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In vitro antimicrobial, antioxidant, and anti-helminthic activities and GC-MS analysis of fractions of the leaves of *Aspilia silphioides*

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Abstract
The leaves of *Aspilia silphioides* are effective for wound healing, stomach aches, headaches, worm infestations, and birth control. The purpose of this study was to determine the phytochemical components of the bulked fractions of *A. silphioides* and evaluate the antimicrobial, anti-helminthic, and antioxidant activities of various solvent fractions. Standard methods were employed for the phytochemical screening. The methanol extract was fractionated and bulked to obtain bulked fractions A, B, C and D. The bulked fractions contained phytoconstituents including tannins, flavonoids, phenolics, steroids, polyphenols, phytosterols, and terpenoids. GC-MS analysis of the fractions revealed various compounds including 9-octadecenamide, O-methyl- (+)-a-tocopherol, and a-Amyrin as being present in the bulked fractions. The IC_{50} of the fractions B, C, D and the reference drug with regards to the DPPH scavenging activity were 15.47±0.61 µg/mL, 28.16 ±0.61 µg/mL, 19.62 ±0.61 µg/mL, and 40.29±0.62 µg/mL respectively. At 20 µg/mL, the paralysis and death time for both fraction C and Mebendazole drug were 19.45±0.15 min, 31.60±18 min, and 52.06±2.89min, 82.03±47.37min respectively. The minimum inhibitory concentrations of fractions A, C, and D against the tested organisms were 25±0.01-50±0.01 mg/mL, 6.25±0.02-25±0.01 mg/mL, and 12.5±0.01-25±0.01 mg/mL respectively. The minimum bactericidal concentrations of fractions A, C, and D against the tested organisms were 25±0.01-50±0.01 mg/mL, 6.25±0.01-25±0.01 mg/mL, and 12.5±0.01-50±0.02 mg/mL respectively. The bulked fractions of the methanol extracts of *Aspilia silphioides* possess antioxidant, anti-helminthic, and antimicrobial activities.

Keywords: *Aspilia silphioides*, anti-helminthic, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), antimicrobial, and antioxidant.

1. Introduction
Human survival directly hinges on plants and their derivatives, which serve as essential sources of sustenance, shelter, medicinal solutions, and clothing (Girot PO, (2012) [12]. Moreover, plants play an indirect, yet crucial, role in ecosystem preservation, further emphasizing the necessity of effectively managing plant resources with contemporary technology and knowledge (Rai PK, & Singh JS, 2020) [21]. The escalating global demand for medicinal plants is fueled by their accessibility, minimal side effects, and their occasional status as the only available remedy (Lucas, 2010) [14]. Traditional medicine, deeply rooted in centuries-old practices, has been pivotal in the treatment, prevention, and diagnosis of ailments (Rai *et al*., 2016; Schulz *et al*., 2011) [20], serving as the primary healthcare system for approximately 80% of rural populations in many developing nations, particularly across Africa (Appiah *et al*., 2018). Traditional medicinal approaches persist as integral components of healthcare across various nations, including Ghana (Abel *et al*., 2005) [1].
Contemporary research initiatives are directed towards pinpointing active compounds within herbal medicine, thereby facilitating advancements in disease management, drug development, and the formulation of herbal remedies (Newman *et al*., 2007; Asase, A., 2011) [17, 8]. Furthermore, specific medicinal plants demonstrate pharmaceutical properties that augment their effectiveness in traditional medicine practices.
While medicinal plants are abundant sources of diverse chemical compounds, the efficacy and safety of some phytochemicals remain unconfirmed under scientific scrutiny. Nevertheless, numerous plants exhibit antimicrobial, antioxidant, and anti-inflammatory properties (Acheampong *et al*., 2018; Osei Akoto *et al*., 2019) [3, 6]. Research into medicinal plants has yielded groundbreaking drug discoveries, such as digoxin, morphine, vincristine, vinblastine, and taxol, which are indispensable in treating various ailments.

Additionally, drugs like quinine and artemisinin, derived from medicinal plants, are crucial in combating malaria, whether used alone or in combination therapies. Artemisinin, sourced from the Chinese herb Artemisia annua, stands as a pivotal antimalarial agent (Cheuka *et al*., 2016) [10].

