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CHARACTERIZATION AND MICROBIAL ACTIVITIES OF B-SITOSTEROL AND B-SITOSTENONE MIXTURE ISOLATED FROM THE STEM BARK OF METHANOL FRACTION OF S*ARCOCEPHALUS LATIFOLIUS* (SMITH BRUCE)

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ABSTRACT: *Two terpenoids,* β*-Sitosterol (**1**) and* β*-sitostenone (**2**) mixture were isolated from the methanol fraction of the stem bark of Sarcocephalus latifolius (Smith Bruce).The structures of the compounds were established by spectroscopic studies and by comparison with existing data from literature. The antimicrobial properties of the isolated mixture of compound was carried out on some clinically selected microorganism and the result compared with a standard drug. Although these compounds had been in existence and isolated from various plant species, but this is the first time the mixture will be isolated from the stem bark of Sarcocephalus latifolius.*

KEYWORDS: Sarcocephalus latifolius, methanol stem bark extract, Rubiaceae, β-Sitosterol, β-Sitostenone.

1. Introduction

Medicinal plants have been used virtually in all cultures as a major source of medicament. This usage has been traced to the occurrence of natural products present in them with medicinal properties. Ethnobotany and ethno-medicinal studies are today recognized as the most viable methods of identifying new medicinal plants or refocusing on those earlier reported for bioactive constituents (Adjanahoun *et al*., 1991; Farnsworth, 1966). Scientific investigations of medicinal plants have been initiated in many countries because of their contributions to health care. The continual search for, and the interest in natural plant products, for use as medicines has acted as the catalyst for exploring methodologies involved in obtaining the required plant materials and hence probing into their constituents for possible medicinal usage.

Sarcocephalus is a genus of tropical evergreen trees and shrubs belonging to the Rubiaceae family. This plant family is one of those frequently used by Ethno medicinal practitioners in Sierra Leone and its neighboring countries. It is a shrub or small spreading tree that is widely distributed in the Savannah and tropical Africa. It is known by the Hausa as *Tafashiya* or *tuwon biri* called *Ubuluinu* in Igbo land in Nigeria. The current folk medicinal applications of *Sarcocephalus latifolius are* varied and numerous, for example the bark and the root are said to be used in malaria treatment (Akubue and Mittal 1982; Oye; 1990). It is used as a tonic and remedy for treating fever, toothaches, dental cures, septic mouth, and diarrhea and in dysentery treatment and also used as chewing stick (Etkin *etal*,1990; Lamidi *etal*, 1995) . In Nigeria the bark is said to be useful in the treatment of wounds, cough and gonorrhea in Nigeria (Madibunyi,

1995) while the leaves are claimed to be used in the treatment of fever, and the roots and bark are also used for the treatment of venereal disease, wounds and as odontalgic remedy (Pedro and Antonio, 1998). The crude root extract had been reported to have antihypertensive effect (Nworgu *etal*, 2009). New Indole alkaloids, 21-O-methylstrictosamideaglycone, Augustine, nauclifine, augustidine, 19-0-ethylaugustoline, naucleidinal, 19-epi-naucleidinal, quinovic acid-3β–O- β-D-fucopyranoside, quinovic acid-3β-O-β-D-rhamnopyranoside, scopoletin, and β-Sitosterol were isolated from the root. The strictosamide isolated from it was reported to show moderate antiplasmodial activity against plasmodium falciparum (Pedro and Antonio, 2001). Betunilic acid had been isolated from the the bark of this plant which was shown to have MBC against *Staphylococcus aureus, Streptococcus pyrogenes, Bacillus subtilis, Escherichia coli, proteus vulgaris, salmonella*typhi, Shigellia dysenteric and Candida virusei at concentration of 25 µg/ml and 50 µg/ml for Methicillin Resistant Staphylococcus Aureus (MRSA), Proteus mirabilis, Pseudomonas aeruginosa, Candida albicans and Candida tropicalis respectively (Isah *etal*, 2012).

