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Cannabinoid Dronabinol alkaloid with antimicrobial activity from *Cassia alata* Linn.

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ABSTRACT

Chemical investigation of the bioactive constituents from the seeds of Cassia alata Linn. (ringworm plant) resulted in the isolation of a new cannabinoid alkaloid (4-butylamine 10-methyl-6-hydroxy cannabinoid dronabinol). The structure was elucidated using NMR spectroscopy in combination with IR and MS spectral data. Antimicrobial studies showed that the isolated compound successfully inhibited Pseudomonas aeruginosa, Klebsiella pneumonia, Escherichia coli, Staphylococcus aureus Candida albicans and Aspergillus niger. The antimicrobial observation of the above compound against these pathogens showed that the bioactive compound could be responsible for the activity of C. alata and its use in herbal medicine in Nigeria.

Keywords: antibacterial activity, antifungal activity, bioactive compound, herbal medicine.

INTRODUCTION

In Nigeria, many plant extracts have been used successfully in phytomedicine to cure diseases and heal injuries. These plants have various effects on living systems. They are sedative, analgesic, cardio-protective, anti-inflammatory, oxytocic, anti-spasmodic and immune modulators [1,2] The utilization of plants against diseases such as cancer, parasitic infection, rheumatism, arthritis, wound treatment, tumor growth, stroke, jaundice, typhoid, fibroid, syphilis and gonorrhea have been well documented [3-5].

There is increasing development of drug resistance in human pathogens as well as the appearance of undesirable side effect of certain synthetic antimicrobial agents. It is this background that necessitated the need for the extensive and intensive screening of plants for

more safe, selective and efficacious natural products. Many reports have shown that some *Cassia* species contain anti-microbial, bioactive compounds, particulary *Cassia alata* [4, 6-11]

Cassia alata Linn (Ringworm plant, Leguminosae) is a shrub that grows 2-3 m high. It is widely distributed in the tropical countries/areas, particularly America, India, Fiji, Indonesia, Malaysia and Africa [12]. *C. alata* is popularly known as ringworm shrub or ringworm *Cassia* in many countries due to the utilization of its fresh leaves in the treatment of skin diseases such as ringworm, eczema, pruritis, itching, scabies, ulcers and other related disease [3-5]. The leaf extract not only exhibits various pharmacological properties ranging from antibacterial to antifungal activities [10,11], but also perform anti-inflammatory and antioxidant functions [5].The extracts of *C. alata* have been used in cosmetic formulation for dermatological skin care products [13]. Chemical investigation on ethnolic and water extracts of the leaves and stem back from *C. alata* revealed the presence of flavonoids [14, 15] and anthraquinones [16-19]. Several studies [20-23] have documented the scientific basis for the efficacy of plants in phytomedicine. This study seeks to ascertain the usefulness of C. alata in the treatment of infections conditions caused by common pathogens. The study involves the isolation, structural elucidation and characterization of the bioactive constituents in the plant and consequently evaluates the antibacterial and antifungal activity against some pathogenic bacterial and fungi for possible development of new drugs for the prevention and treatment of infections. Herein we report for the first time the isolation, characterization and structural elucidation of cannabinoids dronabinol alkaloid from the seeds of *C. alata*.

MATERIALS AND METHODS

General experimental procedure

The IR spectra were determined on a Thermo Nicolet Nexus 470 FT_IR spectrometer. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avnce 400FT NMR spectrometer using TMS as internal standard. Chemical shifts are expressed in part per million (ppm). LC-ESIMS spectra were determined in the positive ion mode on PE Biosystem API 165 single quadruple instruments.

HRESIMS (Positive ion mode) spectra was recorded on a Thermo Finniga Mat 95xL mass spectrometer, column chromatography was carried out with silica gel (200-300 mesh) and to monitor the preparative separations, analytical thin layer chromatography (TLC) was performed at room temperature on pre-coated 0.25 mm thick silica gel $60F_{254}$ aluminum plates 20 × 20 cm Marck Darmstadt, Germany. Reagents and solvents like ethanol, chlorofoam, diethylether, hexane were all of analytical grades and procured from Merck. TLC aluminum sheets silica gel $60F_{254}$ was also purchased from Merck. The nutrient agar was purchased from Scharlan Chemie APHA Spain.

Plant materials

Mature seeds of *C. alata* were harvested from the Botanical Garden of Michael Okpara University of Agriculture Umudike, Abia State Nigeria, on 6th February 2007. The plant samples fruits, seeds and leaves were identified by Dr. A. Nmeregini of Taxonomy Section, Forestry Department of the University. A voucher specimen No CA/225 has been deposited at the Forestry Department Herbarium of the University.