Aspilia silphioides, a member of the Asteraceae family commonly known as "MFOFO Sika", exhibits therapeutic properties. Species within the *Aspilia* genus, including *Aspilia africana*, are utilized in treating a variety of ailments such as tuberculosis, coughs, measles, diabetes, malaria, and wounds (Okello, D. *et al*., 2021; Okello, D., Lee, J., & Kang, Y., 2020) [19, 19]. Among the Akan community in Ghana, *Aspilia silphioides* is traditionally used for promoting wound healing and alleviating conditions such as stomach aches, severe pains, blood clots, headaches, worm infestations, and even for birth control.

The methanolic extract of the leaves of *Aspilia silphioides* has been found to exhibit pharmacological properties including anti-helminthic, antioxidant, and antimicrobial activities (Acheampong *et al*., 2024) [2]. The purpose of this work is to fractionate the methanolic leaf extract of *Aspilia silphioides*, screen for the phytochemicals present, and determine the pharmacological activities of the fractions.

2. Materials and Methods

2.1 Chemicals and Reagents

Analytical grade organic reagents were acquired from BDH Laboratory Supplies in England. All the other chemicals, except the standard drugs, were acquired from Sigma Aldrich Co. Ltd, Irvine, UK.

2.2 Sample collection and authentication

As discussed in our previous publication, the leaves of *Aspilia silphioides* were harvested from Bekwai in December 2021 and authenticated at Herbal Medicine Department of KNUST. (Acheampong *et al*., 2024) [2].

2.3 Sample preparation and extraction

The sample preparation and extraction were performed as discussed in our previous work (Acheampong *et al*., 2024) [2]. Phytoconstituents were extracted through cold maceration. Dried leaves were soaked in methanol (97%) for three days at room temperature for 72 hours. The mixture was intermittently shaken and the container was occasionally opened to release pressure, and the mixture was stirred until the soluble components were fully extracted. Following this, the damp solid residue underwent filtration using a funnel with Whatman filter paper. Finally, the resulting extract was concentrated under reduced pressure utilizing a rotary evaporator to obtain the plant extract.

2.4 Fractionation of methanol extract column chromatography

Column chromatography was employed to fractionate the methanolic leaf extract of the *A. silphioides*. The glass column was set up with a piece of cotton placed at one end, followed by tightly packing the silica gel, functioning as the stationary phase, into the column. The mobile phase, composed of ethyl acetate and hexane in a 2:8 ratio (predetermined using TLC monitoring), was then introduced into the column. Another piece of cotton was placed atop the silica gel to secure it in position. Next, the methanol extract was delicately applied onto the cotton at the column's upper part, followed by the gentle pouring of the mobile phase over it. A collection container was positioned beneath the column tap to gather the fractions, which were then concentrated using a rotary evaporator.

2.5 Qualitative phytochemical analysis of Fractions

The screening of the fractions of the leaf extracts of *Aspilia silphioides* was performed using standard protocols by Trease and Evans (2009) [25].

2.6 *In vitro* anthelmintic assay

2.6.1 Worm collection and Authentication

The earthworms were collected and identified as indicated in our previous work (Acheampong *et al*., 2024) [2]. The earthworms, *Eudrilus eugeniae*, were collected and identified at the Zoology Unit, Department of Theoretical and Applied Biology, KNUST.

2.6.1.2 Anthelminthic Assay

The anthelmintic assay was performed according to the standard methods set out by Ajaiyeoba *et al*. (2001). First, stock solutions of the fractions were prepared at a concentration of 20 mg/mL using sterile distilled water. From these stock solutions, four further concentrations—1.250, 2.50, 5, and 10 mg/mL were prepared. Mebendazole was also prepared at 1.250, 2.50, 5, 10, and 20 mg/mL as a reference standard, with sterile distilled water serving as the negative control. All solutions for testing and standards were freshly made prior to the experiment. Each Petri dish, containing 50 mL of the respective test solutions, was seeded with four worms of approximately equal size. The times of paralysis and death were meticulously recorded. Paralysis was indicated by the absence of movement, except when the worms were shaken vigorously. Death was noted when worms did not move after vigorous shaking or immersion in warm water (50°C) and their body color faded. The experiment was conducted in triplicate, and results were presented as mean ± standard error of the mean (SEM), (Akoto *et al*., 2019) [6].

2.7 *In vitro* Antioxidant activity

Antioxidant activity tests were done using DPPH scavenging and hydrogen peroxide assays.

