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Isolation and Characterization of Phytosterols from Stem Bark of *Aubrevillea kerstingii*

[*1]Ahmed AH, [2]Ahmed A, [2]Musa KY, [1]Lawal M, [3]Alkali YI.

[1*]Department of Pharmacognosy and Ethnopharmacy, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto- Nigeria

[2]Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical sciences, Ahmadu Bello University, Zaria-Kaduna-Nigeria

[3]Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto- Nigeria.

***Correspondence:** aliyu.hamidu@udusok.edu.ng

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Abstract

This study was carried out in order to isolate stigmasterol and β-sitosterol. The powdered stem bark of *Aubrevillea kerstingii* was extracted with n-hexane, ethyl acetate and methanol using successive maceration. A small portion of the ethyl acetate extract was tested for the presence of steroids/triterpenes using Lieberman-Buchard and Salkowski test. The ethyl acetate extract (5g) was pre-adsorbed on silica gel and loaded into a column pre-packed with 100g silica gel (60-120 Merck). The extract was eluted with n-hexane: ethyl acetate in a gradient elution starting from n-hexane (100%). The evaluates were collected in 30ml fractions. A total of 180 fractions were collected and pooled together on the basis of their thin layer chromatographic profile (TLC). The pooled fractions were labelled E1-E12. The TLC analysis of fraction E4 using n-hexane: ethyl acetate (9:1) revealed a single spot. This was coded AK1 (10mg). The TLC analysis of fraction E7 after using n-hexane: ethyl acetate (7:3) revealed a single spot. This was coded AK2 (20mg). The compounds were identified as stigmasterol and β-sitosterol based on the ^{1}H NMR and ^{13}C NMR data compared to literature.

Keywords: *Aubrevillea kerstingii*, ethyl acetate stigmasterol, β-sitosterol,

1.0 Introduction

Medicinal plants have been found to contain molecules of high drug values and are considered as herbs of great significance [1]. About 25% of the contemporary pharmacological drugs are derived directly or indirectly from plants [2]. Majority of the plant derived drugs were related to their initial ethnopharmacological purpose. Therefore, screening of medicinal plants is required for the discovery and development of useful bioactive agents for treatment of ailments [3]. Modern searches for bioactive molecules typically make use of sophisticated bioassays and bioassay-guided fractionation of medicinal plants used. This has led to the isolation of several new therapeutically important compounds. There is about 500,000 plant species around the world, most of which have not been investigated for their medicinal uses. Therefore medicinal plants have a promising future role to play in treatment of diseases [4]. *Aubrevillea kerstingii* (Harms) Pellegr is a large tree belonging to the family fabaceae. It is used in West Africa for treatment of oedema, kidney diseases and is also used as diuretics, laxatives and for treatment of gout [5]. Phytosterols are important constituents of plant cell. They are reported to have numerous biological activities including, obesity, hypocholesterolemic, antidiabetogenic, and antioxidant activities [6, 7, 8]. This study was carried out in order to isolate two phytosterols; stigmasterol and β-sitosterol.

2.0 Materials and Methods

2.1. Collection and Identification of Plant Material

The stem bark of *Aubrevillea kerstingii* was collected from Gwargwaji Zaria Local Government Area of Kaduna State, Nigeria. The plant was identified at the Herbarium unit of the Department of Biological science Ahmadu Bello University Zaria, Nigeria and was given a voucher number. The fresh stem bark of *Aubrevillea kerstingii* was air dried under shade at room temperature, pulverized and stored in an airtight container for further use.

2.2. Extraction

The powdered stem bark of *Aubrevillea kerstingii* (1.5kg) was extracted with n-hexane, ethyl acetate and methanol using sequential maceration [9].

2.3. Test for Steroids/Triterpenes

2.3.1 Lieberman-Buchard Test

A small portion of the extract was dissolved in chloroform. Equal volume of acetic anhydride was added, followed by concentrated sulphuric acid down the side of the test tube. The mixture was observed for the presence of a brown ring at the interphase which indicates the presence of steroids/triterpenes [10].

2.3.2. Salkowski Test

A small quantity of the extracts was dissolved in 1ml chloroform and to it 1 ml of concentrated sulphuric acid was added down the side of the test tube. Formation of red coloration at the interphase was taken as an indication for the presence of sterols [11].

