PHYTOCHEMICAL SCREENING AND ISOLATION OF STEROLS FROM *Cassytha filiformis* LINN

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Abstract: *Cassytha filiformis*, a leafless and perennial vine with small scales as a replacement of the leaves is currently being used in the treatment of various disease conditions such as jaundice, fever and malaise. Preliminary phytochemical screening of various extracts (Petroleum ether, Ethyl acetate and methanol) showed that the whole plant contains alkaloids, steroidal nucleus, phenolic nucleus, terpenoids, flavonoids and saponins. Isolated compound 5B from petroleum ether extracts was analyzed using various test ranging from physical (Melting point 144-146^{o}C uncorrected and Solubility in ethyl acetate and chloroform), chemical (Positive reaction using Libermann-Buchard test) and spectroscopic techniques (NMR, FT-IR and MS) in order to determine the identity of the isolated compound. Based on the result obtained from these analyses and comparing the data with that reported in the literatures, 5B contained β-sitosterol and stigmasterol.

Keywords: β-sitosterol, *Cassytha filiformis*, FT-IR, NMR, *RumfarGada*, stigmasterol

Introduction

Plants of the Lauraceae are nearly all woody trees and shrubs comprising 32 genera and about 2,000 – 2500 species. An exception is the vining, leafless, parasitic genus *Cassytha* (Watson and Dallwitz, 1993). This plant is considered to be unique in the family of Lauraceae as it is a parasite. The genus derived its name, *Cassytha*, from the Greek name of *Cuscuta* (meaning dodder). The vine has several common names in the regions of the tropics. For example, South Sea Islanders called this vine as *"tentanini"* which has the meaning "to go round and round," and this seems to be a true descriptive adjective for the plants entwining habit (Mythili *et al.,* 2011). Hausas in northern Nigeria called the plant as "RumfarGada".

*Cassytha filiformis*is a plant used for its various ethnomedical purposes in Nigeria. The plant is used in traditional treatment of many diseases such as vermifuge, kidney ailment, gonorrhea and also in the suppression of lactation after still birth by several tribes in Nigeria (Burkill, 1995). The plant (stem and leaves) is boiled in water and administered for varying lengths of time to treat Jaundice (Personal communications). Men were also reported to use it in love magic while women used the extracts of the vine as a colouring agent or as a dye to provide a black color for the fabrics (Schroeder, 1967).

In the traditional Ayurveda, *Cassytha filiformis*is used as the major substitute for *Cuscuta* (Sakshy *et al.,* 2010). The brown colour of the stem is used as the colouring agent and hence possesses a major application in the dyeing industries. Several aporphinoid alkaloids were isolated from the samples originating from Taiwan, Brazil, Australia and New Guinea but compositions were found to be quite variable among the different origins. Six aporphines from *C. filiformi* swere shown to have *in vitro* cytotoxic properties out of which actinodaphnine, cassythine, and dicentrine, also show *in vitro* antitrypanosomal properties against *Trypanosoma bruceibrucei* (Quetin-Leclercq *et al*., 2004). Aqueous and alcoholic extracts of *C. filiformis* were tested for their diuretic activity in Wister rats. Total urine output volume and the concentration of Na^+, K^+ and Cl^- ions excretion in the urine were finally estimated. Aqueous and alcoholic extracts of *C. filiformis* were found to exhibit significant diuretic activity by causing a marked increase in the Na^+ and K^+ excretion (Sharma *et al.,* 2009).

This study was designed to isolate addition compounds from the petroleum ether extract of *Cassyttha filiformis.*

Materials and Methods

Materials

The chemicals (ethyl acetate, methanol, acetone and petroleum ether) used during the study were of analytical grade. The instruments were well calibrated before use (EP, 2011). All ^{1}H and ^{13}C NMR were recorded using a Bruker AVENGE 500 MHz spectrometer and data were processed by ACD/NMR Processor (Academic Edition). Samples were made as dilutions of $CDCl_3$. FT-IR spectra were obtained with an ALPHA Bruker Optics FTIR spectrophotometer equipped with ZnSe ATR crystal. The samples were scanned from 400 – 4000 cm^{-1} wavenumber with a 32 scan per sample circle and a resolution of 4.

