STIGMASTEROL AND β-SITOSTEROL FROM THE ROOT OF *Mangifera indica* AND THEIR BIOLOGICAL ACTIVITIES AGAINST SOME PATHOGENS.

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Authors' contributions

This work was carried out in collaboration between the authors. All authors read and approved the final manuscript.

ABSTRACT

Aim: *Mangifera indica* is a plant with several phytoconstituents which accounts for its trado-pharmacological uses such as treatment of syphilis, anemia, diabetes, diarrhea, mouth wound, ulcer, leucorrhea, fever, liver diseases, urinary tract infection, asthma, rheumatism, cough, colon cancer and dysentery. The present study was aimed at identifying and characterizing some of the active principles from root of the plant.

Places and Duration of study: The isolation and characterization of the compounds was carried out at the Strathclyde Institute of Pharmacy and Biomedical Sciences (SIPBS), University of Strathclyde, Glasgow, United Kingdom between October, 2018 to February, 2019 while the bioassay analysis was done at Ahmadu Bello University, Zaria, Nigeria.

Methodology: The root powder was subjected to Soxhlet extraction with ethyl acetate to obtain the crude extract, which was fractionated on column using hexane, and ethyl acetate in increasing ratios. The isolated components were tested for their antimicrobial activities against some plants and animal pathogens at Ahmadu Bello University, Zaria, Nigeria.

Results: White crystals were obtained which on spectra analysis (IR, 1H-NMR, 13C-NMR, 2D-NMR) were identified as mixture of stigmasterol and β-sitosterol. The isolated compounds exhibited appreciable antimicrobial activities against some microbes, thus confirming the many ethnomedical uses of the plant.

Conclusion: The compounds isolated were identified as stigmasterol and β-sitosterol. They showed moderate sensitivity towards the pathogens tested in the study.

KEYWORDS: *Mangifera indica,* Stigmasterol , β-sitosterol, zone of inhibition, MIC, MBC, MFC and pathogen.

Introduction

Medicinal plants are a rich source of drugs that form a vital component in traditional and modern medicines as well, serve as pharmaceutical intermediates in drug production. This accounts for the usefulness of plants in the treatment of diseases and play vital roles in the world's pharmaceuticals due to chemical substances found in them [1]. *Mangifera indica* belong to the genus *Mangifera* of the family *Anacardiaceae,* commonly known as mango family. The genus Mangifera consists of about 30 species of tropical edible fruit trees in the flowering plant from the family *Anacardiaceae* with *Mangifera indica* being the most common species. Taxonomically, *Mangifera indica* belongs to the kingdom-plantae, class-*Mangoliopsida*, phylum-*Mangoliophyta,* order-*Sapindales*, family-*Anacardiaceae*, genus-*Mangifera* and species-*Indica* [2]. *Mangifera indica* plant is an evergreen broad canopy tree which grows to a height of 8-20 m, brown-gray colour thick bark and superficially cracked [3]. Leaves are 10-15 cm in length with variable sizes. *Mangifera indica* species have a long history of medicinal use in the treatment of syphilis, anemia, diabetes, diarrhea, mouth wound, ulcer, leucorrhea, fever, liver diseases, urinary tract infection, asthma, rheumatism, cough, colon cancer and dysentery. Other trado-medicinal uses of the plant root have been reported [4]. Plant sterols have been reported for their antitumor effects and cholesterol lowering [5]. Thus, this study investigated the root of *Mangifera indica* with a view to identifying some of the bioactive principles responsible for its ethnomedical applications.

Materials and Methods

Plant collections

Roots of *M. indica* were collected in April, 2018 from Bunu Tai, Tai Local Government Area, Rivers State and authenticated at the Department of Forestry, Rivers State University, Port

Harcourt by Dr. Sarogoro David and deposited at the Department Herbarium with Voucher specimen No: RSU/2018/M.I/039. The root was washed, air dried at room temperature for three weeks, pulverized with the aid of pestle and mortar and stored in air tight container until required.

