EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 8
Issue: 11
319-322
Year: 2021

ISOLATION AND CHARACTERIZATION OF BETA –SITOSTEROL FROM THE ETHYL ACETATE EXTRACT FRACTION OF DRIED LEAVES OF *SARCOCEPHALUS LATIFOLIUS*

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Article Received on 20/09/2021 **Article Revised on 11/10/2021** **Article Accepted on 01/11/2021**

ABSTRACT

Background: Local fish farmers use the leaves of *Sarcocephalus latifolius* in rural areas of Southern Nigeria for the treatment of microbial infections of fishes. The plant has diverse folk medicinal uses in medicine was omitted, however the main focus of this work is to isolate and spectroscopically characterize the bioactive constituents of the dried leaves of the plant. *Sarcocephalus latifolius*. **Method:** The air dried leaves were pulverized to powder and extraction was maintained in soxhlet extractor using methanol solvent. The crude extract was further fractionated using varying solvents of n-hexane, and using vacuum liquid chromatography(VLC). The most sensitive fraction (ethyl acetate) based on the antibacterial sensitivity test was subjected to column chromatography and thin layer chromatography. **Results:** From antibacterial activity, a significant zone of inhibitions at 14mm and 20 mm were recorded against *E. coli* and *P.aeruginosa* when compared with the standard drug, tetracycline at 20 mm and 28 mm respectively. A positive test was indicated for the presence of steroid and a white powder compound was isolated which was characterized using combined spectroscopic techniques such as UV, FT-IR, ^{1}HNMR and ^{13}CNMR. The proposed compound was β-sitosterol, a steroid molecule with pharmacological significance in medicine.

KEYWORDS: *Sarcocephalus latifolius*, steroids, Antimicrobial, β-sitosterol.

1.0 INTRODUCTION

Plants and herbs have been in use in traditional for a long time. Plants are reservoir of a wide variety of phytochemicals like alkaloids, tannins, saponins and flavonoids, which act against different human and animal diseases (Pandey and Madhuri, 2010). Though there is availability of a large number of synthetic antibacterial agents, there is still need for more effective antibacterial drugs. This is because most of the drugs available are either having toxicity for ecosystem or bacteria developed some kind of resistance against them. Plants are reservoir of many phytochemicals and can therefore provide valuable sources of new drugs which possess antimicrobial properties. Plants are however known to contain naturally occurring valuable chemical compounds that have some kind of activities against bacteria.

Sarcocephalus latifolius is multi-stemmed perennial shrub or a small tree. It is a small tree found in the tropical humid rainforest zone and austral regions of Africa (Arbonnier, 2009). *Sarcocephalus latifolius* (Sm.) E.A. Bruce is known in English as African peach. It is called Tafashia (Hausa), Ubuluinu (Igbo), Ogbasin (Yoruba), Mbom-ibong (Ibibio) and Itu (Itsekiri) Arise *et al*., 2012). The plant belongs to the family called Rubiaceae. Other related species are *Sarcocephalus pobeguini*, *Sarcocephalus diderichii*, and *Sarcocephalus vanderguchtii*. According to Arbonnier (2009), the plant has flexible and drooping branches. Its leaves are opposite, green, shiny, and greasy to touch. Its ball-like inflorescence is composed of numerous flowers. The fruit is red in colour when ripe and smells like strawberries. It has an irregularly shape but containing thousands of minute seeds (Stangeland *et al*., 2007).

1.1 Medicinal uses

This species has medicinal uses that are much more known in sub- Saharan Africa in the traditional pharmacopoeia (Badiaga, 2011). Many studies demonstrated that *S. latifolius* has some medicinal properties (Yesufu *et al*., 2010; Badiaga, 2011). Karou *et*

al., 2011 reported that the plant is used in the treatment certain diseases like diabetes. Lamorde *et al*., 2010) reported that it is used for the treatments of Acquired Immune Disease Syndrome (AIDS). Sicknesses such as fever, gonorrhoea, coughs, stomach aches and disorders of gastrointestinal tract system have been reportedly treated by the use of the leaves and roots of this plant or its combination in equal proportions (Abreu and Pereira, 1998). *Sarcocephalus latifolius* exhibits antimicrobial and anti-parasitical activities (Abreu and Pereira, 2001; Onyeyili *et al.*, 2001). Cold infusion of the stem bark of the plant is commonly taken as a diuretic and anti-worm drug (Ademola *et al*., 2007). Other local uses of *Sarcocephalus latifolius* include treatment of sicknesses such as hypertension, tuberculosis and dysentery (Okieemy-Andissa *et al.*, 2004).

