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Isolation, Characterisation and Antimicrobial Activity of a Steroidal Ester from the Leaves of *Cassia nigricans* Vahl.

[1]R.G. Ayo, [2]J.O. Amupitan and [2]A.O. Oyewale
[1]Samaru College of Agriculture,
[2]Department of Chemistry, Ahmadu Bello University, Zaria, Nigeria

Abstract: The aim of the study was to scientifically validate the claims that *C. nigricans* is used in traditional medicine for the treatment of skin diseases, infections and wounds. The leaves of *Cassia nigricans* is said to be used in traditional medicine for the treatment of peptic ulcer, gastro-intestinal disorders, diarrhoea and skin diseases. The glycoside present in the methanol extract of the leaves was hydrolysed using dilute hydrochloric acid. A silica gel column of the resulting aglycone (using petroleum ether:ethyl acetate mixtures) gave a white amorphous powder, identified as steroidal ester by means of spectral analysis. The antimicrobial activity of the steroidal ester was investigated against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Corynebacterium pyogenes*, *Bacillus subtilis*, *Salmonella typhi*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Neisseria gonorrhoeae* and *Klebsiella pneumoniae* using agar diffusion technique. The results showed that the compound was effective against all the test organisms and the minimum inhibitory concentration was found to be 2×10^3 μg mL^{-1}.

Key words: *Cassia nigricans*, leguminosae, methanol extract, steroidal ester, antimicrobial activity

INTRODUCTION

Plants of *Cassia* genus (Leguminosae, sub-family: Caesalpiniacea) are known to be sources of anthracene derivatives, polyphenols, polysaccharides, flavonoids and steroids (Nageswara Rao *et al*., 2000; Bahorun *et al*., 2005). *Cassia* species are well known in folk medicine for their laxative and purgative uses as well as for treating skin diseases such as ringworm, scabies and eczema (Elujoba *et al*., 1999).

Cassia nigricans, a member of Leguminosae family, is of high therapeutic value in ulcers, gastro-intestinal disorders, diarrhoea and skin diseases (Akah *et al*., 1998; Nwafor and Okwuasaba, 2001; Jacob *et al*., 2002). The *C. nigricans* leaves also showed good analgesic, anti-inflamatory, larvicidal and anti-plasmodial activities (Chidume *et al*., 2001; Yang *et al*., 2003; Obodozie *et al*., 2004). The antimicrobial activity of the leaves has been reported by Ayo and Amupitan (2004). The plant extracts also have the potential to be used as an organic approach to the management of some of the agricultural pests (Georges *et al*., 2008). A perviously isolated compound from the leaves of *C. nigricans* was emodin (Obodozie *et al*., 2004; Ayo *et al*., 2007). Recently, citreorosein, emodic acid and luteolin were also identified (Georges *et al*., 2008).

The aim of the study was to scientifically validate the claims that *C. nigricans* is used in traditional medicine for the treatment of skin diseases, infections and wounds. In addition, we isolated and characterised an active constituent of the methanol extract of the leaves of the plant and evaluated its activity against some common pathogenic microorganisms.

Corresponding Author: R.G. Ayo, Samaru College of Agriculture, Ahmadu Bello University, Zaria, Nigeria

MATERIALS AND METHODS

General Experimental Procedures

The experiment was carried out in the Organic Research Laboratory, Department of Chemistry, Ahmadu Bello University, Zaria, Nigeria between September, 2006 and March, 2007.

All chemicals used were of analytical grade. Thin Layer Chromatography (TLC) was carried out on plates coated with silica gel having gypsum binder and fluorescent indicator and viewed under ultraviolet lamp (254 and 366 nm). Precoated plates silica gel $60F_{254}$ were used. Column chromatography was carried out using silica gel (60-120 mesh). Infrared (IR) spectrum was recorded on a Perkin-Elmer IR spectrophotometer 1600 series. The solid sample was run as Nujol mull on sodium chloride plates.

