Isolation, Characterization and Antimalarial Activity of β-Sitosterol from the Leaves of *Alchornea cordifolia* (Schumach. & Thonn.) Mull. Arg

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Abstract

Alchornea cordifolia is well-known for its phytotherapeutic benefits which include potential antimalarial effects. In this study, bioactive components from A. cordifolia leaves were isolated and characterized. Additionally, the antimalarial effects of its crude methanol extract and ethyl acetate fraction were examined in vitro on human whole blood infected with Plasmodium falciparum. Standard techniques were employed for extraction, column chromatography and in vitro antimalarial activity. Salkowski and Liebermann-Burchard reactions were performed. The isolated fraction was characterized using FTIR, ^{1}H NMR, ^{13}C NMR (DEPT 135) and mass spectroscopy. Salkowski and Liebermann-Burchard reactions showed the presence of steroids. The compound interpreted from the spectral results was β-sitosterol. On antimalarial activity, the % parasitaemia after 72 hours was 0.1±0.0 and 0.1±0.6 for crude methanol extract and ethyl acetate fraction respectively.

The results compared well with the parasitaemia rates of the standard drugs (chloroquine, 0.1±0.0) and ACT (0.1±0.0). The isolated compound was identified as β-sitosterol, which showed significant antimalarial activity against Plasmodium falciparum.

Keywords: *Alchornea cordifolia,* β–Sitosterol, spectroscopy, antimalarial, isolation, extraction

Introduction

Four Plasmodium species including the most lethal *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae and Plasmodium ovale*, infect people and spread malaria through the bites of anopheles mosquitoes. Poor populations in tropical and subtropical regions, where the climate is conducive to the growth of parasites and vectors, are the main victims[8]. The disease poses a threat to more than 40% of the world's population[29]. Half of those at high risk of transmission (1.2 billion people) live in Africa; 80% of these cases are concentrated in 13 countries with more than half occurring in Nigeria, Congo, Ethiopia, Tanzania and Kenya[31].

Over the past ten years, there has been a resurgence of interest in indigenous medicine due to the limited use of orthodox medicine. According to the World Health Organization[31], traditional medical practices are still used by 80% of Africans for their primary healthcare needs. The US Agency for International Development asserts that the expanding global demand for medicinal plants and their byproducts in recent decades, as well as the rising number of users and the variety of applications for which they are employed, have increased the importance of medicinal plants[6]. At least 25% of all modern medicines are thought to be produced from medicinal plants today, either directly or indirectly. This is largely due to the integration of modern technology with traditional knowledge.

Alchornea cordifolia is a shrub with a height of up to 8 meters. It is widespread in tropical Africa. Traditional African medicine uses the plant to cure diseases[5]. "Christmas bush" is its common name. It is an evergreen, dioecious shrub or small tree that can grow up to 8 metres tall and has loosely branching stems[26]. This species is common in riverine woodland, especially in swampy places but occasionally in drier sites[26]. The stem bark, roots and leaves are rich in guanidine alkaloids, terpenoids, tannins, saponins, flavonoids and carbohydrates[5]. Additionally, the leaves contain a variety of hydroxybenzoic acids including gentisic acid, anthranilic acid, protocatechuic acid, gallic acid and ellagic acid (alizarine yellow)[7]. The seed oil contains alchornoic acid, a vernolic acid (C20 homolog)[5]. Its dried leaves are frequently used as tea[30].

The plant's roots are used to cure amoebic dysentery, diarrhoea and venereal disorders[2]. Conjunctivitis and other eye conditions are treated using drops made from it[26]. *A. cordifolia* is a crucial medicinal plant in traditional African medicine and extensive pharmacological research into its antibacterial, antifungal and antiprotozoal characteristics, as well as its anti-inflammatory activity, has been conducted with remarkably encouraging outcomes[26]. The leaves or leafy stems are also thought to have other medicinal properties including those of an abortifacient, antispasmodic, blood purifier, diuretic, emmenagogue, oxytocic, purgative, sedative and tonic[26,30]. *A. cordifolia* leaf extract has been shown to have antibacterial activity against many bacterial strains[20]. At 2.1 g/mL, an extract from *A. cordifolia* displayed 50% 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity[20].

Recently, there has been a surge in the use of *A. cordifolia* extract for the treatment of infectious disorders [7]. The relationship between its antimalarial activity and its phytochemical components is often unclear. More research is needed to elucidate the structures of this plant.