General Experimental Procedure

Nucleomagnetic Resonance (NMR) analysis were recorded on a Bruker Avance 3 spectrometer at 400 MHZ (^{1}H), 376 MHZ (^{19}F) and 101 MHZ (^{13}C). All spectra were measured in $CDCl_3$ to which chemical shifts in parts per million (ppm) were adjusted accordingly at 7.26 ppm. Coupling constants (J) were measured in $Hert_Z$ (H_Z). Infrared (1R) spectroscopy was recorded by Agilent Technologies ATR-FT 5500 series FTIR instrument with 32 scans and frequencies are reported in cm^{-1}. Melting points (Mp) were determined with a SMPI stuart Scientific melting point apparatus. Column chromatography was performed on silica gel 60H, 200-400 mesh. Thin layer chromatography (TLC) was performed on pre-coated aluminum backed (silicycle), Tlc plate silica gel (0.2 mm) and Visualized by spraying with concentrated sulfuric acid and then heated to $105^{0}C$

Sample Extraction

Pulverized *Mangifera indica* root (1 kg) was sequentially extracted using Soxhlet (n-hexane and ethyl acetate). Excess solvent was removed under reduced pressure and residue stored in the refrigerator. The ethyl acetate extract was dissolved in n-hexane and chloroform (1:1), and refluxed for three hours then filtered.

Chromatographic Separation

The filtrate was subjected to column chromatography using silica gel as stationary phase. The solvent system consisted of n-hexane in combination with increasing percentage of ethyl acetate.

About 35 mL fractions were collected by gradient elution technique and elutes were monitored using thin layer chromatography using 30:70 of ethyl acetate and hexane solvent ratio. The fractions showing similar R_f value after visualizing by UV-lamp were pooled. Obtained fractions were verified with thin layer chromatography by spraying with anisaldehyde-Sulphuric acid and the plate the purple colour spots were confirmed as sterols. Fractions corresponding to R_f values were pooled, washed with acetone and subjected to TLC with hexane-ethyl acetate 8:2 (V/V). Fractions 18-24 (18.9 mg) were discovered to have crystals. The crystals were washed separately and repeated with Hexane (40 ml) TLC with hexane and dichloromethane (1:2) gave a single spot with Rf 0.64. The purified fraction was labeled NPJ-68. This was confirmed using Salkowski reaction and Leibermann-Burchard reaction as described by [6].

Spectroscopic characterization

Different spectroscopic methods such as FTIR, LCMS, MP, ^{1}H-NMR and ^{13}C-NMR were used to elucidate the structure of stigmasterol and β-sitosterol. The spectra (^{1}H, ^{13}C, 2D NMR were recorded using $CDCl_3$ as solvent on a Bruker Avance 3 spectrophotometer at University of Strathclyde, Glasgow, Scotland, United Kingdom.

Antimicrobial Screening

The antimicrobial activities of the isolated compound from the plant under investigation were determined using some animal and plant pathogens. The animal pathogens were obtained from the Department of Medical Microbiology Ahmadu Bello University teaching hospital, Zaria. The plant pathogens were obtained from I.A.R A.B.U. Zaria. The animal pathogens (bacteria) used for the antimicrobial assay include*; Methicillin Resist staph aureus, Vancomycin resist enterococci, Staphylococcus aureus, Escherichia coli, Salmonella tryphi, Pseudomonas aeruginosa.* The plant pathogens (fungi) used in this study were, *Aspergillus flavus, Aspergillus fumigatus, Aspargillus nigre, Coniophora puteana, Fibrophoria vaillentii, Fomitopis pinicola,*

Fusarium oxysporum, Fusaruim proliforatum, Rhizopus sp, Sclerotium rofsii and Serpula lacrymans. All micro-organisms were checked for purity and maintained in slants for agar [7].

Cultivation and standardization of test organism.

A loop full of test organisms were taken from the agar slant and sub culture into test tubes containing sterile nutrient agar for bacterian and subourand dextrose medium (20 ml) for for fungi, the test tubes were incubated for 48hrs at 37 ^{0}C. The broth cultures were standardized using sterile normal saline to obtain a density of 106 cfu/mL for bacteria. A sporulated test fungal spore was harvested with 0.05 % Tween80 in sterile normal and standardized to 106 spores/mL.