The ^{1}H NMR and ^{13}C NMR spectra were obtained at 400 MHz on a INOVA-400 NMR instrument using tetramethylsilane as internal standard. The sample was run in deuterochloroform ($CDCl_3$). The Gas Chromatography/Mass Spectrometry (GC/MS) spectra were recorded on GC/MS ATVRIN 2000R Varian spectrometer interphased with Varian Model 3800 GC/MS. The scan range was 1-1680.

Plant Material

The leaves of *C. nigricans* Vahl were collected in September, 2006 from Jama'a Village near Ahmadu Bello University dam, Zaria (11° 10' N, 7° 38' E), Nigeria. The plant was identified and confirmed in the Herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria, where a Voucher Specimen Number 613 was deposited. The leaves of *C. nigricans* were air-dried and powdered.

Extraction and Isolation

The extraction and the hydrolysis of the leaves were carried out using known standard procedures (Harborne, 1984). The powdered leaves (250 g) were extracted by Soxhlet extraction method with petroleum ether (60-80°C) and methanol. Each extract was concentrated and evaporated to dryness on a rotary evaporator. The crude methanol extract (20.0 g) was treated with 3.0 M HCl (200 cm^3). The mixture was cooled and filtered. The hydrolysate was washed with chloroform. The chloroform extract was then concentrated at a reduced pressure to give the aglycone (1.55 g).

The aglycone (0.5 g) was loaded on silica gel column. The column was developed using mixtures of petroleum ether (60-80°C) and ethyl acetate of increasing polarity from petroleum ether to petroleum ether:ethyl acetate (1:3). Fractions were collected in 20 cm^3 aliquots. The elution was monitored by TLC. Similar fractions were pooled together, washed, dried and evaporated on rotary evaporator and finally combined into seven fractions. Fraction 2, the major fraction from the column, was further purified on silica gel using Preparative Thin Layer Chromatography (PTLC).

Test Microorganisms

Standard strains of 10 pathogenic microorganisms were obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Zaria, Nigeria. *Staphylococcus aureus* (Sa) ATCC 13709, *Streptococcus pyogenes* (Local strain), *Corynebacterium pyogenes* (Local strain), *Bacillus subtilis* NCTC 8236, *Salmonella typhi* (St) ATCC 9184, *Escherichia coli* (Ec) NCTC 10418, *Pseudomonas aeruginosa* (Pa) NCTC 6750, *Candida albicans* (Ca) ATCC 10231, *Neisseria gonorrhoeae* (Local strain) and *Klebsiella pneumoniae* ATCC 10031 were used for the experiment.

Antimicrobial Activity Assay

Antimicrobial activity was carried out using paper disc diffusion method (Ericsson *et al*., 1960; Bauer *et al*., 1966). Solutions of steroidal ester of varying concentrations, ranging from 1.0×10^3 to 5.0×10^3 μg mL^{-1} were prepared. Nutrient agar was prepared, sterilised and used as the growth medium for the microorganisms. Twenty milliliters of the sterilised medium was poured into each sterilised Petri dish, covered and allowed to solidify. The Mueller-Hinton sensitivity agar (oxoid) plate was then seeded with the test microorganisms by the spread plate technique and was left for about 30 min to dry. The sterilised paper discs were soaked in the prepared solutions of the extracts with varying concentrations and were dried at 50°C. The dried paper discs were then planted on the nutrient agar seeded with the test microorganisms. The plates were incubated at 37°C for 24 h, after which they were inspected for the zones of inhibition of growth. The zones of inhibition of growth produced by the Minimum Inhibitory Concentrations (MICs) were measured in millimeters and the values obtained were recorded. A control experiment was also set up using pure dimethyl sulphoxide for each of the test organisms.