Spectroscopic methods have been used to determine the structures of plant extracts[1,3,10-12,14-18,22-26]. We hereby report the isolation, characterization and antimalarial activity of β-sitosterol from the leaves of *A. cordifolia.*

Material and Methods

Plant Materials: New leaves of *A. cordifolia* were collected from the surrounding forest at the Michael Okpara University of Agriculture in Umudike, Abia State. A plant taxonomist from the Department of Forestry and Environmental Management at Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria, identified the plant and the voucher number was given to the plant. The leaves were collected and fragile leaves were chosen for the best phytochemical extraction. The leaves were powdered after being air dried at room temperature.

Extraction, column chromatography and thin-layer chromatography analysis: The method of extraction was maceration. 1000 g of the powdered leaves were soaked in methanol in a stoppered bottle for 4 days with frequent agitation until soluble matter dissolved and then filtered using Whatmann no. 1 filter paper. The filtrate was concentrated by evaporation using a rotary evaporator at 40 ^{o}C. The crude methanol extract collected was greenish-brown in colour and weighed 30 g.

Merck's silica gel 60 GF 254 (70–230 mesh) served as the stationary phase for column chromatography (CC). Thin-layer chromatography (TLC) analysis was conducted using silica gel 60 F254 pre-coated aluminium plates (0.2 mm, Merck). Through exposure to iodine vapour, UV light and cerric sulphate solution spraying, spots were made visible. The crude extract was eluted by Hex:CH_2Cl_2 (100→0), CH_2Cl_2:$CH_3COOC_2H_5$ (100→0) and $CH_3COOC_2H_5$: MeOH (100→0), using CC in silica gel (150 g). Ninety-one (91) fractions of 125 mL each were obtained. TLC was used to analyze the faint green fraction that was eluted from $CH_3COOC_2H_5$: MeOH (100:0). The TLC mobile phases were hex: CH_2Cl_2 (1:2). A single spot with an Rf value of 0.61 was obtained. Hence, it was labelled EAF3.

Salkowski reaction: A few EAF3 crystals were dissolved in chloroform (5 mL) and the solution was then treated with 5 mL of strong sulfuric acid. The presence of steroids was indicated by a reddish colour in the upper chloroform layer [9].

Liebermann-burchard reaction: Chloroform (5 mL) was used to dissolve a few EAF3 crystals and then strong sulfuric acid (5 mL) and acetic anhydride (5 mL) were added. The formation of violet, blue and then green colours denoted the presence of steroids[9].

Spectroscopic characterization: The NMR Bruker AV 400 WB spectrometer was used to measure the ^{1}H NMR and ^{13}C NMR-DEPT 135 of the EAF3 at 400 MHz and 100 MHz respectively in $CDCl_3$. The chemical shift was measured in ppm with tetramethylsilane serving as the internal reference. At 70 eV, electron impact-mass spectra were recorded using a Jeol AX-505 HA mass spectrometer. The infrared spectrum was recorded on a Bruker Tensor 27 spectrometer on NaCl film. Each compound's spectroscopic data (^{1}H and ^{13}C NMR-DEPT 135) was compared to those that had previously been reported in the literature[13,19,21].

Antimalarial studies

Collection of blood and preparation of medium: Exactly 5 mL of venous blood was drawn from the infected patients after screening and with their full consent. The following criteria were used to select blood samples: blood film containing only *P. falciparum* and no other Plasmodium species, parasitemia of less than 0.02 % and negative Saker-Solomon urine test results.

Incubation: The blood medium was distributed into test plates containing EDTA as an anticoagulant. The blood parasites were incubated in a candle jar. Incubation was done for 48 hours to ensure maximum parasite development. This was done for morphological assays based on Schizont counts.

Determination of the end point: The developmental stage of each isolate was quickly assessed by microscopic analysis of Giemsa-stained blood smears after 48 hours of incubation. Blood samples with less than 10% schizogony were used for the *in vitro* assay.

Blood medium: Each test plate received exactly 0.5 ml of blood, distributed into 7 rows and 5 columns. There were "drug-free blood control wells, BC; crude methanol extract CME, ethyl acetate fraction EAF3, chloroquine CQ and coartem ACT" in the first five rows of the test plate. The last two rows were for "supplementary drug-free control wells SW" and "wells for precautionary purposes PW."