METHODS

Plant Materials and method

The stem bark was collected from the vicinity of the Federal College of Education, Okene, in Kogi state, Nigeria in March 2010.The plant was identified by a taxonomist, Mallam Musa Abdullahi of the Herbarium of the Department of Biological Sciences, Faculty of Science, A.B.U, Zaria, Nigeria and a voucher specimen of number 1268 was deposited at the Herbarium. The sample was air-dried and pulverized using wooden pestle and mortar. Finally, they were stored in an air-tight polythene bag and kept away from moisture until when needed for analysis.

Extraction procedure

The pulverized stem bark of *Sarcocephalus Latifolius* weighing 320g was exchaustively extracted in a soxhlet extractor using 2litres of redistilled methanol. The extract was concentrated in vacuo at 40^{o}C using rotary evaporator and this afforded 56g (17.5%) of it with respect to the plant material. The crude extract was subjected to exhaustive solvent partitioning using petroleum ether (600ml), chloroform (400ml), dichloromethane (400ml), ethyl acetate (600ml) and Methanol respectively.

Isolation of the pure compounds

The Methanol fraction was column chromatographed under vacuum and eluted with Petroleum ether and ethyl acetate respectively. The fractions collected were pooled together based on similarities of TLC plates to yield four fractions as ETCI, ETC2, ETC3 and ETC4 respectively. The ETC1 was further cleaned up using VLC to obtain 30 different fractions coded ETCC1 to ETCC30.From the TLC result it was found that fractions ETCC 6, 7, 8 and 9 had the same R_f value of 0.9 using the solvent mixture Petroleum ether : Ethyl acetate (1:9) and they were pooled together to yield EFSB.

Spectral analyses: The component was studied spectroscopically using Nuclear Magnetic Resonance Spectroscopy (NMR), both 1D-NMR and 2D-NMR inclusive, Fourier Transformed

Infrared Spectroscopy (FTIR) and also by comparing the obtained data with already existing data.

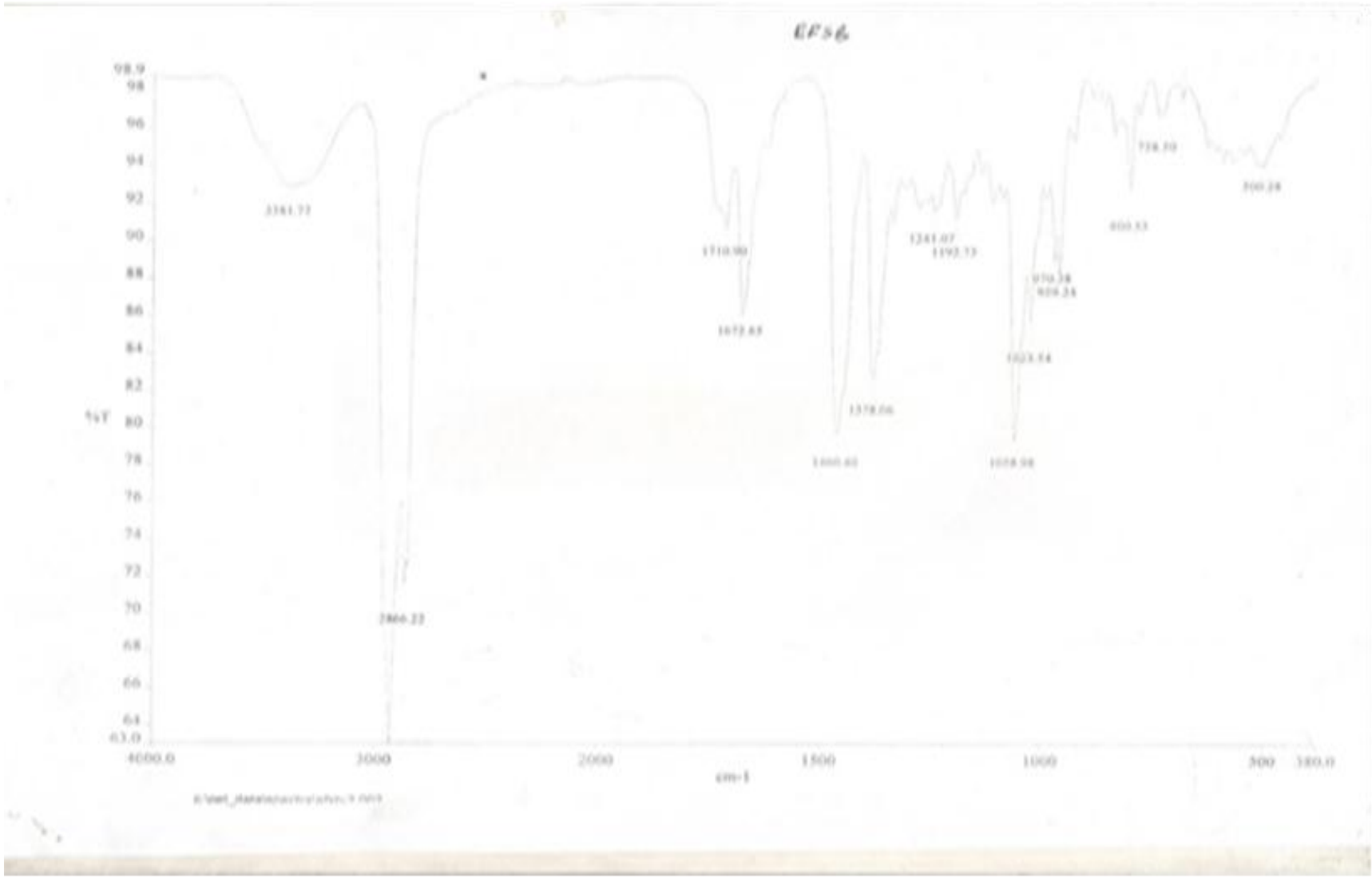
The IR (KBr) λ_{max} (cm^{-1}): Absorption peaks recorded for EFSB were**:** 3381.77, 2866.22, 1710.90, 1672.65, 1460.40, 1378.06, 1241.07, 1192.73, 1058.98, 1023.54, 970.38, 959.24, 800.53, 738.50 and 500.28.