2.0 MATERIALS AND METHODS

2.1 Plant Samples Collection, Identification and Preparation

Fresh leaves of s*arcocephalus latifolius* were collected from around Ekiadolor, Ovia Northeast Local Government Area, Edo State. The plant was taxonomically identified at Department of Plant Biology and Biotechnology, faculty of life sciences University of Benin, Benin City. The leaves were cleaned, washed and shade dried at room temperature for two weeks and then ground into coarse powder using a mechanical grinding machine. The dried powder was used for extraction

2.2 Soxhlet Extraction of Plant Leaves

1000g of ground dried leaves of *Sarcocephalus latifolius* was weighed and placed in a thimble-holder in a soxhlet extractor. A 5liter capacity round bottom flask, which was at the base of the soxhlet extractor, was placed on a heating mantle. The leaves powder was first extracted with 3.5L of n-hexane for 48hrs nearer to the boiling point of the solvent to defat the leaves. The process was repeated using methanol as extraction solvent. The crude extracts were then concentrated using rotary evaporator. The weight of the extracts was taken and the extracts kept in a sealed glass bottle in a refrigerator till needed for fractionation and isolation.

2.3 Vacuum liquid chromatography (VLC) of SL crude methanol extract

The vacuum bed was prepared by using dry packing method. 200g silica gel (60-230 mesh) was used. The silica gel was introduced into 350ml size sintered glass and uniform surface level was maintained by gentle tapping. 30g dried crude methanol extract sample of *Sarcocephalus latifolius* dried leaves was loaded onto 350ml sintered glass bed. The sample was covered with cotton wool. The sintered glass was connected to 1000ml suction glass, which was then connected to a suction pump. The elution of the extract was done with 1000ml each of solvents of gradually increasing polarity starting from n-hexane followed by chloroform, ethyl acetate. The three fractions were concentrated separately to dryness on digital steam bath. The concentrated fractions were kept in screwed labelled glass bottles and stored in deep freezer for further analysis.

2.4 Column chromatography of the most active fraction.

The ethyl acetate fraction of the methanol extract of dried *Sarcocephalus latifolius* leaf that showed the most significant antibacterial activity was subjected to silica gel column chromatography to isolate the active compounds. Silica gel was used as the stationary phase while varying solvent mixtures of increasing polarity was used as the mobile phase. Glass column was used. 250g silica gel (60 -120 mesh size) was activated by placing it in hot air oven overnight at 110^{o}c.It was allowed to cool down and then suspended in n-hexane overnight.

Small piece of cotton wool, which had earlier been washed in n-hexane was tamped into the bottom of the column to prevent leakage of the silica gel. The column was fixed to stand vertically using clamps. About one-third of the column was filled with n-hexane. The column was then carefully packed with silica gel slurry with gentle tapping till all the gel was packed. The solvent was allowed to drain as the silica gel packs tightly. When the solvent just barely becomes level with the silica gel, the sample was carefully loaded into the column. Then small cotton wool was used to cover the sample. The bottom outlet tap was opened. The column was first eluted with 100% n-hexane. Then the polarity was gradually increased by 5 % increments of ethyl acetate in n-hexane. Fractions were collected into separate test tubes. The fractions were monitored with Thin Layer Chromatography (TLC). Fractions with same retention values (R_f) were pooled together and concentrated.

2.5 Thin layer chromatography

TLC was carried out on the fractions collected from the column chromatography. A drop of each fraction was carefully applied onto a thin layer chromatographic plate using a thin walled glass capillary tube and left to dry. After drying, the plate was developed in a suitable solvent in TLC chamber. The compounds in the spot move upwards by capillary movement. The plate was then removed from the solvent and the solvent front marked. The solvent was allowed to dry. The positions of different compounds were observed under ultra violet light (UV) after spraying with 5% concentrated H_2SO_4 in methanol. Fractions that have same TLC profiles were combined and concentrated.