Preparation of stock solutions and drug administration: To make a stock solution, 0.15 mg of each extract or drug (CME, EAF3, CQ, ACT) was dissolved in 1 ml of 70% ethanol (used as a solvent; chloroquine was dissolved in distilled water because it is water soluble). This yielded a concentration of 0.15 mg/mL. The stock was diluted to 1:30 to provide a working solution with a concentration of 0.005 mg/mL or 5.0 g/mL. Then, using the ACT dosage interval as a benchmark, a fixed dose of 5.0 g/ml was administered to each blood test plate at time interval which is: 0-8, 8-24, 24-36, 48-60 and 60-72 hours for three days. A microscopic examination and parasite count were performed prior to each treatment. The positive controls were ACT and CQ, while the negative control was BC.

Microscopic examination and parasite count: The percentage of parasitaemia was calculated using equation 1. The concentration (IC_{50}) at which growth was 50 % inhibited, was calculated [21]. A one-way ANOVA with

Turkey HSD was used in statistical analysis to categorize the activity levels of the extract and fractions.

$$\%\ Parasitaemia = \frac{No\ of\ infected\ erythrocytes}{Total\ No\ of\ uninfected\ and\ infected\ erythrocytes} x\ 100\ \% \quad (1)$$

Results and Discussion

Figures 1-4 depict the FTIR spectrum, ^{1}H NMR, ^{13}DEPT-135 and mass spectrum of EAF3. The DEPT, ^{13}C NMR and ^{1}H NMR chemical shift values and spectral data of EAF3 are shown in table 1. The FTIR spectrum (Figure 1) showed a broad absorption peak at 3470 cm^{-1}, indicating the presence of the OH group. The vibration frequencies of 2922 and 2852 cm^{-1} indicated the presence of aliphatic C-H stretching. The vibration frequency of 1660 cm^{-1} was assigned to the C=C stretch. The peaks at 1162 and 1097 cm^{-1} indicated the presence of the C-O stretch. The wavenumber at 1463 cm^{-1} indicated the presence of C-H bending while the peak at 1376 cm^{-1} was due to the C-H bending of the gem-dimethyl group. The wavenumber at 974 cm^{-1} was due to alkene bending. The FTIR spectrum of the isolated compound compared well with the FTIR spectrum of β-Sitosterol.

The positive tests for steroids revealed that the compound contained a steroidal skeleton. The ^{1}H NMR data showed two olefinic protons at δ 5.35 and δ 5.75 (H-5 and 6). Multiplets were observed at δ 3.55 (H-3), which indicated the presence of OH. Six methyl protons with their signals at δ 1.50, 1.60, 1.17, 1.30, 1.30, 0.80 and 0.70 were assigned to H-18, 19, 21. 26. 27 and 29 respectively. These signals and assignments agreed with reported values[13,19,28].

The ^{13}C NMR (DEPT 135) showed two quaternary carbons, eleven methines, nine methylenes and six methyl carbons. The quaternary carbons C-10 and C-13 were observed at δ 36.50 and 40.50 respectively. Similar reports have also been reported in the literature[13,19,28]. Olefinic carbons at C-5 and C-6 were observed at δ 141.00 and 121.50 [13, 19, 28]. C-1, C-2, C-4, C-7, C-11, C-12, C-15, C-16, C-22, C-23 and C-28 were observed at δ 37.20, 31.60, 38.20, 31.80, 24.30, 38.70, 23.06, 28.90, 31.60, 28.90 and 25.40 respectively. Methine protons C-8, C-9, C-14, C-17, C-20, C-24 and C-25 were assigned 30.90, 50.10, 56.70, 57.00, 39.60, 51.20 and 36.10 respectively. The chemical shift values at δ 17.05, 23.41, 24.00, 25.90, 25.70 and 25.90 were attributed to carbon atoms C-18, C-19, C-21, C-26, C-27 and C-29, respectively [17]. A signal at 67.80 (C-3) indicated a CH-OH [13, 19, 28]. Notably, these compounds were obtained from 100 percent ethyl acetate as a single solvent.