Antimicrobial profile (Sensitivity test)

0.002 mg of the compound was weighed and dissolved in 10mls of DMSO obtain a concentration of 20 µg/ml. Diffusion method was used for screening the compound. Mueller Hinton agar sabouraud dextrose agar were the media used as the growth media for the microbes. The media were prepared according to the manufacturer's instructions sterilized at 121 °C for 15 minutes, poured into sterile petri dishes and were allowed to cool and solidify. The Mueller Hinton agar was seeded with 0.1 ml of the standard inoculum of the test bacteria while the sabouraud dextrose agar was seeded with 0.1 ml of the test fungi. The inoculum was spread evenly over the surface of the medium by the use of a sterile swab. By the use of a standard sterile cork borer of 6mm in diameters, well was cut at the centre of each inoculated media. 0.1 ml of the solution of the compound of the concentration of 20 µg/ml was then introduced into the well on the inoculated media. Incubation was made at 37 °C for 24hrs for the bacteria and at 30 °C for 1-7 days for the fungi after which the plates of the media were observed for the zone of inhibition of growth, the zone was measured with a transparent ruler and the result recorded in millimetres.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibition concentration of the compound was determined using the broth dilution method. Mueller Hinton broth and sabouraud dextrose broth were prepared, 10 mls of the broth was dispensed into test tubes and were sterilized at 121°c for 15 minutes, the broth was allowed

to cool. Mc-farland's turbidity standard scale number 0.5 was prepared to give turbid solution. Normal saline was prepared, 10 mls was dispensed into sterile test tube and the test microbe was inoculated and incubated at 37 °C for 6hrs. Dilution of the test microbe was done in normal saline until. The turbidity marched that of Mc-farland's scale by visual comparison at this point the test microbe has a concentration of 1.5×10^8 cfu/ml. Two-fold seriel dilution of the compound was done in the sterile broth to obtain the concentrations of 20 µg/m/l, 10 µg/ml, 5 µg/ml, 2.5 µg/ml and 1.25 µg/ml. the initial concentration was obtained by dissolving 0.002 mg of the compound in 10mls of the sterile broth.Having obtained the different concentrations of the compound in the sterile broth, 0.1 ml of the test microbe in the normal saline was then inoculated into the different concentrations incubation was made at 37 °C for 24 hours for the bacteria and at 30 °C for 1-7 days for the fungi, after which the test tubes of the broth were observed for turbidity (growth) the lowest concentration of the compound in the sterile broth which shows no turbidity was recorded as the minimum inhibition concentration (MIC).

Determination of minimum bactericidal/ fungicidal concentration (MBC/MFC)

MBC/MFC was carried out to determine whether the test microbes were killed or only their growth inhibition. Mueller agar and sabouraud dextrose agar were prepared, sterilized at 121°C for 15 minutes, poured into sterile petri dishes and were allowed to cool and solidify.The contents of the MIC in the seriel dilutions were then sub-cultured onto the prepared media, the bacteria were sub-cultured onto the Mueller agar while the fungi were sub-cultured onto sabouraud dextrose agar, incubation was made at 37 °C for 24 hours for the bacteria and at 30 °C for 1-7 days for the fungi, after which the plates of the media were observed for colony growth MBC/MFC were the plates with lowest concentration of the compound without colony growth. Results were recorded after 24 hours [8].

RESULTS AND DISCUSSION

The results of the spectroscopic characterization and antimicrobial activities of stigmasterol and β-sitosterol from *Mangifera indica* root are reported in Tables 1 and 2,3,4, and 5 respectively.