The plant material

The whole fresh plants of *C. filiformis* were collected from ABU Dam area and identified and confirmed by a Taxonomist at the Department of Biological Sciences, Ahmadu Bello University, Zaria, the voucher specimens were preserved at the Department herbarium library (2314). After the identification, the samples were dried under shed for 1 week prior to extraction.

Extraction of powdered C. filiformis

Powdered *C. filiformis* (1.5 kg) was extracted using cold maceration with pet ether (40-60^{o}C) (2.5 L) for 4 h. The extracts obtained were evaporated under reduced pressure using Rotary evaporator to brown residue and stored at room temperature.

Qualitative phytochemical screening

Qualitative phytochemical analysis was carried out on the extracts (petroleum ether, ethyl acetate and methanol) to determine the presence of alkaloids, glycosides, phenolic compounds such as flavonoids and saponins, sterols, etc. by following standard procedures (Khandelwal, 2005; Chulet *et al*., 2010).

Unsaponifiable fraction

The unsaponifiable fraction was determined using the method described by Pearson (1991) as:

$$\text{Unsaponifiable fraction (\%)} = \frac{Weight of residue}{Weight of sample} \times 100$$

Chromatographic and Spectroscopic Studies

Thin layer chromatography of the extracts

Thin layer chromatography analysis of the three extracts were carried out on silica gel pre-coated plates (MERCK® GF 254, 0.25 mm) in order to establish profile of the major constituent in each extract. Suitable solvent systems used were EtOAc:$CHCl_3$:MeOH:H_2O (15:8:4:1), BuOH: Acetic acid: H_2O (6:1:1) and Hexane: Ethyl acetate (2:1). Visualization was achieved using anisaldehyde/H_2SO_4 (general detecting reagent), $FeCl_3$ (for phenolic compounds) and Dragendoff'sragent (for alkaloids). The experiment was carried out following standard procedure described by WHO (2011).

Column chromatographic separation of petroleum ether extract

The petroleum ether extract was subjected to column chromatography using silica gel (60-100 mesh, Sigma-Aldrich, Germany) as stationary phase and ran by gradient elution technique where n-hexane and ethyl acetate were employed as the mobile phase. The silica gel (100 g) was packed in a glass tube (100 cm long X 2 cm diameter) with hexane using wet packing method. The column was allowed to stabilize for 3 h before the extract (2 g) was loaded on it. Elution began with hexane (100%) and the followed by gradual introduction of ethyl acetate (5, 10, 15%, etc.) until ethyl acetate (100%) was used. 50 ml aliquots were collected and analyzed using TLC visualized by UV light and 10% H_2SO_4 solution. Similar fractions were pooled together for further purification (Chulet *et al*., 2010).

Melting point analysis

The melting points of the isolated compound 5B were recorded on a Stuart Scientific SMP3 system.

Spectroscopy of isolated compound 5B

The isolated compound was subjected to proton NMR (^{1}H, NMR) using Bruker AVANCE 500 MHz spectrometers. Data was manipulated directly using BrukerXwinNMR (version 2.6) and the samples were made as dilute solutions of $CDCl_3$ unless otherwise stated. All chemical shifts (δ) are reported in parts per million (ppm) relative to residual solvent peaks δ 7.27 for 1H NMR in $CDCl_3$. COSY spectrum was obtained to aid in the structure elucidation.

FT-IR spectra were obtained with an ALPHA Bruker Optics FTIR spectrophotometer equipped with ZnSe ATR crystal. The samples were scanned from 400 – 4000 cm^{-1} wavenumber with a 32 scan per sample circle and a resolution of 4.

Results and Discussion

Preliminary phytochemical screening of various extracts (Petroleum ether, Ethyl acetate and methanol) showed that the plants contain alkaloids, steroidal nucleus, phenolic nucleus, terpenoids, flavonoids and saponins. This is presented in Table 1.