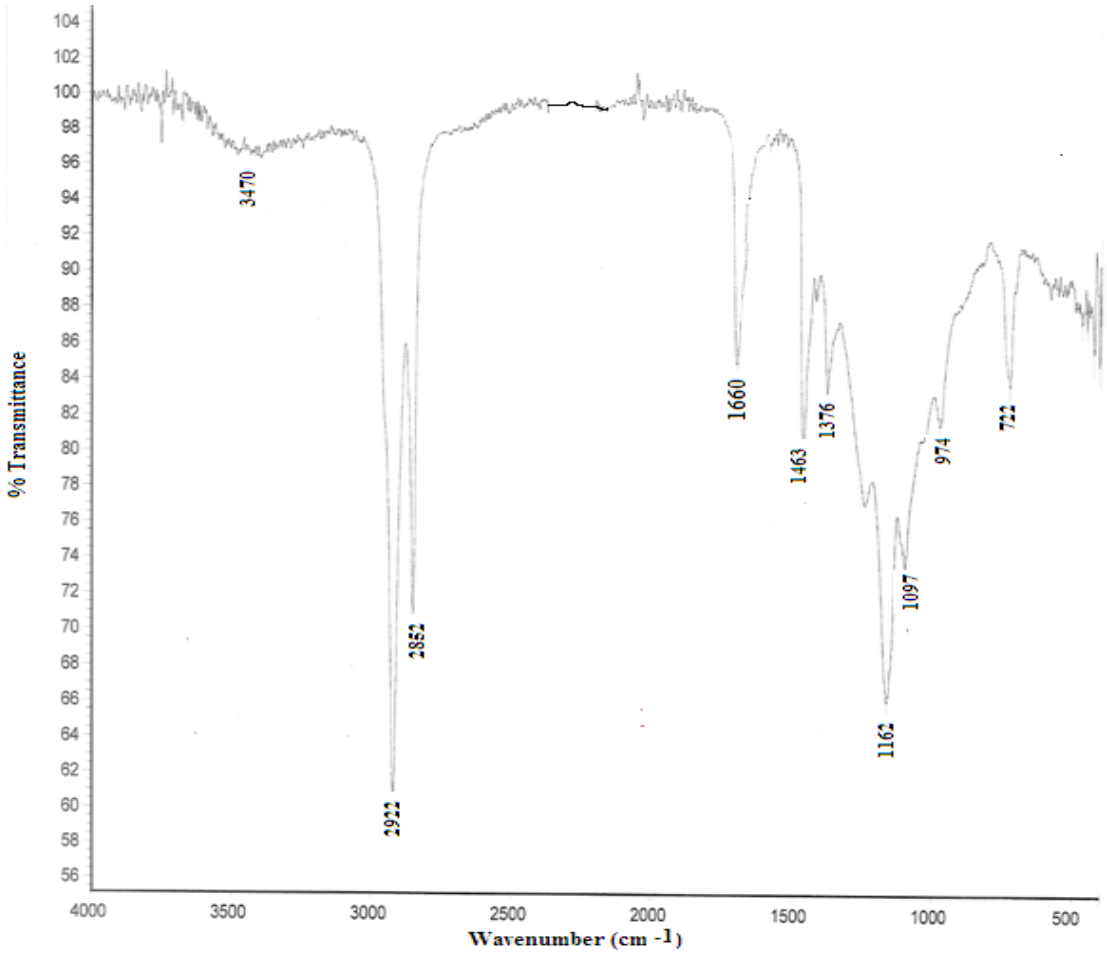

Figure 1: FTIR spectrum of EAF3

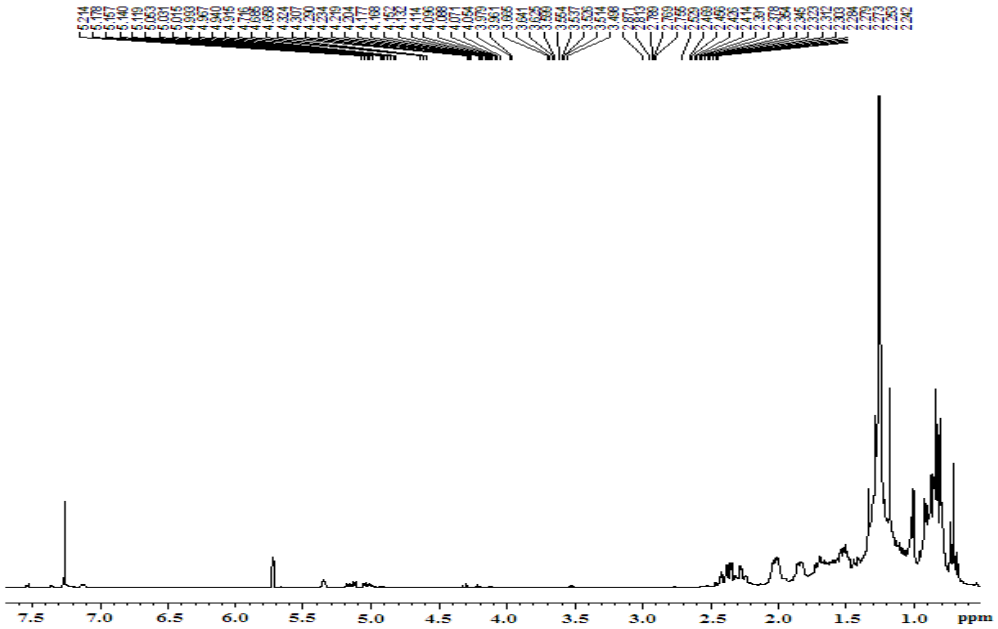

Figure 2: ^{1}H NMR spectrum of EAF3

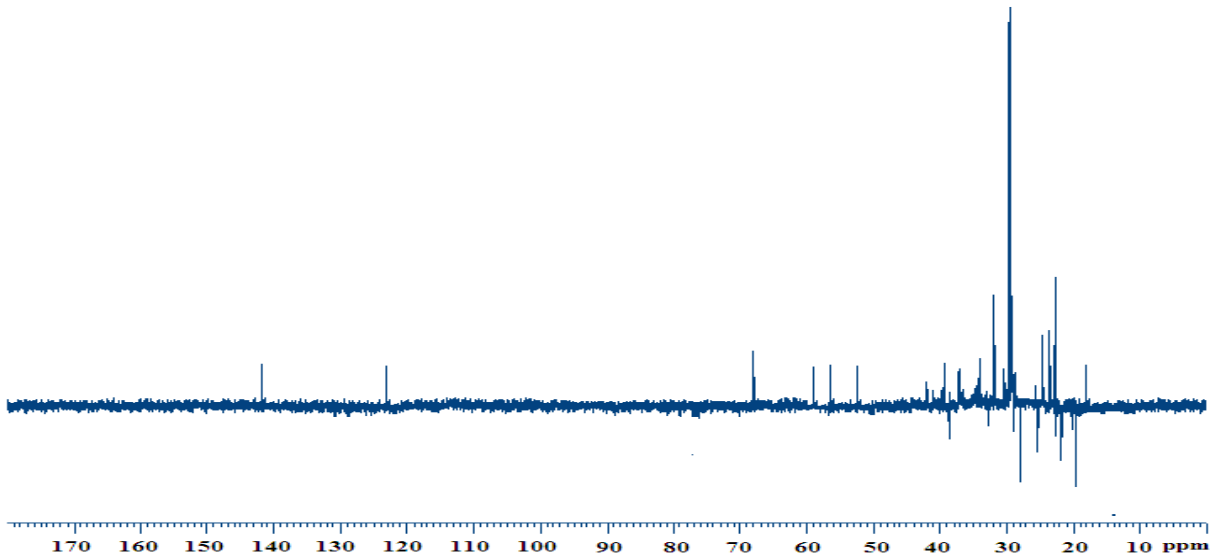

Figure 3: DEPT-135 spectrum of EAF3

Based on the Salkowski reaction, Liebermann-Burchard reaction, FTIR, ^{1}H NMR, DEPT-135 and mass spectra, the structure of β-sitosterol (Figure 5) has been proposed for EAF3. The mass spectrum fragmentation pattern of β-sitosterol is shown in fig. 6. A molecular ion peak was observed at m/z 414.70. This is in agreement with the molecular formula $C_{29}H_{50}O$. The loss of H_2O molecules from the molecular ion (414.70 m/z) resulted in a peak at m/z of 396.69. Loss of C_3H_6 yielded a peak at m/z 354.61. Loss of C_5H_6 gave a peak at m/z 288.51. Dealkylation (-CH_3) of m/z 288.51 yielded a peak at m/z 274.48. Dealkylation (-CH_3) and dehydrogenation (-3H) yielded a peak at m/z 256.42.

Regarding antimalarial action (Tables 2 and 3), the percentage of parasitemia after 72 hours was 0.1±0.0 for CME and 0.1±0.6 for EAF3. The outcomes were compared to the percent parasitemia of the common medications, chloroquine and ACT (0.1±0.0 and 0.1±0.0 respectively). CME, EAF3, CQ and ACT all demonstrated good antimalarial action with mean IC_{50} values of parasitemia of 12.2±0.8, 12.9±1.2, 13.3±0.9 and 9.2±0.7 µg/ml respectively. It was found that the antimalarial activity of CME, EAF and CQ did not significantly differ from each other ($P>0.05$) whereas ACT showed a significant difference ($P<0.05$). CME and EAF3's IC_{50} antimalarial activity values were comparable to CQ's but not as potent as ACT's.