^{1}H NMR of (δ): 5.7009, 5.3369, 5.3238, 3.6439, 3.5021, 24036, 2.3910,2.3678, 2.3552, 2.3410, 2.3323, 2.3210, 2.2973, 2.2884, 2.2791, 2.2627, 2.2563, 2.2503, 2.2426, 2.2370, 2.2144, 2.0238, 2.0149, 2.0040, 1.9900, 1.9822, 1.9763, 1.9478, 1.9409, 1.9349, 1.9288, 1.8610, 1.8441, 1.8357, 1.8294, 1.8211, 1.8116, 1.7896, 1.7079, 1.6661, 1.6548, 1.6499, 1.6376, 1.6327, 1.6207, 1.6157, 1.6038, 1.5938, 1.5859, 1.5695, 1.5507, 1.5192, 1.5102, 1.4920, 1.4823, 1.4748, 1.4555, 1.4451, 1.4260, 1.4142, 1.3964, 1.3864, 1.3510, 1.3367, 1.3279, 1.2924, 1.2813, 1.2625, 1.2325, 1.2140, 1.1966, 1.1782, 1.1717, 1.1591, 1.1400, 1.1304, 1.1110, 1.0941, 1.0775, 1.0615, 1.0505, 1.0360, 1.0256, 1.0169, 0.9983,0.9875, 0.9776, 0.9611, 0.9453, 0.9359, 0.9286, 0.9078, 0.9056, 0.8980, 0.8918, 0.8817, 0.8690, 0.8585, 0.8427, 0.8237, 0.8058, 0.8013, 0.7840, 0.7723, 0.7633, 0.7599, 0.7532, 0.7457, 0.7072, 0.6890, 0.6774, 0.6717 and 0.6589.