Table 1: ^{1}HNMR and ^{13}C NMR chemical shifts for NPJ 68

Spectroscopic technique	Data
Mp	132 – 134^{0}C
Rf	0.53
1R(cm^{-1})	3547, 3230, 3025, 2850, 1640
^{13}C NMR	δ 36.67(C-1), 29.86(C-2), 71.97(C-3), 42.47(C-4), 146.93(C-5), 121.86(C-6), 31.83(C-7), 29.32(C-8), 46.01(C-9), 37.42(C-10), 18.94(C-11), 39.94(C-12), 41.42(C-13), 57.09(C-14), 25.03(C-15), 28.40(C-16), 56.93(C-17), 12.72(C-18), 19.97(C-19), 42.49(C-20), 23.09(C-21), 142.92(C-22), 129.87(C-23), 46.01(C-24), 34.12(C-15), 21.25(C-26), 23.24(C-27), 26.26(C-28), 12.02(C-29).
^{1}HNMR	δ 1.42(H-1), 1.82(H-2), 3.52(H-3), 2.25(H-4), 5.35(H-6), 1.98(H-7), 1.20(H-8), 2.00(H-9), 1.80(H-11), 1.40(H-12), 1.35(H-14), 1.12(H-15), 1.07(H-16), 0.90(H-17), 1.21(H-18), 0.81(H-19), 1.55(H-20), 1.01(H-21), 4.17(H-22), 5.35(H-23), 2.05(H-24), 1.04(H-25), 0.85(H-26), 0.96(H-27), 1.04(H-28), 1.01(H-29).

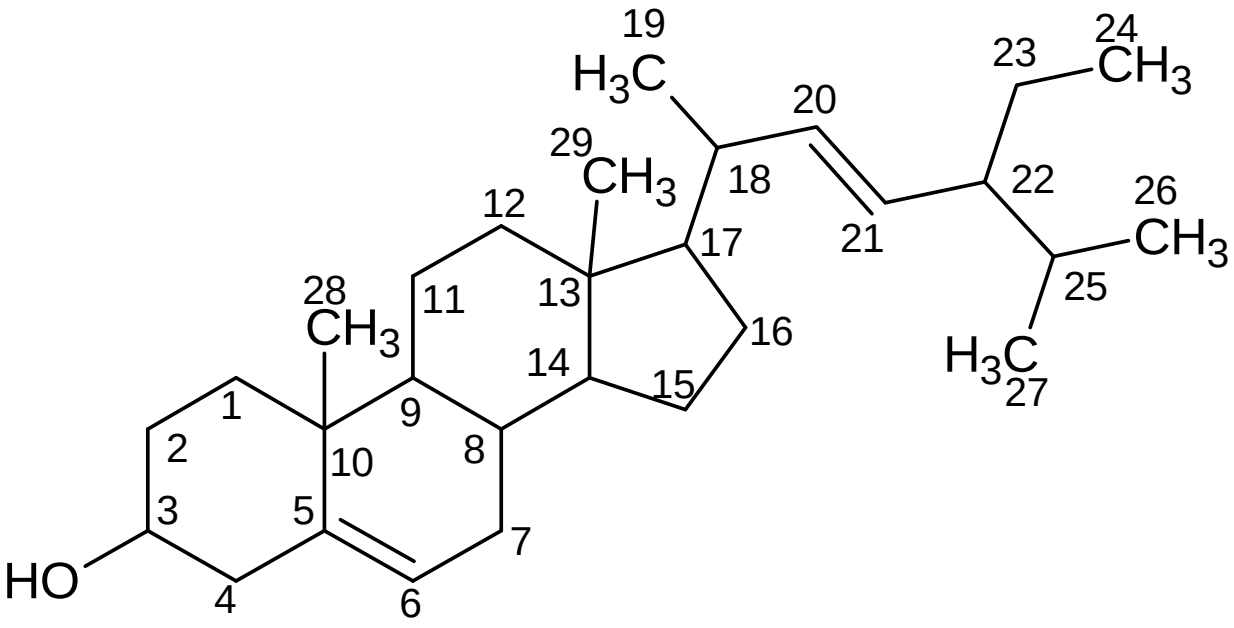
Characterization of Compound NPJ 68 as a mixture of stigmasterol and β-sitosterol