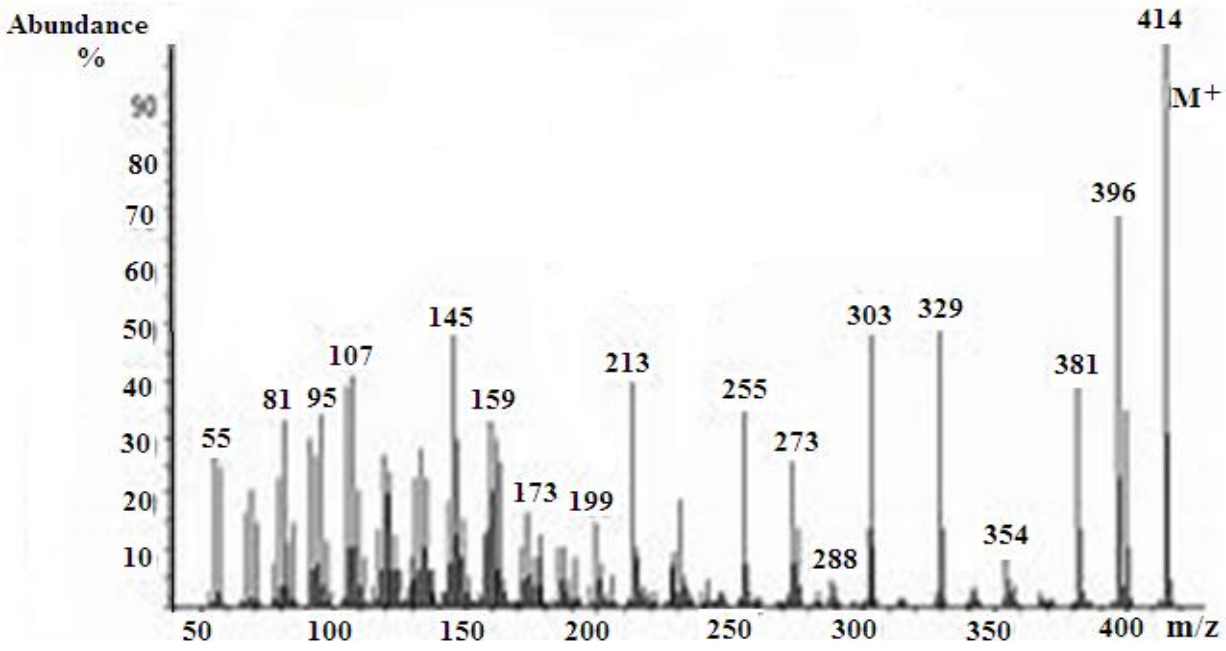

Figure 4: MS Spectrum of EAF3

Table 1
^{13}C NMR (DEPT 135) and ^{1}H NMR chemical shift values of compound 2 (β-sitosterol) recorded in $CDCl_3$

Carbon Atoms	Type of Carbon	^{13} CNMR (ppm)	^{1} HNMR (ppm)
C – 1	CH_2	37.20	
C – 2	CH_2	31.60	
C – 3	CH-OH	67.80	3.55 (m,1H)
C – 4	CH_2	38.20	
C – 5	C=C	141.00	5.35 (d, 2H)
C – 6	C=CH	121.50	5.75(t, 1H)
C – 7	CH_2	31.80	
C – 8	CH	30.90	
C – 9	CH	50.10	
C – 10	C	36.50	
C – 11	CH_2	24.30	
C – 12	CH_2	38.70	
C – 13	C	40.50	
C – 14	CH	56.70	
C – 15	CH_2	23.06	
C – 16	CH_2	28.90	
C – 17	CH	57.00	
C – 18	CH_3	17.05	1.50(s, 3H)
C – 19	CH_3	23.41	1.60(s, 3H)
C – 20	CH	39.60	
C – 21	CH_3	24.00	1.17(d, 3H)
C – 22	CH_2	31.60	
C – 23	CH_2	28.90	
C – 24	CH	51.20	
C – 25	CH	36.10	
C – 26	CH_3	25.90	1.30(d, 3H)
C – 27	CH_3	25.70	1.30(d, 3H)
C – 28	CH_2	25.40	0.80(M, 2H)
C – 29	CH_3	25.90	0.70(t, 3H)