Table 1: Preliminary phytochemical screening of *C. filiformis*

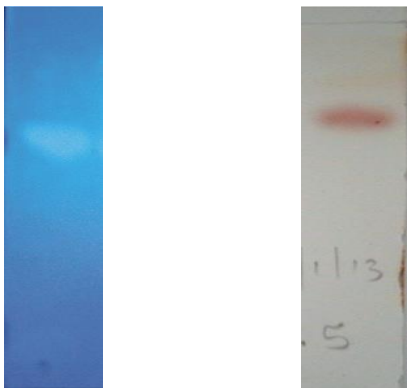
Test	Observation	Inference
Dragendoff's reagent	Orange red precipitate	***Alkaloids present***
Mayer's reagent	Creamy white precipitate	***Alkaloids present***
Wagner's reagent	Brown precipitate	***Alkaloids present***
Guinard Test	Brick red colour	***Cyanogenic present***
Liebermann-Burchard'sreagent	No green colouration	***Steroidal nucleus present***
Kella-Killiani'sreagent	No reddish brown at interphase	***Deoxy-sugars absent***
Kedde'sreagent	No purple blue colouration	***Lactone ring absent***
Lead acetate Test	Buff precipitate	***Tannins present***
Bromine water Test	Blue colouration	***Tannins present***
Ammonia solution Test	Green colouration	***Tannins present***
Borntrager'sreagent	Pink colour	***Anthraquinones present***
$FeCl_3$ Test	Greenish	***Phenolic nucleus present***
NaOH Test	Yellow colouration turns colourless with HCl	***Flavonoids present***
Shinoda Test	Pink colouration	***Flavonoids present***
Amyl alcohol Test	Yellow colouration	***Flavonoids present***
Frothing Test	Persistent froth	***Saponins present***
Haemolysis Test	Haemolysis	***Saponins present***
Salkowski's Test	Brown colour at interface	***Terpenoids present***

Isolation of compound 5B

Column fractions showing single spots (plate X) was re-crystalized using ethyl acetate and afforded white crystalline powder coded 5B (10.2 mg) which was subjected to physical, chemical and spectral analysis for identification

Thin layer chromatographic analysis of 5B

The result of thin layer chromatographic analysis of 5B revealed a single spot with $R_f$value of 0.70 when developed with hexane: ethyl acetate (2:1) as solvent system (Plate 1).



A **B**

Plate 1: TLC plates of 5B developed in Hexane:Ethyl acetate (2:1)

Solubility profile of 5B

The compound 5B was found to be soluble in ethyl acetate and most soluble in chloroform.

Melting point of 5B

The sample was found to have a melting point range of 144 -146^{o}C.

Spectroscopic analysis

The results of the spectroscopic analysis of compound 5B are presented in Table 2.The ^{1}H-NMR and ^{1}H-H COSY spectra of **5B** spectrum of compound 5B (Figs. 1, 2 and Table 2) exhibited clusters of signals at lower field (0.02 ppm) which corresponded to signals of CH_3, CH_2 and CH of steroids and terpenoids. Analysis of the proton NMR revealed the presence of a signal at 3.52 ppm which is a typical characteristic of a proton at C-3 of steroid. The proton NMR also revealed the presence of a proton at 5.35 broad singlet (brs) which is assigned to proton attached to unsaturated carbon (olefinic) at position H-8. The appearance of another two olefinic proton signals at 5.12 and 5.15 both double doublet (dd) revealed the presence of stigmasterol in 5B. This established that 5B is a mixture of *β*-sitosterol and stigmasterol. All the protons in 5B were assigned as shown in Table 2 and are similar with the spectral data of *β*-sitosterol and stigmasterol isolated from curcurbitaceae (Anjoo and Ajay, 2011).

Table 2: Some NMR Signals of Compound 5B Measured in $CDCl_3$ at 500 MHz

δH^1 (ppm)	Multiplicity	No. of Protons	Assignment
3.52	m	1H	H-3
5.36	brs	1H	H-6
0.70	brs	3H	H-18
1.01	brs	3H	H-19
0.92	brs	3H	H-21
5.02	dd	1H	H-22
5.15	dd	1H	H-23
0.82	brs	3H	H-26
0.83	Brs	3H	H-27
0.86	Brs	3H	H-29