Extraction and isolation of plant materials

Plant materials were treated and analyzed at the Chemistry laboratory, Michael OKpara University of Agriculture Umudike, Nigeria. Mature seeds (1 kg) of *C. alata* were dried on the laboratory bench for 10 days. The dry samples was milled and ground into powder (850 g) using Thomas Wiley machine (model 5 USA). The powdered plant samples (500 g) were packed into a Soxhlet apparatus (2 L) and extracted exhaustively with 1000 ml ethanol for 24 h. The ethanol extract was concentrated using a rotary evaporator at 45°C and left on the laboratory bench for 2 days to obtain a dark brown pigment (48 g). The column was packed with silica gel and 30 g of the brown pigment isolated placed on top of silica gel and eluted with methanol, chloroform and petroleum ether (20: 30: 50) to afford a brown solid (0.88 mg). The brown solid was re-crystallized from hexane to afford compound 1 brown solid (0.52 mg). Thin layer chromatography (chloroform: methanol 7: 3) iodine vapor shows the presence of one spot (R_f 0.82), IR Vmax 3397 cm-^{1} (OH), 1648 cm-^{1} (C=C aromatic) and 1095 cm-^{1} (C-O ether). HREIMS m/z 284.2716 [M^+] calculated for m/z 285 ($C_{18}H_{23}O_2N$) and m/z 73.0288 base peak calculated for m/z 72 $C_4H_{10}N$. ^{1}H NMR and ^{13}C NMR is presented in **Table 1**.

Bioassay

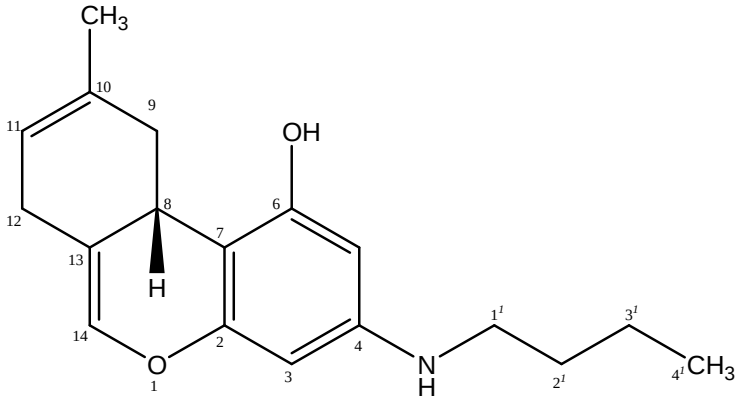
The *in vitro* antimicrobial activity of compound I was carried out for 24 h culture of four bacteria and two fungi. The bacteria used were three Gram-negative organisms comprising *Escherichia coli*, *Pseudomonas aeruginaosa*, and *Klebsiella pneumonia* and Gram-positive *Staphylococcus aureus*. The two fungi used were *Candida albicans* and *Aspergillus niger*. All test organisms are clinical isolates of human pathogens obtained from the federal medical centre (FMC) Umuahia, Nigeria, cultures were broth to labouratory conditions by resuscitating the organisms ion buffered peptone broth (Seharlan Chemie) APHA Spain and there after nutrient agar (peptone 5 g/l and meat extract 3 g/l) and incubated at 37°C for 24 h.

The antimicrobial activity was performed by filter paper disc diffusion technique. The medium (7 g nutrient agar in 250 ml distilled water, autoclaved at 115°C for 15 min) was cooled to 50°C. 20 ml of the medium was poured into a sterile Petri dish and allowed to solidify.

It was allowed to stay for 8h and observed for contamination. The sterility of the medium was tested using autoclave at 121°C 15 psi for 15 min. Nutrient agar (Scharlan Chemie) APHA Spain was used for bacteria while subourands agar (Scharlan Chemie) APHA Spain was used for fungi. Compound **1** (1 g) was dissolved in 1 ml of absolute ethanol and made up to 10 ml with distilled water to give a concentration of 100 mg/ml (10% dilution). A colony of each organism was sub-cultured on nutrient broth which contains peptone (5 g/l and meat extract (3 g/l) and incubated aerobically at 37°C for 8 h, 30 mls of the nutrient broth was used to flood the agar plates. A sterilized what man No **1** filter paper disc socked in compound **1** (0.02 ml) was used to test for the sensitivity or anti-microbial effect of compound **1**. The plates were incubated at 37°C for 24h. After incubation, plates were observed for zones of inhibition (in mm diameter). The minimum inhibitory concentration was determined. Plates containing agar medium without the addition of compound **1** were used as control, each test tube was replicated three times.