RESULTS

The extraction of the leaves of *C. nigricans* gave 9.6 g (3.84%) of crude petroleum ether extract and 21.40 g (8.56%) methanol extract. The crude methanol extract (20.0 g) when hydrolysed gave the aglycone (1.55 g). The resulting aglycone was subjected to column chromatography on silica gel column using petroleum ether:ethyl acetate mixtures. The fraction 2 from the column, when further purified on silica gel PTLC using petroleum ether:ethyl acetate (1:2) as mobile phase, gave white amorphous powder. The spectral data, IR, ^1H – NMR, ^{13}C-NMR and GC/MS of the white powder are shown below:

- **IR: υ_{max} (cm^{-1}):** 467, 604, 720, 862, 1036, 1114, 1240, 1376, 1463, 1738, 2852, 2922, 3443
- **^1H NMR ($CDCl_3$) ppm:** δ 0.8 (6H), 1.2 (m, ring protons), 1.58 (s, 1H), 2.0 (m, 1H), 2.25 (t, 3H), 4.1 (q, 2H)
- **^{13}C NMR ($CDCl_3$) (ppm):** δ_C 14.04, 14.14, 14.18, 22.63, 24.93, 29.08, 29.08, 29.20, 29.30, 29.46, 29.6, 29.6, 33.34, 60.09, 173.90
- **GC/MS:** m/z 318 (15), 272 (10), 242 (5), 210 (27.5), 147 (40), 103 (10), 73 (100)

The results of the antimicrobial activity of the steroidal ester isolated from the methanol extract of *C. nigricans* leaves were summarised in Table 1 and 2. Table 1 shows the diameter of the zone of inhibition for the steroidal ester. *Staphylococcus aureus* had the highest zone of inhibition (48 mm), while *B. subtilis* had the lowest zone of inhibition (18 mm). Table 2 shows the results of the MIC test. The MIC value was found to be 2×10^3 μg mL^{-1} against *S. pyogenes* and *E. coli*, while for *C. pyogenes*, *S. typhi*, *P. aeruginosa*, *C. albicans* and *N. gonorrhoea*, the value was 3×10^4 μg mL^{-1}. For *B. subtilis* and *K. pneumoniae*, the MIC value was 4×10^3 μg mL^{-1}.

Table 1: Zone of inhibition of the isolated steroidal ester

Test organism	Diameter of zone of inhibition (mm)
Staphylococcus aureus	48
Streptococcus pyogenes	32
Corynebacterium pyogenes	30
Bacillus subtilis	18
Salmonella typhi	26
Escherichia coli	22
Pseudomonas aeruginosa	24
Candida albicans	27
Neisseria gonorrhoea	21
Klebsiella pneumoniae	24

Table 2: Minimum inhibitory concentration of the isolated steroidal ester

	Concentrations of emodin (μg mL^{-1})				
Test organism	1×10^3	2×10^3	3×10^3	4×10^3	5×10^3
S. aureus	–	0+	+	+	+
S. pyogenes	–	0+	+	+	+
C. pyogenes	–	–	0+	+	+
B. subtilis	–	–	–	0+	+
S. typhi	–	–	0+	+	+
E. coli	–	0+	+	+	+
P. aeruginosa	–	–	0+	+	+
C. albicans	–	–	0+	+	+
N. gonorrhoea	–	–	0+	+	+
K. pneumoniae	–	–	–	0+	+

+: Inhibition, 0+: Minimum inhibition, –: No inhibition, no inhibition with negative control

DISCUSSION

The structure of the steroidal ester, possessing antimicrobial activity, isolated from the methanol extract was established by the spectral data shown above. The IR spectrum of the compound indicated the presence of $>CH_2$, CH_3 stretching at 2852 and 2923 cm^{-1} for the alkanes and O-H stretching and bending at 3443 and 1463 cm^{-1}, respectively. The strong band at 1738 cm^{-1}was characteristic of unconjugated carbonyl of saturated ester function (-CO-O-) and the absorption at 1240 cm^{-1} was for the carbon-oxygen bond (-C-O). These infrared absorption bands agreed reasonably well with those previously reported by Williams and Fleming (1989).