^{13}CNMR of (δ): 199.7092, 171.7615, 140.7743, 123.7451, 121.7273, 77.2202, 71.8223, 56.7799, 37.2636, 36.5173, 36.1547, 36.1247, 35.6965, 35.6353, 33.9946, 33.9567, 33.8885, 32.9659, 32.4274, 32.0602, 31.9198, 31.6775, 29.7068, 29.1590, 28.2558, 28.2010, 26.0812, 24.3120, 24.1957, 23.0750, 21.0922, 21.0352, 19.8244, 19.4063, 19.0352, 18.7878, 18.7088, 17.3952, 15.4507, 14.1233, 11.9861, 11.8681, -0.0033 and 209.6076.

RESULTS AND DISCUSSION

The Compound was obtained as a white amorphous solid. From the IR (Fig1) the signal observed at 3381.77 may be due to hydroxyl group (OH), while the signal at 2866.22 is assignable to carbon-carbon single bond. The peak at 1710.90 cm^{-1} is due to carbonyl signal (C=O) and the signal at 1460.40 is due to the methylene groups while the one at 1378.06 is typical of carbon-carbon double bond (C=C).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3361.77
2866.22
1710.90
1672.83
1378.06
1241.07
1193.13
1023.54
1078.58
970.38
939.24
838.53
738.59
700.24