2.6 Test for steroids

2.6.1 Salkwoski test: Few crystals of the isolated compound were dissolved in about 2ml of chloroform and 2ml of conc.H_2SO_4 was carefully added down the side of the inner test tube. A reddish brown colour was seen at the inter-phase.

2.7 Spectroscopic characterization: The spectroscopic characterization of the isolated bioactive constituent(s)

was carried out using combined spectroscopic techniques ultra violet (UV), infra-red (IR), gas chromatography mass spectrometry (GC-MS), proton nuclear magnetic resonance (^{1}HNMR) and carbon-13 nuclear magnetic resonance (^{13}C NMR) spectroscopy.

3.0 RESULTS AND DISCUSSION

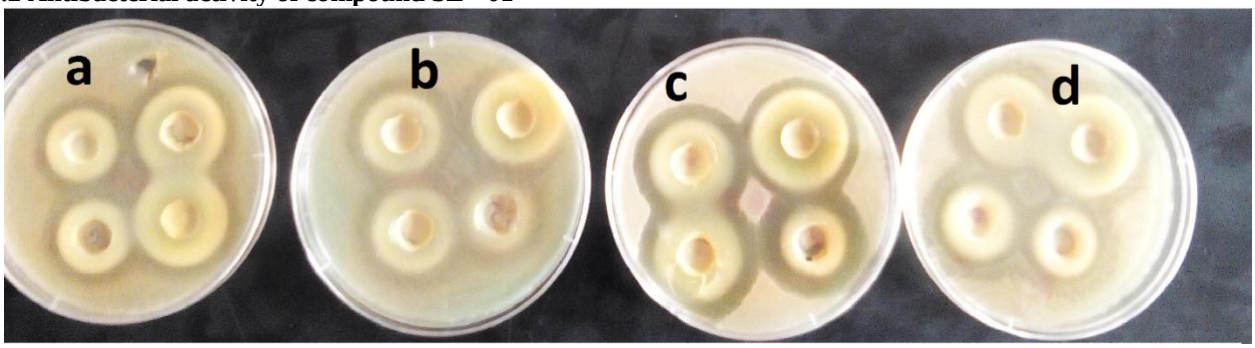
3.1 Physical characteristics of SL -01: A light brown solid obtained from the column with 9:1 (n-hexane: ethyl acetate) solvent gave a melting point range of 139-141^{o}C.

3.2 Thin Layer Chromatography (TLC)

Table 1: TLC investigation of fractions eluted using column chromatography.

S/N	Test tube no.	Description	Number of spots	Figures
1	14- 34	Eluting of light yellow band	No spot	Oil
2	69- 98	Colourless band eluting	one brownish spot after spraying with 5% conc. H_2SO_4 in methanol, air dried for 5mins and heated in an oven at 110^{0}C,viewed at 254 nm. (R_f = 0.53)	SL-01

3.2 Antibacterial activity of compound SL - 01



Growth inhibition *of (a)E coli (b) P. aeruginosa (c) B. subtilis and (d) S. aureus* by SL01

Table 2: Diameter of zones of inhibition of tested bacteria against the isolated compound SL01.

Test samples	Concentration mg/ml	Bacterial Zone of inhibition (mm)			
		E. coli (-) **(a)**	*P.aeruginosa (-)* **(b)**	*B. subtilis (+)* **(c)**	*S. aureus(+)* **(d)**
SL01	20	14	20	16	18
Tetracycline	10	20	28	28	26
Distilled water	20	N	N	N	N
DMSO	20	N	N	N	N

Key:
-: gram negative bacteria. **SL01**: isolated compound.
+: gram positive bacteria. **N:** no inhibition.

The antibacterial activity (Table 2) of SL01 against four different bacteria strains (two gram positive and two gram negative) was investigated. The compound showed activity against all tested bacteria with different degrees of sensitivities. The antibacterial result indicated that SL01 exhibits relatively higher zone of growth inhibition against two bacteria – *P. aeruginosa* (-) (20mm) and *S. aureus* (+) (18mm). This is an indication that the compound is broad spectrum in its activity. Comparing the compound with the standard drug, the standard showed better sensitivity, which may not be unconnected to the purity of the standard drug.