The ^{13}C- NMR revealed the presence of twenty-two signals. The signals at δ_C 14.04, 14.14 and 14.18 ppm were assigned to the three methyl groups. The signal at δ_C = 173.90 ppm was assigned to the carbonyl carbon (C = O) of the acetate, while the δ_C = 60.1 ppm was assigned to carbon of the ester linkage (-OCH_2CH_3). The δ_C 21.86 and 34.34 were assigned to C-17 and C-12, the carbons bearing the esters and hydroxyl groups. C-1 and C-3 were likely to be equivalent and appeared as one peak. The C-5, C-8, C-9 and C-10 were also equivalent as a result of overlapping and also gave rise to one peak. C-6 and C-7 were also equivalent and appeared as one peak. The remaining five carbon signals were due to C-2, C-4, C-14, C-15 and C-16.

The compound was found to have signals at δ 0.8, 1.2, 1.58, 2.0, 2.25 and 4.1 ppm. There were broad complex bands of absorbance which appeared as large multiplets at 1.2 ppm for the protons of the ring system. The protons of two methyl groups attached to C-4 appeared at signal 0.8 ppm. There was a broad signal at 1.5 ppm for the proton of the hydroxyl attached to C-12. The methine proton at C-12 appeared as a multiplet at signal 2.0 ppm. The protons of the methyl group of the ethyl group of the ester absorbed at 2.25 ppm as triplet, while the methylene protons appeared as quartet at 4.1 ppm. The nmr spectral patterns observed for the compound were in agreement with the structure of a saturated steroidal ester (Bhacca *et al*., 1962; Reich *et al*., 1969; Smith, 1978).

The GC/MS spectrum of the compound exhibited mass fragments at m/z 318, 272, 242, 210, 147, 103 and 73. The expected molecular ion peak at m/z 348 was absent. It was the peak m/z 318 that appeared. This signal, m/z 318 (M^+ - 30) indicated simultaneous loss of ethyl group of the ester and probably the proton of the hydroxyl group.

Based on the available spectroscopic data, the compound was interpreted as hydroxyestranic acid ethyl ester and the proposed structure is shown in Fig. 1.

The results of the zone of inhibition demonstrated that the steroidal ester exerted high inhibitory effect on the growth of the pathogenic microorganisms, thus providing a scientific basis for the use of the plant extracts in the treatment of wounds and skin diseases in folk medicine. From these results, it was evident that the isolated compound was active against all the test microorganisms, but at different concentrations. The above results are comparable with the significant antimicrobial and

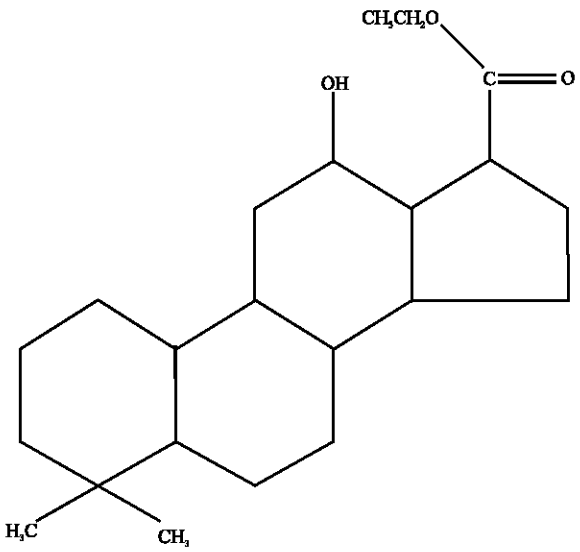

Fig. 1: Hydroxyestranic acid ethyl ester

antifungal activities of *C. fistula* (Duraipandiyan and Ignacimuthu, 2007) and *C. alata* (Nebedum *et al*., 2009). The isolation and characterisation of the steroidal ester from the leaves of *C. nigricans* and the antimicrobial activity of the ester are reported for the first time in the present study.

CONCLUSION

A steroidal ester was isolated from the methanol extract of the leaves of *C. nigricans* Vahl., characterised and found to be active against common pathogenic microorganisms. This compound may be a source of drugs that could improve the treatment of infections caused by microorganisms.

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