2.4. Isolation

The ethyl acetate extract (5g) was pre-adsorbed on silica gel and loaded into a column pre-packed with 100g silica gel (60-120 Merck). The extract was eluted with n-hexane: ethyl acetate in a gradient elution starting from n-hexane (100%). The eluates were collected in 30ml fractions. A total of 180 fractions were collected and pooled together on the basis of their thin layer chromatographic profile (TLC) (Table 1). The pooled fractions were labelled E1-E12 (Table 2).

2.5. Nuclear Magnetic Resonance (NMR)

The isolated compounds were identified by using NMR spectroscopy. The ^{1}H-NMR and ^{13}C-NMR spectra were obtained in $CDCl_3$ with NMR spectrometer (BrukerAvance III 400MHZ). The chemical shifts (δ) were reported in parts per million (ppm).

Table 1: Fractions Collected from Column Chromatography of Ethyl acetate extract

S/N	Fractions	Mobile for column chromatography	Mobile phase for TLC
1	1-6	n-hexane 100%	n-hexane: ethyl acetate 9:1
2	7-13	n-hexane: ethyl acetate 95:5	n-hexane: ethyl acetate 9:1
3	14-25	n-hexane: ethyl acetate 90:10	n-hexane: ethyl acetate 9:1
4	26-38	n-hexane: ethyl acetate 85:15	n-hexane: ethyl acetate 9:1
5	39-51	n-hexane: ethyl acetate 82.5: 17.5	n-hexane: ethyl acetate 7:3
6	52-94	n-hexane: ethyl acetate 80:20	n-hexane: ethyl acetate 7:3
7	95-114	n-hexane: ethyl acetate 75:25	n-hexane: ethyl acetate 7:3
8	115-138	n-hexane: ethyl acetate 70:30	n-hexane: ethyl acetate 1:1
9	139-159	n-hexane: ethyl acetate 65:35	n-hexane: ethyl acetate 1:1
10	160-174	n-hexane: ethyl acetate 60:40	n-hexane: ethyl acetate 4:5
11	175-182	n-hexane: ethyl acetate 50:50	n-hexane: ethyl acetate 4:5

Table 2: Pooled Fractions Collected from Column Chromatography of Ethyl acetate extract

Pool Name	Pool formed
E1	1-4
E2	5-14
E3	15
E4	16-24
E5	25-38
E6	39-51
E7	52-94
E8	95-114
E9	115-119
E10	120-124
E11	125-138
E12	139-182

3.0. Results

3.1. Test for Steroids/ Triterpenes

3.1.1 Liebermann-Burchard Test

The presence of a brown ring at the interphase which indicated the presence of steroids/ triterpenes.

3.1.2 Salkowski's Test

Formation of red coloration at the interphase was taken as an indication for the presence of sterols.

3.2 Isolation

3.2.1. TLC of Isolated compounds

The TLC analysis of fraction E4 using n-hexane: ethyl acetate (9:1) revealed a single spot. This was coded AK1 (10mg). The compound (AK1) with $R_f$value 0.72 (Plate I) was collected at n-hexane: ethyl acetate (90:10) solvent system from the column.

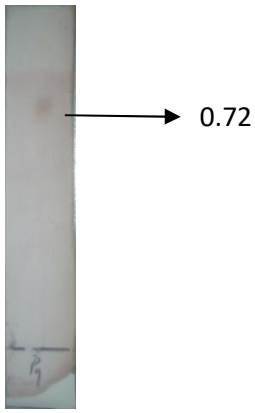


Plate I: Chromatogram of Compound AK1 developed in n-hexane ethyl acetate (9:1) sprayed with 10% sulphuric acid heated at 105°C for 2 minutes

The TLC analysis of fraction E7 after using n-hexane: ethyl acetate (7:3) revealed a single spot (Plate II). This was coded AK2 (20mg). The compound (AK2) with $R_f$value 0.63 was collected at n-hexane: ethyl acetate (8:2) solvent system from the column.

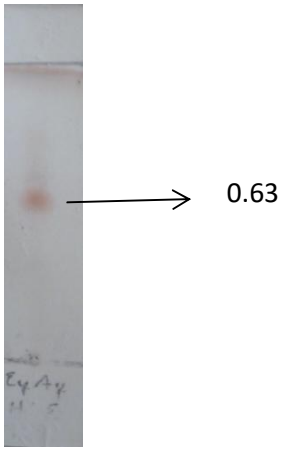


Plate II: Chromatogram of Compound AK2 developed in n-hexane ethyl acetate (7:3) sprayed with 10% sulphuric acid heated at 105°C for 2 minutes