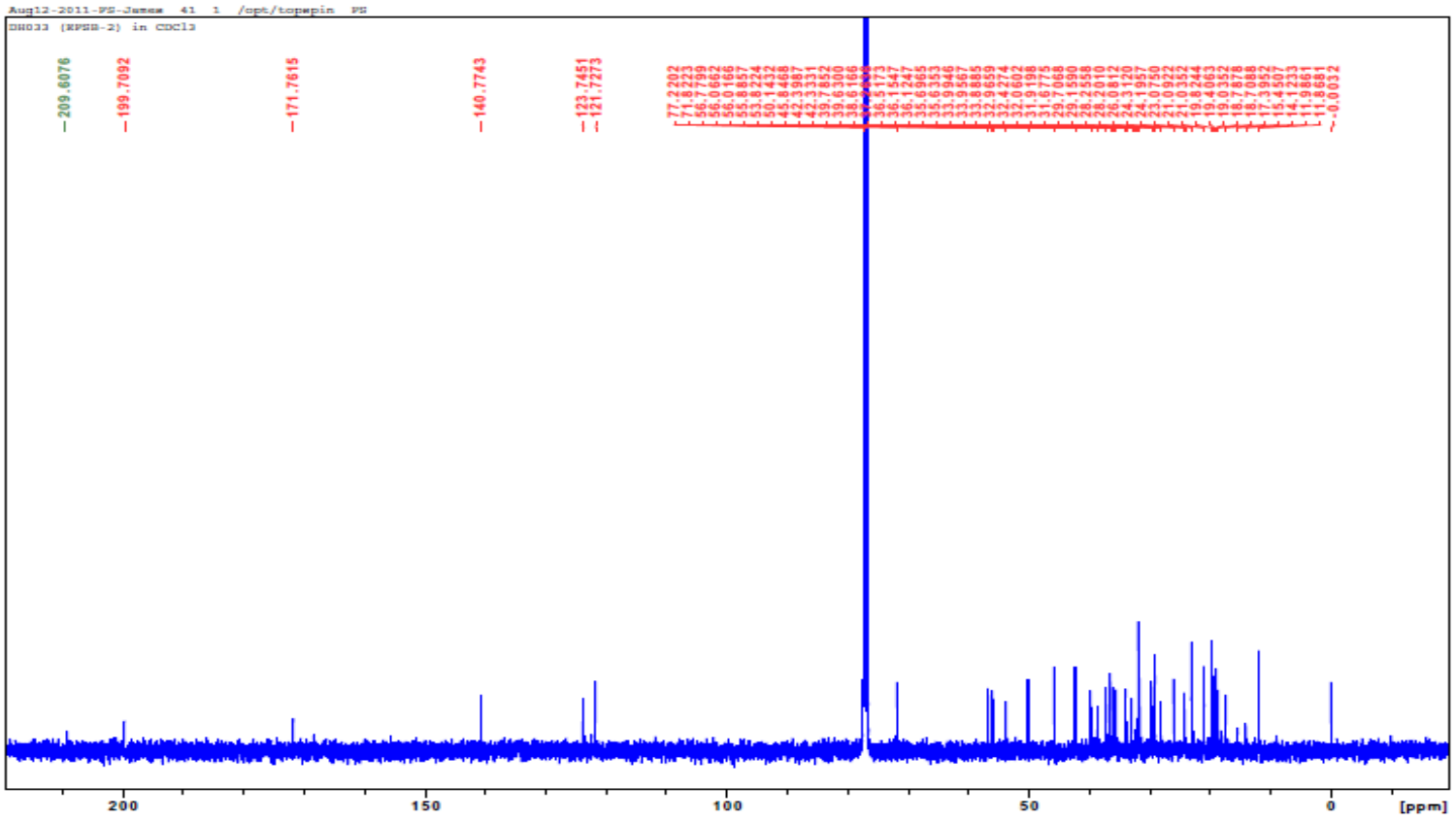
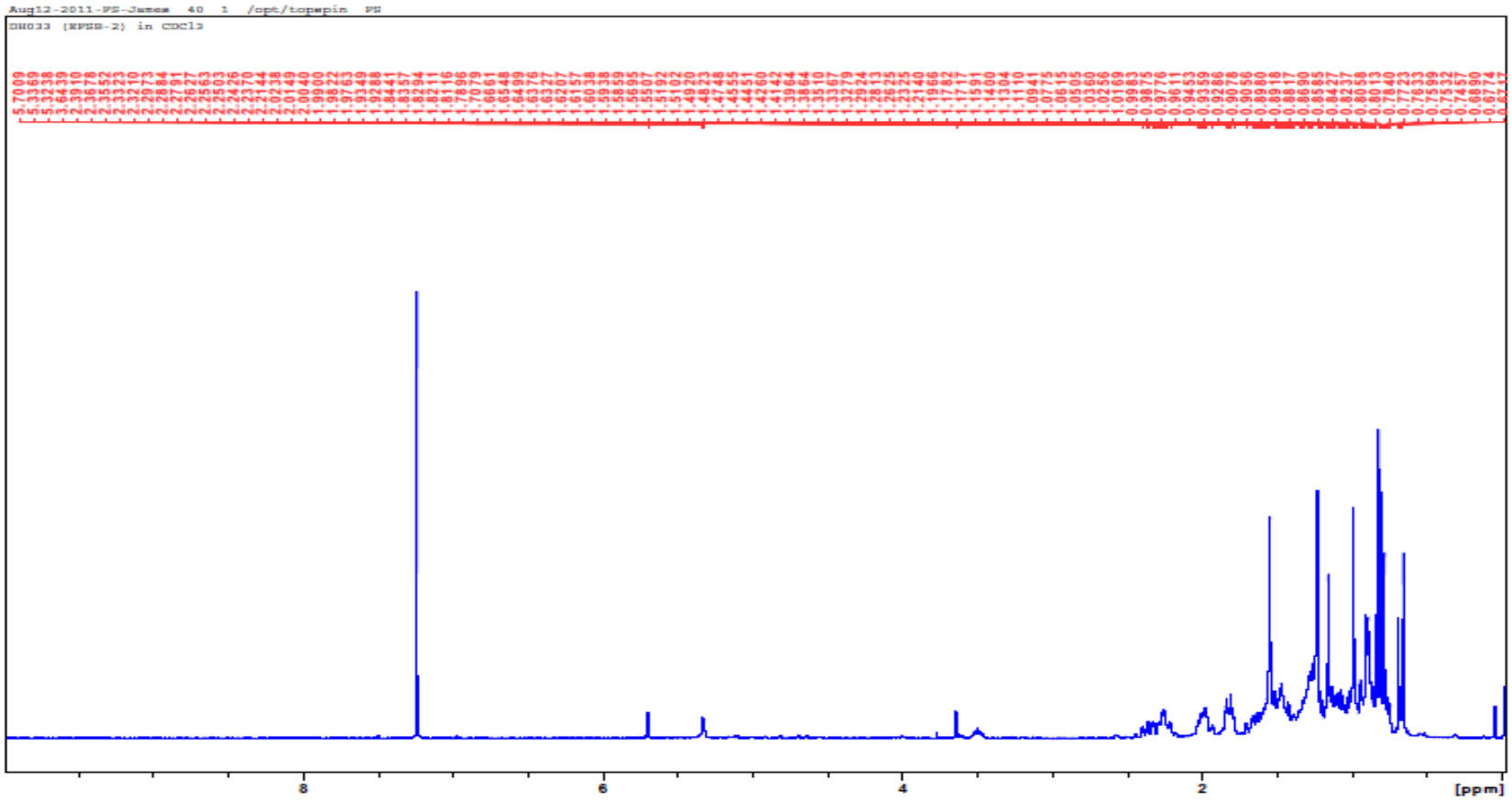