Compound NPJ 68 was obtained as a white crystalline solid. Analysis of the ^{1}HNMR spectrum of the fraction showed a mixture of two compounds. The spectrum indicated signals characteristic to sterols, such as olefinic hydrogens δH 5.35 (H-6) and carbinolic hydrogen δH3.52 (m, H-3), including the accumulation of signals in the region between δH 0.81 and δH 2.25 due to many groups of hydrogens such as methyl, methylene and methine, which characterized the sitosterol. This was demonstrated by the presence of six methyl protons (H-18, H-19, H-21, H-26, H-27 and H-29) with signals at δH 1.25, 0.81, 1.01, 0.85, 0.96, 1.01 respectively. The doublet at δH 4.17 (H-22) and δH 5.35 (H-23) correspond to the olefinic hydrogens of double bond with stereochemistry trans in the side chain of a stigmastane moiety. The DEPT 135 NMR spectrum showed three quaternary carbons, eleven methine, nine methylene, and six methyl carbons [9]. The ^{13}C-NMR spectrum showed characteristic signs of sitosterol and stigmasterol at δC 121.86, 146.93, 129.87 and 142.92 [10]. ^{13}C-NMR spectrum indicated resonance at δc 71.97 attributed to C-3. The hydroxyl group was placed at C-3 due to the correlations observed in the HMBC spectrum the H-3 and carbon resonance (δ_C 146.93 and δ_C 121.86) ascribable to double bonds at C-5 and C-6 [10]. Signals observed at δ_C 142.92 and δ_C129.87 corresponds to C-22 and C-23. The signals relating to the olefinic protons H-22, H-23 and H-6 observed from the ^{1}HNMR spectrum suggested both stigmasterol and sitosterol in an equal proportion in the mixture, this was confirmed by their integration as follows: a doublet of doublet observed at δ 3.52 attributed to H-3 for a mixture of stigmaterol- sitosterol was integrated for 2. The proton at δH 5.35 corresponding to H-6 for sitosterol and stigmasterol. While H-22 and H-23 of stigmasterol with signals at δ_H 4.17 and 5.35 were integrated for 1

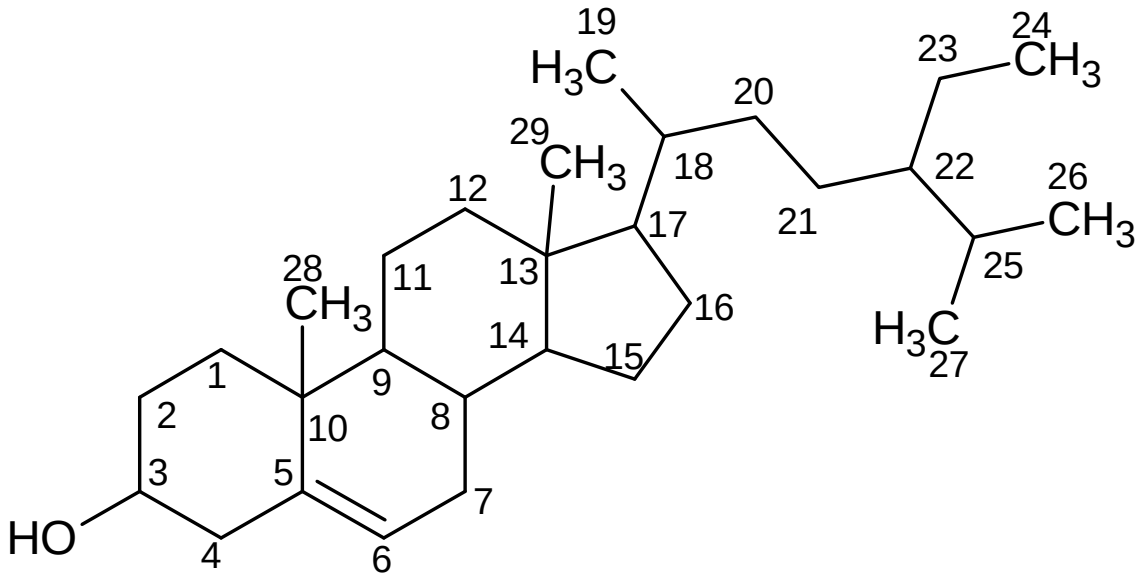
proton each. Therefore, the mixture is in a ratio of 1:1 stigmasterol- sitosterol. The relative configuration of compound NPJ 68 was that expected for a steroidal derivative as it was confirmed by a detailed analysis of COSY and HMBC experiments. In the HSQC spectrum, a single bond correlation was shown by H-20 (δ_H 1.56) and the methyl at C-10 (37.42). While in the HMBC H-21 (δ_H1.01) showed cross-peaks with C-14 (δc 57.09) and C-17 (δc 56.93) and C-24 (δC 46.0) H-12 (δ H 1.40) had correlations with C-11 (δ_C 18.94). Other correlations were observed from H-2 (δH 1.82) to C-1 (δ_C 36.67 and C-3 (δ_C 71.97), from H-4 (δ_H2.25) to C-7 (δ_C 31.83), from H-6 (δH 5.35) to C-4 (δC 42.47) and C-7 (δC 31.83), from H-14 (δH 1.35) to C-15 (δC 25.03), from H-17 (δH 0.90) to C-12 (39.94) and C-14 (δC 57.09), from H-22 (δH 4.17) to C-20 (δC 42.49), from H-25 (δH 1.04) to C-24 (δC 46.01) and C-20 (δC 42.49) and C-26 (δC 21.25), from H-24 (δH 2.04) to C-26 (δC 21.25) and C-28 (δC 26.26) consistent with the proposed A-D ring pattern and confirming identification. The COSY, HSQC and HMBC correlations aided in the assignments of values of all protons for ^{1}H NMR and ^{13}C NMR. The 1D NMR, 2D NMR, 1R and melting point and their comparison [11] identified NPJ 68 to be a mixture of stigmasterol and β-sitosterol.

