability of data and materials

The publisher has the right to make the data public.

Competing interests

The authors declared no conflict of interest.

Funding

The authors declared no source of fundig

Authors' Contributions

Mac Kalunta interpreted the spectra. Ufearoh and Otukere conceived the research while all the authors were involved in writing.