Figure 1: Infrared spectroscopy of EFS

The ^{1}H NMR (Fig.2) had two methyl singlets at δ1.0169 and 0.6890, four doublets of methyl at δ 1.0256, 0.8585, 0.8058 and 0.8013 respectively. There were also three (3) vinylic proton signals at δ 5.7009, 5.3369 and 5.3238. The proton signal at 3.53 may be attributable to the hydroxyl group (-OH).

Figure 2: ^{1}H NMR of EFSB The ^{13}C NMR spectrum showed a total of fifty-five (55) carbon signals. Among them four (4) olefinic carbon signals at δ 140.7743, 123.7451, 121.7273, and 77.2202) and one C-O signal at 71.7615ppm while a keto (C=O) group was



observed at 171.7615ppm.The remaining carbon signals with chemical shifts of 11.8681 and 56.7799 ppm were also recorded (Figure 3).

Figure 3: ^{13}CNMR of EFSB

DISCUSSION

From the above spectroscopic data it was likely that sample EFSB was a mixture of β - sitosterol and β- sitostenone. The only difference between the two compounds is the substituent present at carbon-3 in β- sitosterol (1) and the presence of keto or carbonyl (C=O) at the same carbon (3) position in β- sitostenone (2). Literature has it that β- sitosterol is difficult to be obtained in pure form (Pollock and Stevem, 1965; Fieser and Fieser, 1962). Direct comparison of the ^{13}C NMR data with those that were reported in literature (Rubinstein *etal*, 1976; Sakakibara *etal*, 1983; Kulsum *etal*, 2009 and Wen-Hsin *etal*, 2008) (Tables 1and 2) this was in full agreement. With this it was considered that this compound was a mixture of β -sitosterol and β –sitostenone (Figure 4). Compounds 1 and 2 have been isolated from various plant species including the root of this particular plant. However, this is the first time they are to be isolated from the stem bark of the plant.

The antimicrobial screening of isolated mixture of compound
The isolated mixture of compound was tested against the following microorganisms; *Methycilin Resistant Staphylococcus Aureus (MRSA), Staphylococcus aureus, Streptococcus pyrogenes, Bacillus subtilis, Corynebacterium ulcerans, Escherichia coli, proteus mirabilis, proteus vulgaris, Pseudomonas aureginosa, Salmonella typhi, Shigellia dysenteric, Candida albicans, Candida virusei, and Candida tropicalis* to determine the biological activities. The well- in-plate diffusion (Barry and Thornberry, 1985) method was used and the tested organisms were sensitive with the exception of *MRSA, Corynebacterium ulcerans, Pseudomonas aeruginosa* and *Candida tropicalis* had zones of inhibition ranging from 20 to 25 mm (Table 3) .The MIC values (Table 4) for organisms sensitive to the component were 12.5 µg/ml while the MBC/MFC values (Table 5) were found to be 50 µg/ml with the exception of *Bacillus subtilis* which had MBC of 25 µg/ml. Comparing these values with the values obtain for Sparfloxacin (Table 6) which is a standard drug. It was observed that EFSB has a lower value of MIC when compared to this drug. This also shows that the mixture of isolated compounds are better than Sparfloxacin when combined having a wider broad spectrum of activities over the tested organisms (Table 3).