m= multiplet, brs= broad singlet, dd= double-doublet

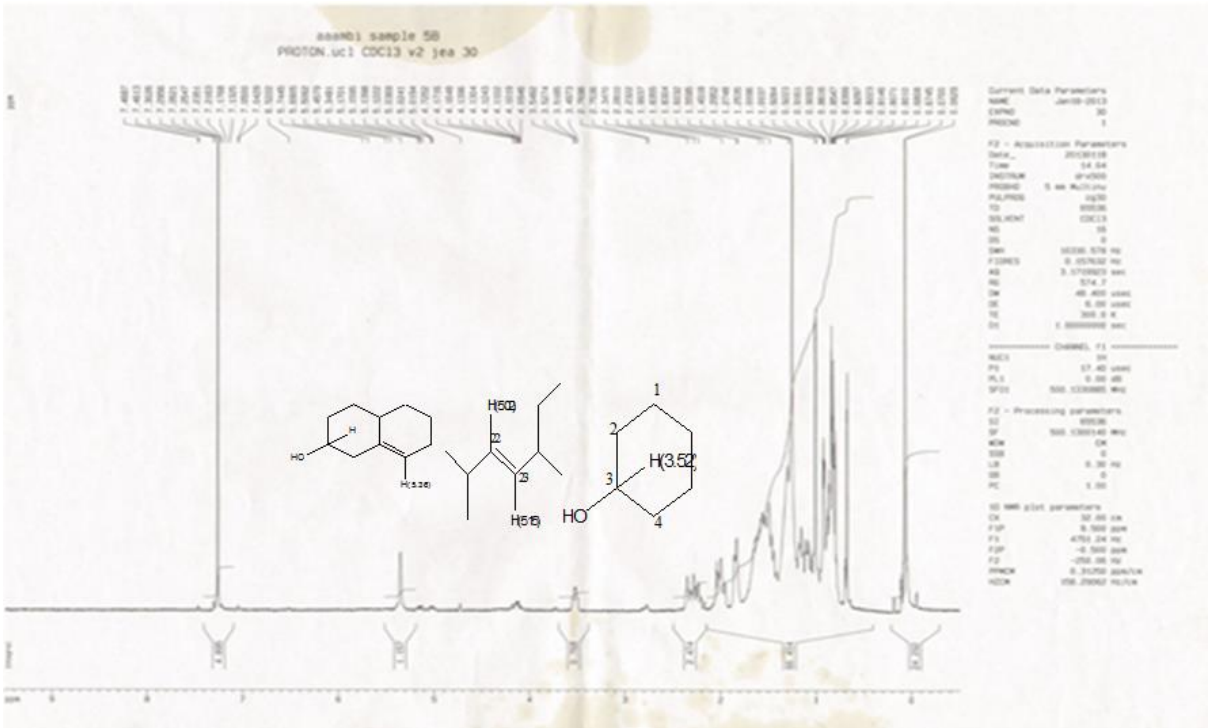


Fig. 1:^{1}H NMR spectrum of 5B in $CDCl_3$ 500MHz

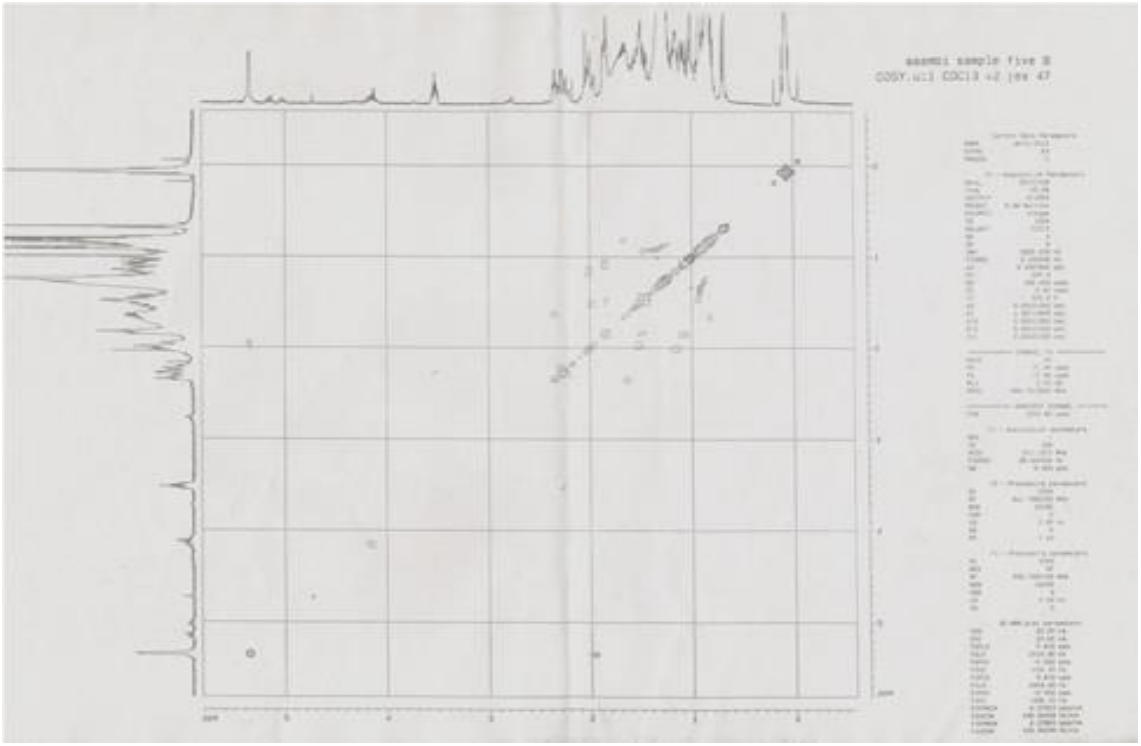


Fig. 2: ^{1}H-H COSY spectrum of 5B in $CDCl_3$ 500MHz

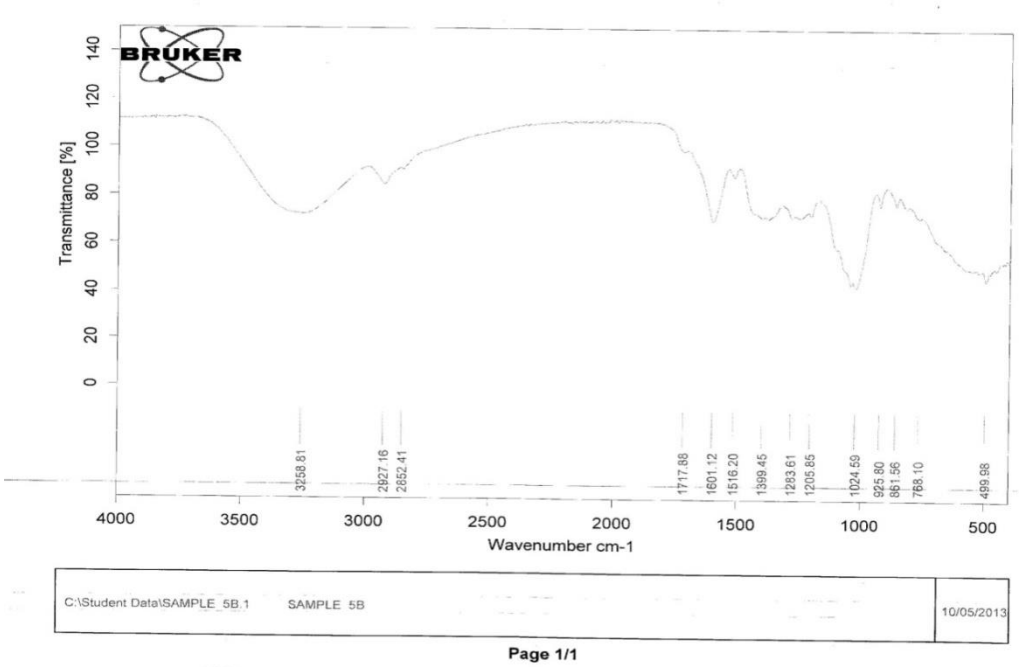


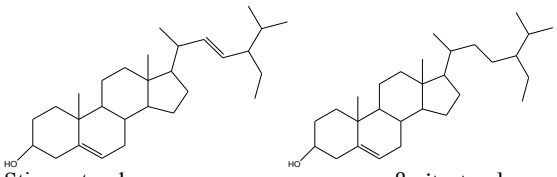
Fig. 3: FT-IR spectrum of 5B

The isolated compound (5B) showed positive result with Libermann-Buchard reagent which suggested that the compound contained steroidal nucleus. FT- IR spectroscopic analysis as revealed in Fig. 3, showed absorption bands at 3258.8 cm^{-1} that is characteristic of O-H stretching, 2927.2 and 2852.4 cm^{-1} are due to aliphatic C-H stretching. Other absorption frequencies include 1601.1 cm^{-1} as a result C=C stretching, the absorption frequency at 1024.6 cm^{-1} signifies cycloalkane. The out of plane C-H vibration of unsaturated part was observed at 861.6cm^{-1}. These absorption frequencies resemble that observed for β-sitosterol and stigmasterol (Jamal *et al*., 2009).