3.3 Spectroscopic results of isolated compound (light brown solid (SL 01) of *Sarcocephalus latifolius*

IR: The compound has major IR bands of medium/strong intensities at 3549cm^{-1} (O−H stretch) indicating the presence of alcoholic functional group; 1641cm^{-1} (C=C stretch) suggesting the presence of unconjugated olefinic bond. The absence of absorption in the area of 1700cm^{-1} indicated that the compound does not contain a carboxylic functional group. The absorption band at 2959cm^{-1}is the CH stretching of the CH_3.The signal at 2868cm^{-1}is the CH stretching of the CH_2 group this was further amplified by absorption in the absorption band at 1464cm^{-1}. The signal at 1370cm^{-1}is the CH bending of CH_3 group. The absorption band at 1640cm^{-1}is the absorption of the C-C stretching of C=C bond. Other peaks at 1050cm^{-1} (C−O stretch) indicated that of acids, esters and or anhydride which is suggestive of the −C−O−C structural feature indicating the glycosidic link while the absorption bands at 950cm^{-1} indicates a steroid property.

^{1}HNMR: The ^{1}HNMR obtained in $CDCl_3$ solvent at room temperature using TMS as standard showed protons signals at δ 0.78, δ 0.87, δ 0.97, and δ 1.14 (3H each), δ 2.26(1H, multiplet) and δ 3.96(1H, multiplet). The signal at 5.33ppm is assignable to olenific proton.

^{13}CNMR: ^{13}CNMR spectra also showed 29carbons: six methyl carbons(6-CH_3), eleven methylene carbons(11-CH_2), nine methine carbons (9 =CH-), and three quaternary carbons (3- =C=). Methyl carbons (-CH_3): C-18, C-19, C-21, C-26, C-27, C-29 and the signals appeared at 12.12, 19.40, 18.92, 19.96, 19.18, and 12.00ppm.

Methylene carbons(=CH_2): C-1, C-2, C-4, C-7, C-11, C-12, C-15, C-16, C-22, C-23, C-28, with the signal at 37.39, 31.76, 42.39, 32.06, 21.22, 39.92, 23.21, 28.39, 34.09, 26.10, and 23.10ppm.

Methine carbons(=CH-): C-3, C-6, C-8, C-9, C-14, C-17, C-12, C-24, C-25, and the peaks appeared at 71.98, 121.88, 31.90, 50.28, 56.91, 56.21, 36.20, 45.91, and 26.23pmm.

Quarternary carbons(=C=): C-5, C-10, C-13 with the following signals at 140.80, 36.65 and 42.39pmm

MS: The MS showed short mass ion at m/z 415.29… $(M-H)^+$, $C_{29}H_{49}O$ indicates m/z 414 of β− sitosterol with major characteristic fragments observed at m/z 346.88, 218.03, 228.69, 206.89, 141.14, 118.85, 87,85, 83.88 (base peak) and 46. From the combined spectroscopic data and comparison with those described in the literature. The compound isolated is thus proposed as β− sitosterol glycoside.

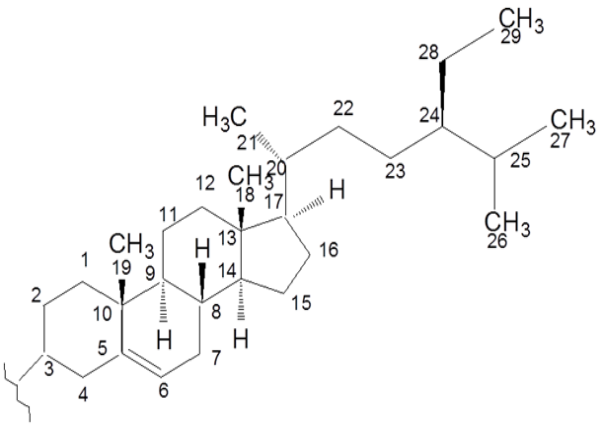


Figure: 1 β-Sitosterol.

CONCLUSION

β-Sitosterol was isolated and characterized from ethyl acetate extract of dried leaves of *Sarcocephalus latifolius*. It is known for cholesterol lowering capacity and also it showed remarkable antibacterial activity against some fresh fish bacteria pathogens like *P. aeruginosa* and *S. aureus*

CONFLICT OF INTEREST

The Authors declare no conflict of interest.

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