β-Sitosterol is a common constituent of herbal plants which has been found to have biological activity, such as anti hypercholestrolaemic and estrogenic effects (Buckingham, 1998). It has also recently been shown to have gastro protective activity in several experimental ulcer models in rats (Navarrete *etal*, 2002). Its antimicrobial, anti-inflammatory, analgesic and anti-pyretic activities of β -Sitosterol have also been reported (Gupta etal, 1980; Salvador etal, 2004)It inhibited neutrophil migration into the inflamed tissue, and in acute topic inflammation it showed decreased myeloperoxidase activity (Gomez *eta*l, 1999). β-Sitosterol has been reported to have quite strong antioxidant activity (Weng and Wang, 2000). So, the use of this plant in the treatment of venereal disease, wounds, diarrhea and dysentery in traditional medicinal practice has been really justified.

Table 1: IR values for β-sitosterol and β-sitostenone

β-sitosterol	β-sitostenone
3428 (OH)	2930(C-H)
2930(C-H)	2871(C-H)
2852(C-H)	1686(C=O)
1657(C=C)	1620(C=C)
1465(CH_2)	1465(CH_2)

*Wen-Hsin Li *et al*., 2008

Table 2: ^{13}C NMR spectra data of β-sitosterol and β-sitostenone (δ ppm)

Carbon	β-sitosterol	β-sitostenone
1	37.24	56.61
2	31.65	32.04
3	71.80	199.71
4	42.29	123.71
5	140.75	171.78
6	121.71	32.94
7	31.90	33.87
8	31.90	35.70
9	50.12	53.80
10	36.49	38.59
11	21.07	21.01
12	39.76	39.61
13	42.31	42.37
14	56.76	56.0
15	24.29	24.17
16	28.24	28.18
17	56.05	55.86
18	11.97	12.0
19	19.39	19.01
20	36.14	36.10
21	19.02	18.68
22	33.93	33.96
23	29.14	29.13
24	45.82	45.81
25	26.06	26.05
26	18.79	17.34
27	19.81	19.80
28	23.06	23.05
29	11.85	12.0

*Wen-Hsin Li *et al*., 2008

Table 3: Zone of inhibitions (mm) of the pure isolated compounds on the test microorganism

Test Organism	EFSB
MRSA	0
Staphylococcus aureus	21
Streptococcus pyrogenes	20
Bacillus subtilis	25
Corynebacterium ulcerans	0
Escherichia coli	20
Proteus mirabilis	21
Proteus vulgaris	20
Pseudomonas aureginosa	0
Salmonella typhi	23
Shigellia dysenteric	20
Candida albicans	20
Candida virusei	20
Candida tropicalis	0

EFSB=Methanol fraction of Sarcocephalus latifolius stem bark

Table 4: Minimum inhibition Concentrations (µg/ml) of EFSB on the test microbes

Test Organism	50	25.5	12.5	6.25	3.125
MR*SA*					
Staphylococcus aureus	_	_	O*	+	++
Streptococcus pyrogenes	_	_	O*	+	++
Bacillus subtilis	_	_	O*	+	++
Corynebacterium ulcerans					
Escherichia coli	_	_	O*	+	++
Proteus mirabilis	_	_	O*	+	++
Proteus vulgaris	_	_	O*	+	++
Pseudomonas aureginosa					
Salmonella typhi	_	_	O*	+	++
Shigellia dysenteric	_	_	O*	+	++
Candida albicans	_	_	O*	+	++
Candida virusei	_	_	O*	+	++
Candida tropicalis					

Key: - =No colony growth, O =MIC, + =light growth, ++ = Moderate colonies growth*

Table 5: Minimum Bactericidal/Fungicidal Concentrations (µg/ml) of EFSB on the test microbes