Stigmasterol and sitosterol are phytosterol commonly found in the plant kingdom. They are obtained in mixture of hard separation, because they show similar physical properties. While stigmasterol has double bond at C-22 and C-23, sitosterol has a single bond. Hence it is difficult to obtain sitosterol in pure state [12]. In ^{1}H NMR spectrum, mixture containing stigmasterol are identified by signals referent to the vinyl, hydrogens (H-22 and H-23) that show up as two doublets between δH 4.17 and δH 5.35. β-sitosterol has its usefulness in the treatment of immune dysfunctions, inflammatory disorders and rheumatoid arthritis breast cancer, colon cancer and benign prostatic hypertrophy [13]. Antioxidant, anti-inflammatory, analgesic and

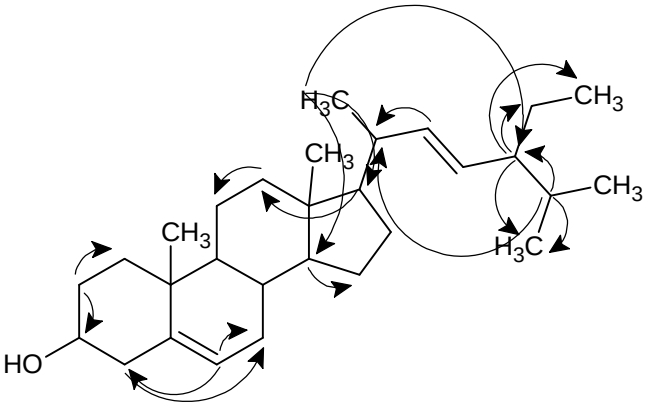
anthelmintic activity. Its hypoglycemic and hypercholesterolemic property as well as its usage as precursor in the synthesis of steroidal drugs has been reported [14].