2.7.1 Hydrogen Peroxide Scavenging Assay

The hydrogen peroxide scavenging activity of the fractions was assessed using a modified procedure based on Mukhopadhyay *et al*. (2016) [16]. This assay involves the formation of a red-orange triphenanthroline complex through the reaction of ferrous ions with 1,10-phenanthroline. Stock solutions of the extract were prepared at a concentration of 1000 µg/mL and then diluted in sterile distilled water to obtain concentrations of 200, 400, 600, and 800 µg/mL. Similarly, gallic acid was prepared at 1000 µg/mL as a standard, and solutions with concentrations ranging from 200 to 800 µg/mL were prepared by dilution. In a series of test tubes, 0.5 mL of ferrous ammonium sulfate (1 mM) was added, followed by 3 mL of the test solutions (which included various concentrations of the extract and gallic acid) and mixed thoroughly. Next, 0.13 mL of 5 mM hydrogen peroxide was introduced to each tube, and the mixtures were incubated at room temperature in the dark for 5 minutes to prevent photo bleaching of hydrogen peroxide. After incubation, 3 mL of 1 mM 1, 10-phenanthroline was added to each tube, mixed, and incubated for an additional 10 minutes at room temperature. The absorbance was then measured at 510 nm using a UV-vis spectrophotometer. The negative control

included ferrous ammonium sulfate (0.5 mL, 1 mM), distilled water (3 mL), and 1, m10-phenanthroline (3 mL, 1 mM). The percentage scavenging activity was calculated using the formula provided below:

$$\%\ Scavenging = [\frac{\text{Atest}}{\text{Acontrol}} \text{ x } 100]$$

Where Atest is the absorbance of the test samples and Acontrol is the absorbance of the negative control. The results were further reported in IC_{50} (A parameter used to assess antioxidant activity of a drug) (Mukhopadhyay *et al*., 2016) [16].

2.8.2 1, 1 Diphenyl-1-picrylhydrazy (DPPH) free radical scavenging assay

The antioxidant activity of the fractions was measured using a common laboratory test called the DPPH assay. Different concentrations of the extracts were mixed with a chemical called DPPH. The amount of DPPH that was neutralized by the extracts was measured to determine their antioxidant power. Ascorbic acid, a known antioxidant, was used for comparison. The antioxidant activity of the fractions was evaluated using the DPPH-free radical scavenging assay, following the procedure outlined by Mahdi-Pour *et al*. (2012) [15] with some adjustments. To perform the quantitative analysis, stock solutions of each extract (100 µg/mL) were diluted with sterile distilled water to produce concentrations of 20, 40, 60, and 80 µg/mL. Ascorbic acid was prepared in a similar manner as the standard, yielding concentrations of 20, 40, 60, and 80 µg/mL through dilution. Reaction mixtures were created by combining 150 µL of 0.2 mM DPPH solution with 50 µL of each test solution at different concentrations, resulting in a total volume of 200 µL. These mixtures were then incubated in the dark at room temperature for 30 minutes. After incubation, the absorbance of each mixture was measured at 517 nm using a V-730 UV-Vis spectrophotometer (Jasco, USA). Distilled water served as the negative control. The DPPH scavenging activity was determined using the formula provided below:

$$DPPH\ scavenging\ activity\ (\%) = \frac{(Ao - A1)}{Ao}\ x\ 100$$

Where A1 is the absorbance of sample containing extract and A0 is the absorbance of the negative control (distilled water and 0.2 mM DPPH solution). Ascorbic acid served as the positive control, and measurements were conducted in triplicate. The concentration necessary for 50% scavenging of DPPH (IC_{50}) was subsequently determined by plotting the percentage of residual DPPH against the sample concentration (Aiyegoro and Okoh, 2010; Torey *et al*., 2010; Basma *et al*., 2011; MahdiPour *et al*., 2012) [4, 24, 15].

2.9 *In vitro* Antimicrobial activity

Broth micro-dilution MIC (minimum inhibitory concentration) assay was employed to assess the antimicrobial activities of the fractions.

2.9.1 Sources of microorganisms

The microorganisms utilized included three Gram-negative bacteria (Escherichia coli, Salmonella typhi, and Pseudomonas aeruginosa), two Gram-positive bacteria (*Streptococcus pyogenes* and *Staphylococcus aureus*), and one fungus (*Candida albicans*). These strains were provided by the Pharmaceutical Microbiology Section of the Department of Pharmaceutics, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST, Kumasi. They were sub-cultured on nutrient broth slants and subsequently incubated at 37 °C for 24 hours (Da Silva *et al*., 2008) [11].