RESULTS AND DISCUSSION

The ethanolic seed extract of *C. alata* was subjected to solvent fractionation followed by solvent chromatography as described in the experimental. The brown pigment obtained after column chromatography was recrystallized from hexane to afford compound **1**. Compound **1** identified as cannabinoid dronabinol alkaloid was assigned the molecular formula m/z 284.2710 calculated for $C_{18}H_{23}O_2N$ (m/z 285) with base peak at m/z 73.028 calculated for $C_4H_{10}N$ (m/z 72) on the basis of HREIMS. The IR spectra revealed the presence of the hydroxyl, aromatic and ether groups at 3397, 1648 and 1095 cm-^{1}, respectively. The relative molecular mass of 284.2710 [M^+] with base peak at 73.0288 confirmed compound **1** as a cannabinoids droabinol alkaloid.



1

The pattern of fragmentation (**Fig. 1**) showed that compound 1 undergoes alpha cleavage to produce the peak m/z 29.039629). Further fragmentation produces the base peak m/z 73.0288 calculated for $C_4H_{10}N$ (m/z 72) and the major fragment m/z 213.1857 calculated for $C_{14}H_{13}O_2$ (m/z 213). Also cleavage at the amine portion produce the peak at m/z 43.0551 calculated for C_3H_7 (m/z 43)

The ^{1}H NMR spectrum (**Table 1**) revealed the presence of a cannabinoid dronabinol alkaloid. The ^{1}H NMR spectrum showed the presence of a methyl group at δH 0.8623 (3Ht) attached to the alkyl side chain. The aromatic proton appeared at δH 7.2618 (1Hs) and 7.712 (1Hs). The ^{1}H NMR spectrum of **1** also showed signals of two olefinic protons at δH 7.5164 (1Hs) and 7.5391 (1Hs) and five methylene protons at δH 1.2344 (2Hd), 1.2558 (2Hd), 1.2884 (2Hs), 1.2916 (2Hd) and 1.3029 (2Hm), respectively. The amine and hydroxyl protons appeared at δH 4.3615 and 4.3794, respectively.

In the ^{13}C NMR spectrum the Sp^2 carbons resonates at δC 128.157 (C_{11}) and 128.942 (C_{14}). The aromatic SP^2 signal were present at δC 131.017 (C_2), 130.307 (C_3), 130.108 (C_4), 129.814 (C_5), 128.942 (C_6) and 131.017 (C_7). There are also seven Sp^3 signals (five CH_2 and two CH_3) were present between δC 24.793 and 29.168. These data were consistent with cannabinoid frame work. All proton and carbon resonances were assigned as reported in Table **1** by careful analysis of ^{1}H NMR and ^{13}C NMR spectra. This analysis confirmed the sample isolated from the seeds of *C. alata* to be cannabinoid dronabinol alkaloid (10-methyl 6-hydroxy 4-propylamine Cannabinoid dronabinol) as the measured spectral properties are in accordance with available

literature data[24, 25]. Cannabinoid dronabinols have been found to exhibit pronounced biological activity such as anti-inflammatory and anticancer activity [24, 25]. Clinical trials revealed cannabinoid dronabinol to be a potent drug with outstanding ability to block the release of the enzymes responsible for pain and inflammation [25]. It may become a very useful treatment for the symptoms of arthritis and muscular dystrophy. It has been used as an adjunctive treatment for the relief of naturopathic pain in multiple sclerosis patients [25]. It was also used as analgesic for patients with advanced cancer who experience moderate to severe pain with the highest tolerated dose of strong opiod therapy [25]. The presence of cannabinoid dronabinol alkaloid may be the reason behind the use of this plant in the treatment of wounds and cancer in herbal medicine in Nigeria. This work therefore shows that cannabinoid dronabinol alkaloid may be one of the physiologically active compounds of *C. alata*. The occurrence of cannabinoid dronabinol in *C. alata* is of significance because this is to the best of our knowledge the first report of its occurrence in any *Cassia* species.