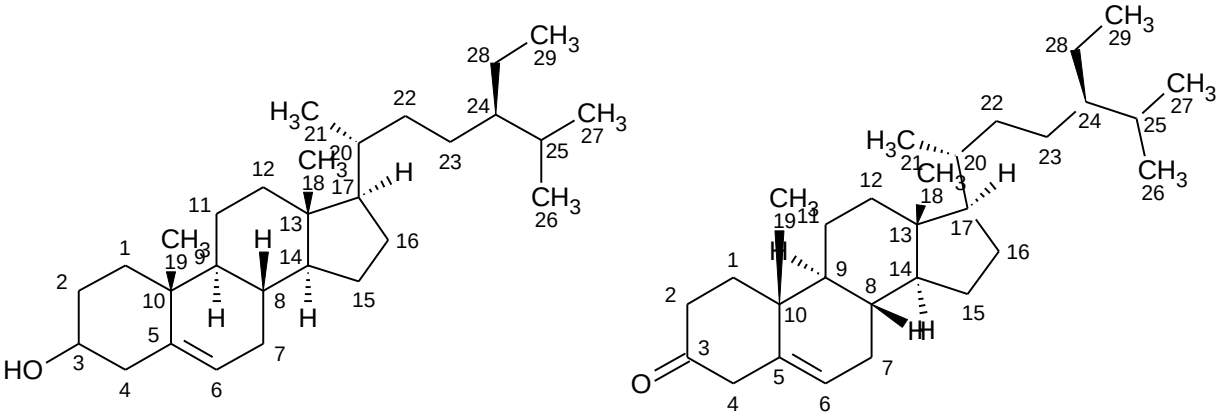
Test Organism	50	25.5	12.5	6.25	3.125
MR*SA*					
Staphylococcus aureus	O*	+	++	+++	++++
Streptococcus pyrogenes	O*	+	++	+++	++++
Bacillus subtilis	_	O*	+	++	+++
Corynebacterium ulcerans					
Escherichia coli	O*	+	++	+++	++++
Proteus mirabilis	O*	+	++	+++	++++
Proteus vulgaris	O*	+	++	+++	++++
Pseudomonas aureginosa					
Salmonella typhi	O*	+	++	+++	++++
Shigellia dysenteric	O*	+	++	+++	++++
Candida albicans	O*	+	++	+++	++++
Candida virusei	O*	+	++	+++	++++
Candida tropicalis					

Key: - =No colony growth, O =MBC/MFC, + = scanty colonies, ++ = Moderate colonies growth, +++= Heavy colonies growth*

Table 6: Minimum Inhibition Concentration of Sparfloxacin against the test microbes

Test Organism	50µg/ml	25.5µg/ml	12.5µg/ml	6.25µg/ml	3.125µg/ml
MRSA	_	O*	+	++	+++
Staphylococcus aureus	_	_	O*	+	++
Streptococcus Pyrogenes	_	_	O*	+	++
Bacillus Subtilis	_	_	O*	+	++
Corynebacterium ulcerans	_*	O*	+	++	+++
Escherichia Coli	_	O*	+	++	+++
Proteus Mirabilis	_	O*	+	++	+++
Proteus Vulgaris	_	O*	+	++	+++
Pseudomonas aeruginosa	_	_	_	_	_
Salmonella typhi	_	O*	+	++	+++
Shigellia dysenteric	_	_	O*	+	++
Candida albicans	Θ	Θ	Θ	Θ	Θ
Candida Virusei	Θ	Θ	Θ	Θ	Θ
Candida Tropicalis	Θ	Θ	Θ	Θ	Θ

Key: - =No colony growth, O =MIC, + = scanty colonies growth, ++ = Moderate colonies growth, +++ = Heavy colonies growth,* Θ = Not tested



Sitosterol (I) Sitostenone (II)

Figure 4.Chemical structure of β-Sitosterol and β-Sitostenone

CONCLUSION

The isolation of Sitosterol and sitostenone mixture from the plant whose bioactivity were established shows that the substances which are better than Sparfloxacin when combined also have wider broad **spectrum of activities over the tested organisms more than justifies why the plant really serves as a general purpose antibiotic in traditional medicine in our society.**

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