3.3 Identification of Stigmasterol and β-Sitosterol

3.3.1 Compound AK1: Stigmasterol

Stigmasterol: white powder (10mg), melting point: 134-136°C. ^{1}HNMR spectrum of AK1 revealed the following chemical shift values of 3.52 (1H, m), 5.35 (1H, t), 0.94 (3H, d), 5.06 (1H,

m), 5.10 (m, 1H), 0.86 (3H, t), 0.83 (3H, d), 0.81 (3H, d), 0.70 (3H, s), 1.03 (3H, s), ^{13}C NMR of AK1 showed 29 signals at^{13}C NMR (200 MHz, $CDCl_3$) δ 140.56, 138.29, 129.29, 122.43, 71.54, 57.05, 55.66, 51.23, 49.68, 43.68, 42.22, 40.03, 38.03, 37.18, 36.69, 32.75, 31.85, 31.67, 31.54, 28.64, 25.40, 24.25, 21.21, 20.83, 20.97, 20.03, 19.28, 12.32, 12.39 (Table 3).

3.3.2. Compound AK2: β-Sitosterol

β- Sitosterol: White crystal (10mg), melting point: 145-148°C. ^{1}HNMR spectrum of AK2 revealed the following chemical shift values of3.52 (t, 1H), 5.35 (m, 1H), 0.92 (m, 1H), 0.86 (t, 3H), 0.85 (d, 3H), 0.83 (d, 3H), 0.68 (s, 3H), 1.01 (s, 3H). ^{13}C NMR of AK2 showed 29 signals at ^{13}C NMR (200 MHz, $CDCl_3$) 140.56, 122.43, 71.54, 57.05, 56.48, 49.68, 45.88, 43.20, 42.22, 38.03, 37.18, 36.69, 36.11, 33.93, 32.75, 32.23, 31.85, 31.53, 29.11, 28.13, 26.21, 24.25, 23.07, 20.97, 19.39, 19.28, 18.59, 12.11, 12.30 (Table 3).

Table 3: ^{1}H and ^{13}C NMR data of AK1and AK2

Carbon position	^{1}HNMR of AK1	^{13}CNMR of AK1	^{1}HNMR of AK2	^{13}CNMR of AK2
1		37.18		37.18
2		32.75		31.85
3	3.52 (1H, m)	71.54	3.52 (tdd, 1H)	71.54
4		42.22		43.20
5	5.35 (1H,t)	140.56	5.35 (t,1H)	140.56
6		122.43		122.43
7		31.67		32.23
8		31.85		31.85
9		51.23		49.68
10		36.69		36.69
11		20.97		20.97
12		38.03		38.03
13		43.68		42.22
14		57.05		57.05
15		24.25		26.21
16		28.64		28.13
17		55.66		56.48
18		40.02		36.11
19	0.94 (3H, d)	21.21	0.92 (d, 1H)	18.59
20	5.06 (1H, m)	138.29		33.93
21	5.10 (m, 1H)	129.29		24.25
22		49.68		45.88
23		25.40		23.07
24	0.86 (3H, t)	12.23	0.86 (t, 3H)	12.30
25		31.54		29.11
26	0.83 (3H, d)	20.83	0.85 (d, 3H)	19.28
27	0.81 (3H, d	20.03	0.83 (d, 3H)	19.39
28	0.70 (3H, s)	19.28	0.68 (s, 3H)	18.59
29	1.03 (3H, s)	12.30	1.01 (s, 3H)	12.11

Lit= Chaturvedula and Prakash, 2012, stigmasterol, AK1= stigmasterol, AK2= β-sitosterol

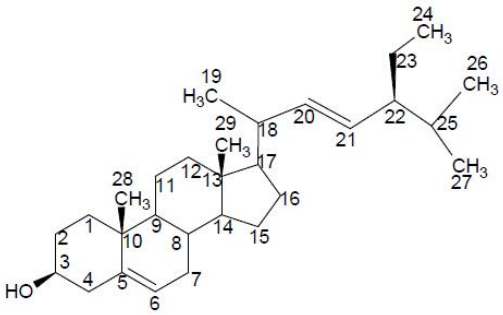


Figure 1: Structure of Stigmasterol (AK1)