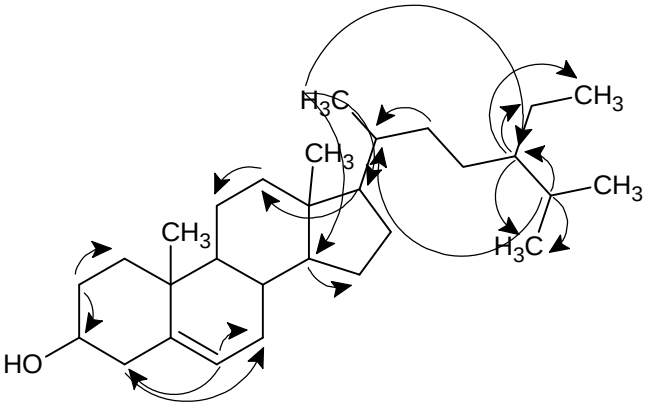
Compound 1 Stigmasterol



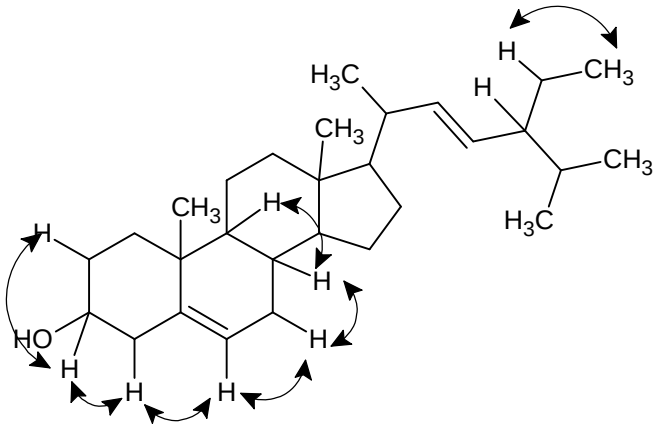
Compound 2: β-Sitosterol



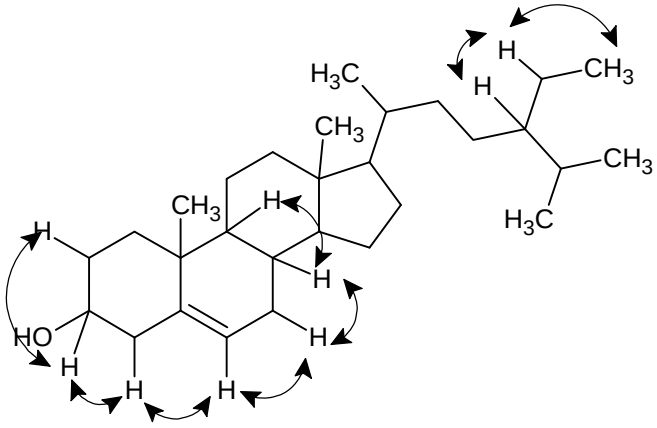
HMBC of Stigmasterol



HMBC of Sitosterol



COSY of Stigmasterol



COSY of Sitosterol

Table 2 shows the activity of the isolated compound on the selected animal pathoges.The compound has inhibition effect against the microbes as it was sensitive to *Vancomycine resist enterococci, staphylococcus aureus, Eucherichia coli and Pseudomona aeruginosa* but was resistance *to methicillin resist staph aureus and salmonella typhi*. [15] reported the ability of *Anogeissus leiocarpus* against some pathogenic microorganisms such as *S. aureus, Klebseilla species, C. albicans, E.coli.* The table also shows the zone of inhibition of the isolated compounds against the clinical isolates. The zones of inhibition range from 28-31 mm which is a strong indication of clearance zone by the compounds. This is an evidence that the root of *Mangifera indica* can be used in the treatment of diseases caused by these pathogens. This is in line with the claims of other researchers that the plant has antimicrobial activity against many pathogens and thus a confirmation of its traditional medical uses. Table 3 shows the antifungal activities of the isolated compounds against some plant pathogens. The result shows that *Aspergillus fumigates, Aspargillus nigre, Coniophora puteana, Fibrophoria vaillentii, Fusarium proliforatum, Rhizopus sp, Sclerotium rofsii and Serpula lacrymans* were sensitive with zone of inhibition ranging from 27-31 mm, while,*Aspergillus flavus, Fomitopsis pinicola, Fusarium oxysporum* were resistant to NPJ-68. This showed that these compounds and the plant Under investigation could be a useful precursor for the production of fungicides which could be used in the management of diseases caused by these tested pathogens.