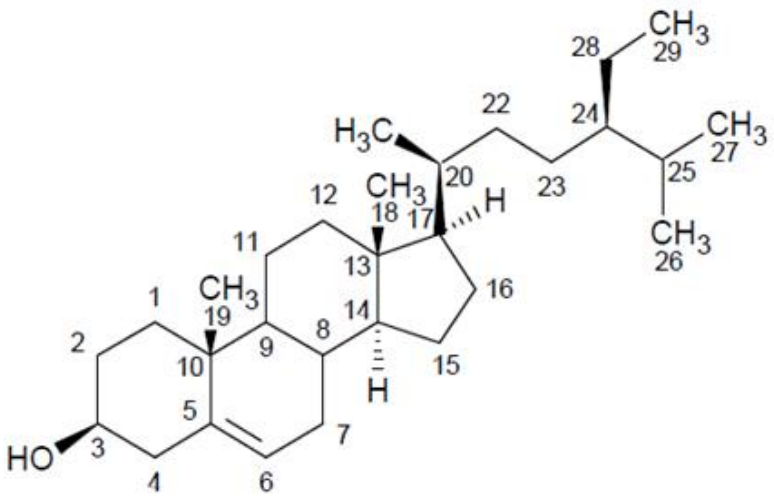

Figure 5: Proposed structure of EAF3 (β-sitosterol)