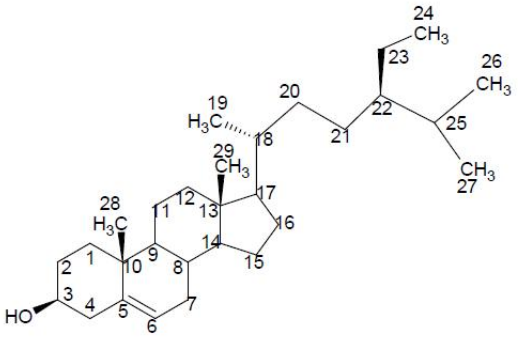


Figure 2: Structure of β-sitosterol (AK2)

4.0. Discussion

Compound AK1 was isolated fromfraction E4 after secondary column chromatography with silica gel using n-hexane: ethyl acetate (9:1). The ^{1}H NMR analysis of AK1 revealed the presence of two methyl singletsat δ 0.70, and 1.03, three methyl doublets that appeared at δ 0.81, 0.83, and 0.94 and a methyl triplet at δ 0.86. AK1also showed protons at δ 5.06, 5.10, and 5.35 suggesting the presence of three protons corresponding to that of a tri-substituted and a di-substituted olefinic bond [12], [13]. The proton corresponding to the H-3 of a sterol moiety appeared as a multiplet at δ 3.52. The ^{13}C NMR spectrum of AK1 showed signals at 140.56 and 122.43 ppm indicating the presence of C5=C6 double bond respectively. The signal at 71.54 shows a β-hydroxyl group at C3 [14]. The signal at δ 31.54 and δ 20.03 ppm corresponds to angular carbon atom at C-25 and C-27 respectively [15]. Chemical shifts of 138.29 and 129.29 indicate olefinic carbons at C-20/21. The NMR spectra of AK1 were consistent with that of stigmasterol reported in the literature [16]. The stigmasterol was isolated as a white powder. This is in agreement with Pratheema et al., [15] who isolated stigmasterol from Alpiniacalcata. The stigmasterol showed an R_f value of 0.72 when developed on TLC usingin n-hexane ethyl acetate (8:2). This was similar to the study of Govindarajan and Sarada [17] who reported that stigmasterol isolated from Acacia niloticahad an R_f value of 0.70 when developed in n-hexane ethyl acetate (8:2). The melting point of stigmasterol was reported to be 134-136°C. This is similar to that reported by Stevens et al., [18].

Compound AK2 was isolated from fraction E7 after secondary column chromatography with silica gel using n-hexane: ethyl acetate gradient.The 1H NMR spectra of AK2showed the presence of six methyl signals that appeared as two methyl singlets at δ 0.68, and 1.01, three methyl doublets appeared at δ 0.83, 0.85, and 0.92 and a methyl triplet at δ 0.86. The 1H NMR spectra AK2also showed one olefinic proton at δ 5.35. The absence of protons corresponding to the double bond between C-20/C-21 in AK2 differentiates it from AK1. The 1H NMR spectra of AK2showed a proton corresponding to the proton connected to the C-3 hydroxy group which appeared as a triplet of doublet of doublets at δ 3.52 [16]. The ^{13}C NMR spectrum of AK2 showed 29 carbons including an oxymethine carbon signal at δ 71·33 and two olefinic carbons at δ 140·56 and δ 122·43. The double bonded unsaturation at δ 140.56 and δ 122.43 while two methylene carbon signals were present at 45.88 and 23.07 for C22 and C23 [19]. The spectral data is in agreement with that of β-sitosterol reported in the literature by Chaturvedula and Prakash [16]. The β-sitosterol appeared as a white crystal this was in agreement with the study reported by Fabunmiet al., [19]who isolated *β*-sitosterol from ethyl acetate fraction of *Acalypha wilkesiana* twigs as white crystals. The β-sitosterol when developed on TLC using the solvent system of n-hexane: ethyl acetate (7:3) appeared as a pink spot with R_f value of 0.63 similar to that reported for β-sitosterol from *Hypoxis hemerocallidea [*20]. The melting point for *β*-sitosterol in this study was between 145-148°C which is similar to that reported by Stevens et al., [18].

5.0. Conclusion

Two phytosterols were isolated from the stem bark extract of *Aubrevillea kerstingii*. The structures of the isolated new compounds were

identified as stigmasterol and β-sitosterol on the basis of spectroscopic data and by compared to that reported in the literature.

6.0. Recommendations

Chronic toxicity studies should be conducted to determine the long term toxicity effect of the plant. Other biological activities should be carried out on *Aubrevillea kerstingii*.

7.0. Acknowledgements

I will like to thank the laboratory staff of the Department of Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria.

8.0. Conflict of Interest

The authors declare that there is no conflict of Interest.

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