The antimicrobial activity of the compound isolated from the seeds of *C. alata* showed potent inhibition on some microorganisms. The cannabinoid dronabinol alkaloid isolated from the seeds of *C. alata* successfully inhibited *A. niger*, *C. albicans*, *S. aureus*, *P. aeruginosa* and *E. coli* (**Table 2**). Many of these organisms are natural flora of the skin and also known etiologic agent of several skin and mucous membranes infections of man [13]. The result obtained from this study show that the isolate from *C. alata* seeds showed inhibition towards pathogenic fungi (*A. niger* and *C. albicans*). These findings confirm the traditional therapeutic claims for the use of this herb for the treatment of ringworm and skin diseases [5]. The activity of compound 1 against *C. albicans* which normally inhibits part of the respiratory, gastrointestinal and female genital tract is also important [13, 26, 32]. Cannabinoid dronabinol alkaloid inhibited the growth of *Candida* cells. *C. albicans* is known to be inherently resistant to many antimicrobial agents. However, inhibition of this micro organism with compound **1** has confirmed the use of *Cassia alata* in herbal medicine for the treatment of *Candida* infections Wounds and boils provide environment conducive for the growth of microbial organism. Usually microbial contaminations of wound and boils involve a variety of organisms such as *P. aeruginosa*, *S. aureus*, *E. coli* and *K. Pneumonia[23]*. Evaluation of the effects of canabinod dronabinol alkaloid isolated from *C. alata* seed on these clinically isolated microbial contaminants of wound and boils showed varying levels of inhibitory activity against these pathogens (Table 2). Microbial infection of wound delays healing [23] and causes a more pronounced acute inflammatory reaction [23] which can lead to further tissue injury and damage. The antimicrobial activity of this compound isolated from *Cassia alata* seeds on these wound pathogens may contribute to wound healing, eliminate infections, thereby resulting to cell proliferation [23]. The minimum inhibitory concentration (MIC) of compound **1** was 6.5-50 mg/ml (**Table 2**). *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus* are common human commensal and have been incriminated in the infection of wounds [29]. These findings also justify the application of *C. alata* in dermatological creams and soaps and indicate that effective skin protection could be achieved at very low concentrations. The mechanism of inhibitory action of this compound in microorganism may be due to impairment of variety of enzyme systems, including those involved in energy production, interference with the integrity of the cell membrane and structural component synthesis [20]. Phenolics form a large group of naturally occurring, diverse and widespread compounds. They are characterized by the presence of aromatic ring with one or more hydroxyl groups. The phenolic ring in compound **1** may be responsible for the antiseptic,

antifungal or bactericide properties of *C. alata.* Phenols and phenolic compounds have been extensively used in disinfection and remains the standard with which other bactericides are compared [20, 21,30]. Phenolic compounds as electron donors are readily oxidized to form phenolate ion or quinone, an electron acceptor [30]. This property bestows upon phenolic compounds enables them to scavenge and trap microorganism [21].