Table 2: Antibacterial Sensitivity test, zone of inhibition (mm) of NPJ-68 versus control (vended) drugs

Test organism	**NPJ-**	**Sporfloxacine**	**Ciprofloxacin**

	68		
Methicillin resist staph aureus	R(0)	S(30)	R(0)
Vancomycine resist enterococci	S(30)	S(29)	S(30)
Staphycococcus aureus	S(28)	S(32)	S(26)
Eucherichia coli	S(31)	S(27)	S(27)
Salmonella typhi	R(0)	R(0)	R(0)
Pseudomona aeruginosa	S(28)	S(28)	S(25)

S= Sensitive, R = Resistance, Conc. of drugs = 5 μg/mL

Table 3: Antifungal sensitivity, zone of inhibition (mm) activity of NPJ-68 VS control drugs (vended)

Test organism	**NPJ-68**	**Fulcin**	**Kefeconazole**
Aspergillus flavus	R(0)	S(32)	R(0)
Aspergillus fumigates	S(30)	S(20)	R(0)
Aspargillus nigre	S(31)	S(30)	S(25)
Coniophora puteana	S(28)	R(0)	S(23)

Fibrophoria Vaillentii	S(27)	S(29)	S(27)
Fomitopsis pinicola	R(0)	S(28)	S(25)
Fusarium oxysporum	R(0)	S(28)	S(25)
Fusarium proliforatum	S(27)	S(28)	R(0)
Rhizopus sp	S(29)	S(27)	R(0)
Sclerotium rofsii	S(30)	S(29)	S(28)
Serpula lacrymans	S(31)	R(0)	S(26)

S= Sensitive; R = Resistance; Conc. of drugs = 5 μg/mL

Table 4: Minimum inhibitory concentration (MIC) (μg/mL) of NPJ-68 against microorganism

Pathogeus	**20 μg/ml**	**10 μg/ml**	**5 μg/ml**	**2.5 μg/ml**	**1.25 μg/ml**
Methicillin resist staph aureus	-	-	-	0*	+
Vancomycin resist enterococii	-	-	-	++	++
Staphylococcus aureus	-	-	-	++	++
Escherichia coli	-	-	-	-	+

Salmonella typhi	-	-	-	++	++
Pseudomonas auruginosa	-	-	-	0*	+

Key: No turbidity (No growth), 0* = MIC, + = Turbid (light growth), ++ = moderate turbidity.

Table 5: Minimum inhibitory concentration of NPJ-68 against test organism

Pathogeus	**20 µg/mL**	**10 µg/mL**	**5 µg/mL**	**2.5 µg/mL**	**1.25 µg/mL**
Aspergillus flavus	-	-	0*	0*	++
Aspergillus fumigates	-	-	0*	0*	++
Aspargillus nigre	-	-	-	+	++

Coniophora puteana	-	-	-	+	+
Fibrophoria Vaillentii	-	-	-	+	+
Fomitopsis pinicola	-	-	-	0*	+
Fusarium oxysporum	-	-	-	0*	+
Fusarium proliforatum	-	-	-	0*	+
Rhizopus sp	-	-	-	0*	+
Sclerotium rofsii	-	-	-	+	++
Serpula lacrymans	-	-	-	+	+

Key: No turbidity (No growth), 0* = MIC, + = Turbid (light growth), ++ = moderate turbidity.

Minimum inhibition concentrations (MICs) of NPJ-68 against the test organisms were shown in tables 4 and 5. The MIC varied from 2.5-5 μg/mL in this study and is lower than that reported [16]. Thus, its inhibition potency against the pathogens account for the various trado-medicinal uses of the plant.

CONCLUSION

The study investigated the isolation and characterization of mixture of stigmasterol and β-sitosterol from *Mangifera indica* root using physical and spectroscopic methods. The isolated compounds exhibited appreciable antimicrobial activities against some microbes, therefore, confirming many ethno-medical uses of the plant. This plant could be a potential candidate for drug development for the treatment of several diseases caused by these pathogens tested in this study for both man and plants.

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