The mass spectra (MS) of 5B revealed a strong molecular ion peak at m/z (mass to charge ratio) 414 and a weak peak at m/z 412 (Fig. 4) which corresponded to the molecular weights of β-sitosterol and stigmasterol respectively. Also, the fragmentation pattern as shown in Fig. 5, exhibited the following *m/z*: 396 [M-H_2O], 381.[M-CH_3,-H_2O], 303

[$C_7H_{11}0$], 273 [M-$C_{10}H_{21}$] and 255 [M-H_2O- $C_{10}H_{21}$]. These two compounds are widely distributed in plants and have closed resemblance in structure but differ in unsaturation at position C-22 and C-23.

Based on the results of FT-IR, NMR, MS and by comparing the data with that reported in the literatures (Vipin and William, 1984), 5B probably contain compounds with the following structures.



Stigmasterol β-sitosterol

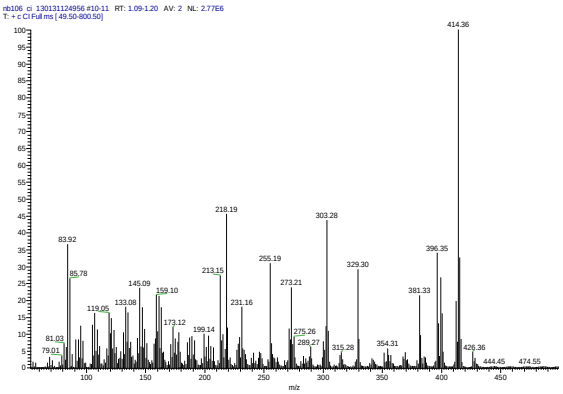


Fig. 4: Mass spectrum of 5B (chemical ionization)

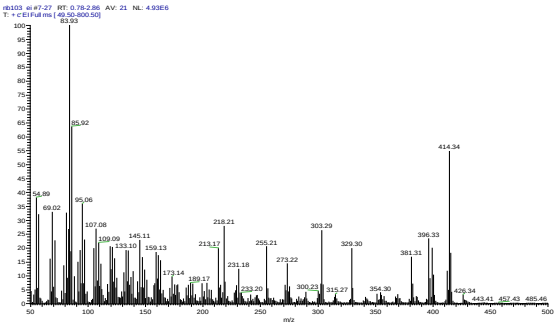


Fig. 5: Mass spectrum of 5B (electron impact)

Sterols such as stigmasterol and β-sitosterol occur in almost all higher plants and have been found very useful in drug development. They acts as a precursor in the synthesis of progesterone and acts as an intermediate in the biosynthesis of androgens, estrogens, corticoids (Sundararaman and Djerassi, 1977), and in the synthesis of vitamin D3 (Kametani and Furuyama, 1987). Stigmasterol was reported to inhibit cholesterol biosynthesis via inhibition of sterol 24-reductase in human Caco-2 and HL-60 cell lines thus suppressing hepatic cholesterol (Batta *et al*., 2006).

Conclusion

The chemical content of the petroleum ether extract of *C. filiformis* were identified based on chemical test and through hyphenated spectroscopic techniques such as FT-IR, MS (both chemical ionization CI and electron impact ionization EI) by comparison with relevant libraries. Attempt to isolate some of the chemical constituents provided a mixture of steroids which were identified by NMR as stigmasterol and β-sitosterol.

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