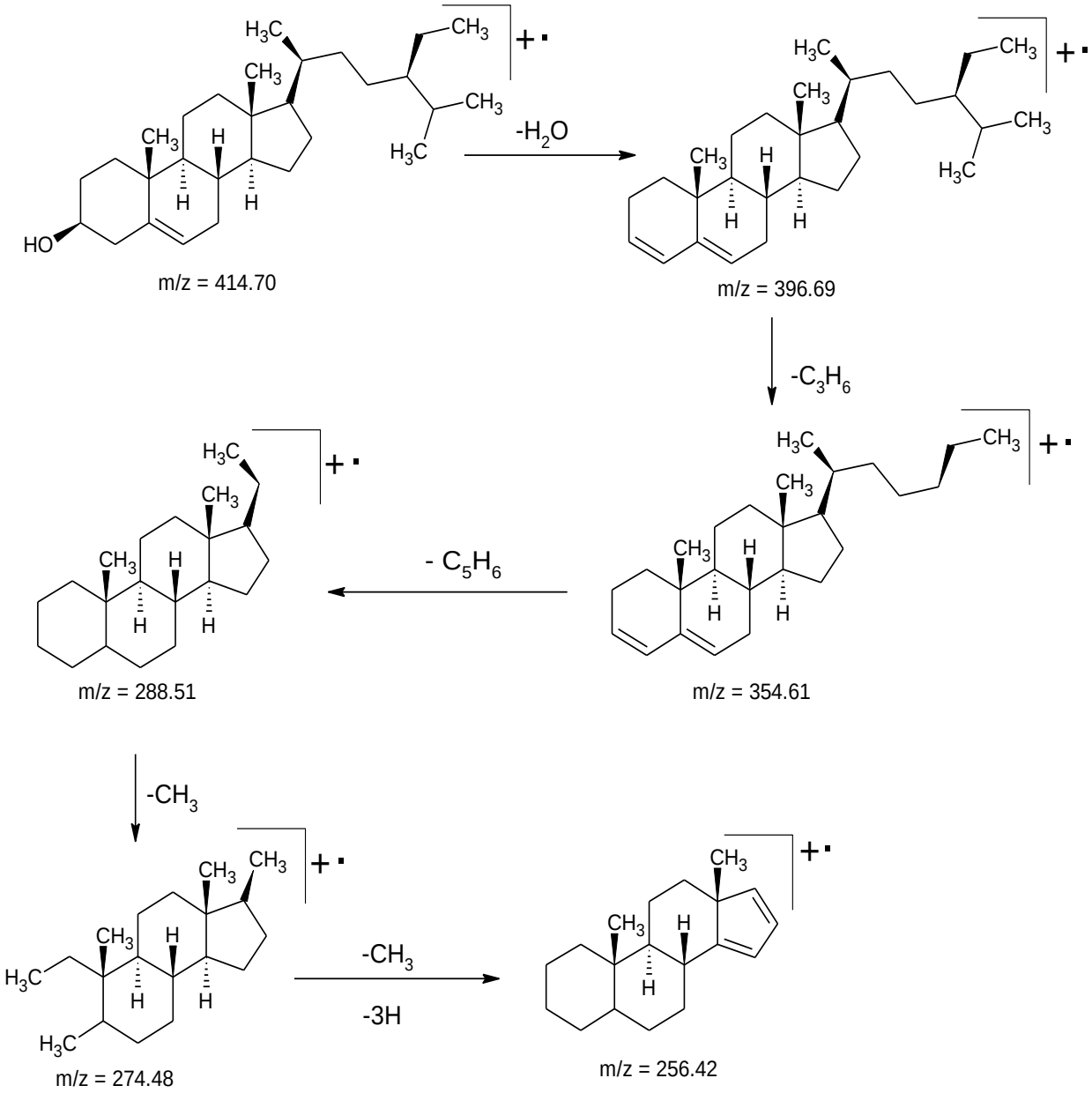

Figure 6: Mass spectrum fragmentation pattern of EAF3 (β-sitosterol)

Table 2
***In vitro* antimalarial activity (% parasitaemia)**

		DAY 1		DAY 2		DAY 3	
Hours	**0**	**0 – 8**	**8 – 24**	**24 – 36**	**36 – 48**	**48 – 60**	**60 – 72**
µg/ml	**0.0**	**5.0**	**10.0**	**15.0**	**20.0**	**25.0**	**30.0**
	% P	**% P**	**% P**	**% P**	**% P**	**% P**	**% P**
BC	81.8±6.6	80.4±7.3	79.2±7. 1	77.7±6.9	75.2±6.2	73.6±5.5	72.1±5.8[a]
CME	81.8±6.6	66.9±6.4	47.3±6.7	32.7±5.4	17.9±5.3	6.9±3.2	0. 1±0.0[b]
EAF3	81.8±6.6	69.5±7.8	48.6±6.6	35.25.2	28.4±4.3	10.4±4. 1	0. 1±0.6[b]
CQ	81.8±6.6	70.5±5.1	49.3±6.0	35.6±4.9	20. 1±6.8	3.5±3.4	0. 1±0.0[b]
ACT	81.8±6.6	62.0±6.9	37.1±6.5	27.3±5.8	16.9±5.2	8.2±2.3	0. 1±0.0[b]

BC=Drug-free blood control wells, CME=crude methanol extract, EAF3=ethyl acetate fraction, CQ =chloroquine, ACT= coartem. Means with different superscripts within the column, differs significantly at (P<0.05) while means with the same superscript at (P>0.05) has no significant difference.

Table 3
Mean IC_{50} values of antimalarial growth inhibition

Sample	CME	EAF3	CQ	ACT
Mean IC_{50} values (µg/ml)	12.2±0.8[a]	12.9±1.2[a]	13.3±0.9[a]	9.2±0.7[b]

BC=Drug-free blood control wells, CME=crude methanol extract, EAF3=ethyl acetate fraction, CQ =chloroquine, ACT= coartem. Means with different superscripts within the row, differs significantly at (P<0.05) while means with the same superscript at (P>0.05) has no significant difference.

The CME and EAF3 demonstrated good antimalarial activity and the relatively polar nature of the antimalarial elements in *A. cordifolia* leaves. The components in the leaves may be polar, as seen by the EAF3's low Rf values. The CME of *A. cordifolia* leaves and EAF3 both showed effective dose-dependent anti-*Plasmodium falciparum* activity in human whole blood medium, with ACT serving as the positive control that reduced parasitemia best.

Pure forms of β-sitosterol were extracted from just one solvent, 100 % ethylacetate, rather than a combination of solvents with Rf values 0.61. One of the phytosterols that can be found in a variety of plants is β-sitosterol. This compound has been isolated from many plant species[9,13,18]. Many health benefits of β-sitosterol have been documented[13,19,28].

Conclusion

β-sitosterol has been isolated from *A. cordifolia* leaves. The strong antimalarial activity of *A. cordifolia* has confirmed its traditional use as an antimalarial drug. Therefore, we suggest using it as a good substitute for current antimalarials, either as a herbal antimalarial or in the continued manufacturing of traditional antimalarials. Patients in isolated places can access A. cordifolia due to its widespread occurrence and it can complement current antimalarials.

Given its demonstrated bioactive components effective in malaria treatment and its significant annual output, *A. cordifolia* can serve as a valuable source of phytochemicals for the ongoing production of traditional antimalarials. The phytochemical marker from *A. cordifolia* may serve as the foundation for concurrent antimalarial medication development.

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