Table 1: ^{1}H (400 MHz) and ^{13}C NMR (75 MHz) data of Compound 1

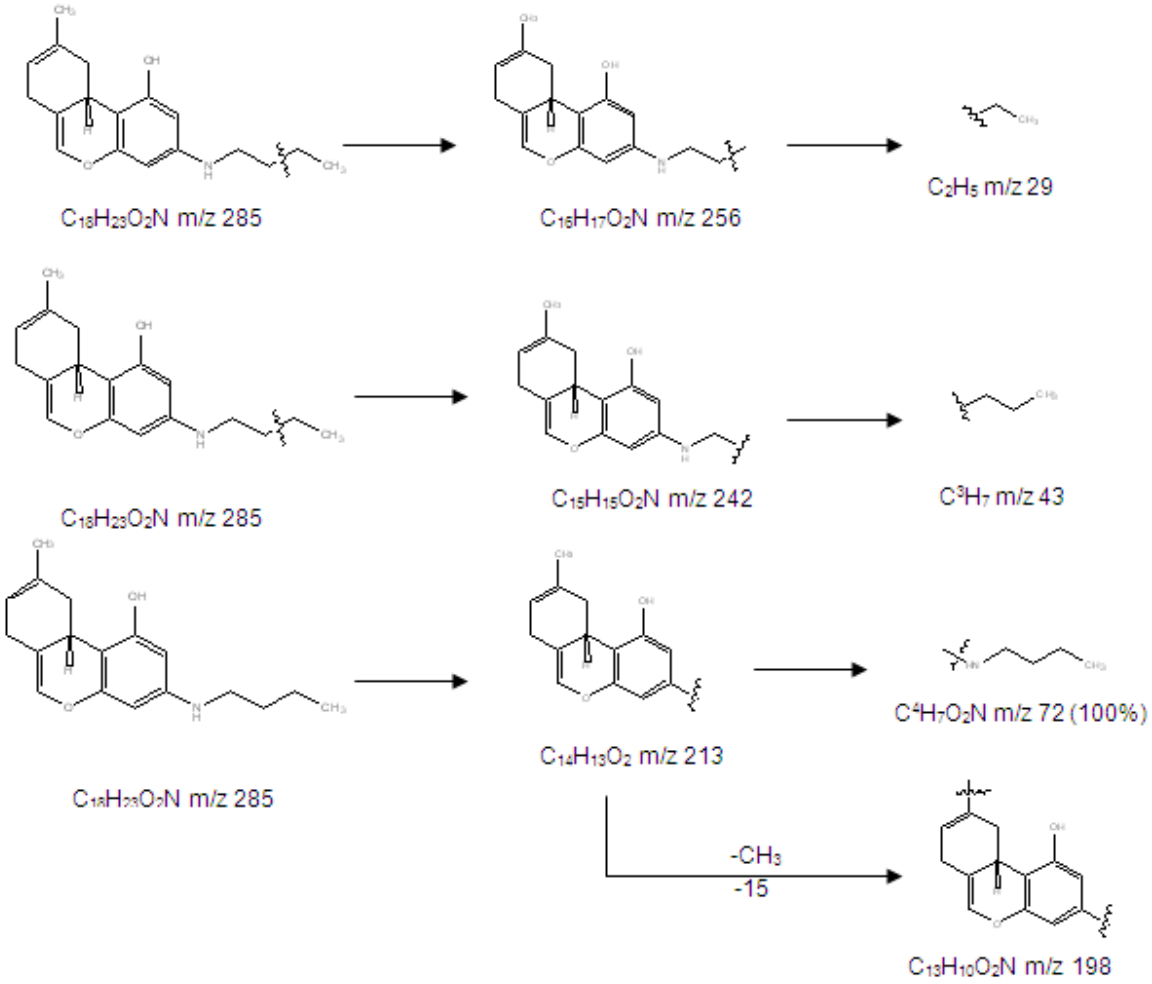
δ C	δ H			
Position	Chemical shift	Chemical shift	Multiciplicity position	
1				
2	131.017			
3	130.307	7.2618	CH	1Hs
4	130.108			
5	129.814	7.7124	CH	1Hs
6	128.942	4.3794	OH	b s
7	131.017			
8	61.718	1.6062	CH	1Ht
9	29.168	1.2344	CH_2	2Hd
10	127.994			
11	128.157	7.5164	CH	1Hs
12	29.343	1.2558	CH_2	2Hd
13	128.157			
14	128.942	7.5391	CH	1Hs
1^{1}	29.408	1.2884	CH_2	2Hs
2^{1}	29.237	1.2916	CH_2	2Hd
3^{1}	29.168	1.3029	CH_2	2Hm
4^{1}	25.720	0.8623	CH_3	3Ht
5^{1}	24.793	0.8896	CH_3	3Hs
NH		4.3615	NH	1Hs

Table 2: Diameter of zones of inhibition and mic values of compound 1 isolated from the seeds of *Cassia alata* (mg/ml)

Pathogens	Concentration of compound 1 mg/ml				
	100	50	25	12.5	6.5
	Zone diameter of inhibition mm				
Staphylococcus aureus	10.10± 0.10^{a}	6.00	3.0	1.0	-
Escherichia coli	7.00 ± 0.10^{c}	2.00	-	-	-
Pseudomonas aeruginosa`	14.00 ± 0.20^{a}	6.00	4.00	2.0	1.0
Klebsiella pneumonia	9.00 ± 0.10^{b}	3.00	2.10	-	-
Aspergillus niger	13.0 ± 0.10^{a}	5.00	2.0	-	-
Candida albicans	12.0 ± 0.20^{a}	10.00	3.0	1.0	-

Data are means ± standard deviation of triplicate determinations. Values with superscript that are the same in each row are not significantly different (P < 0.05). ***-*** *= No inhibition*

The efficacy of compound **1** on these microorganisms may be attributed to the presence of phenolic ring which scavenge for the microorganism or the alkaloid and ether portions which causes impairment of the enzymes in the organism. This compound cause swelling of hyphae, tips, plasma seeping around hyphae, leaking of plasma, cell wall distortion, abnormal branching or fusion of hyphae and consequently wrinkling of hyphae surface [28, 31].



Fragmentation pattern of compound **1** from *C. alata*

These findings further justifies the use of *Cassia alata* in the treatment of skin infections such as eczemas, ringworms, boils, carbuncles, breast abscess, infantile impetigo, sores and wound treatment in herbal medicine and its use as an ingredient in the formulation of medicated and antiseptic soaps. If judiciously taped, extracted and haressed Cassia species could provide raw materials for the pharmaceutical industries in the country.

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