2.9.2 Nutrient broth

13 grams of nutrient broth powder were measured and placed into a beaker. It was dissolved in about 500 ml of distilled water and stirred until completely dissolved. Additional freshly prepared distilled water was added to make a total volume of 1000 mL. Then, aliquots of 10 milliliters were dispensed into test tubes and tightly sealed with cotton wool. These test tubes underwent sterilization by heating in an autoclave at 121°C for 15 minutes.

2.9.3 Inoculum Preparation

Bacterial isolates were streaked onto nutrient agar plates (Oxford, United Kingdom) and incubated for 18–24 hours at 37°C. Employing the direct colony suspension method, suspensions of the organisms were created in nutrient broth and allowed to incubate overnight at 37°C. Subsequently, the colony suspensions from the tests were standardized to a 0.5 McFarland standard in sterile saline and further diluted in sterile double-strength nutrient broth to achieve a concentration of 2×10^5 CFU/mL.

2.9.4 Preparation of Standard Suspension of Microorganism

The standard microbial suspension employed in this investigation was generated by sequentially diluting the cultured microbes in sterile normal saline. These dilutions were adjusted to achieve a suspension with a turbidity comparable to that of 0.5 McFarland standards through visual assessment. This process yielded a dilution containing approximately 1.5×10^8 CFU/mL.

2.9.5 Broth Micro-Dilution

The antimicrobial susceptibility test was conducted following the method outlined by Agyare *et al*. (2004), with slight modifications. Initially, a 100 mg/mL stock solution was made by dissolving 100 mg of the fractions in 1 mL of sterile broth. This solution was then serially diluted to obtain concentrations of 50, 25, 12.5, 6.25, 3.125, and 1.5625 mg/mL in a 96-well microtiter plate. Each well was populated with 85 µL of double-strength nutrient broth, 100 µL of the diluted extract, and 15 µL of a 0.5 McFarland standard. The microtiter plate was incubated for 24 hours. After incubation, MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was added to each well, and color changes were observed. A purple color indicated no microbial growth inhibition, while a yellow color indicated inhibition. The minimum inhibitory concentration (MIC) was recorded, with tests repeated in triplicate and results expressed as mean ± SEM. Gentamicin served as the standard antimicrobial agent (Akoto *et al*., 2019) [6]. To assess the bactericidal and bacteriostatic effects of the extract and fractions, the ratio of Minimum Bactericidal Concentration (MBC) to Minimum Inhibitory Concentration (MIC) was calculated. A ratio of MBC/MIC ≤ 2 indicated a bactericidal effect, while a ratio of ≥ 4 indicated a bacteriostatic effect.

2.10 Fourier Transform Infrared Spectrophotometer (FTIR) Analysis

The fractions were analyzed using a Fourier transform infrared spectrophotometer (FTIR) of the UATR Two model by PerkinElmer. The objective of this analysis was to detect the functional groups present. Scanning occurred across the

region from 4000 cm^{-1} to 400 cm^{-1}, followed by baseline correction.

2.11 Gas Chromatography-Mass Spectrometer (GC-MS) Analysis

The compound analysis of the fractions was carried out using Gas Chromatography-Mass Spectrometry (GC-MS) at the KNUST Central Laboratory in Kumasi, Ghana. The analysis was performed with a PerkinElmer GC Clarus 580 Gas Chromatograph coupled to a PerkinElmer Clarus SQ 8 S Mass Spectrometer. The system utilized a ZB-5HTMS capillary column (5% diphenyl/95% dimethyl polysiloxane) with dimensions of 30 µm x 0.25 mm ID x 0.25 µm DF. The oven temperature program started at 100°C (held for 2 minutes), then increased at 10°C/min to 200°C, followed by a 5°C/min rise to 280°C. For detection, an electron ionization system was used in electron impact mode with an ionization energy of 70 eV. Helium gas (99.9999%) served as the carrier gas at a constant flow rate of 1 mL/min, with an injection volume of 1 µL. The injector temperature was set to 250°C, and the ion source temperature was adjusted to 220°C. Mass spectra were collected at 70 eV with a scan interval of 1 second and a mass scan range of 45 to 500 Da. A solvent delay of 0 to 3 minutes was applied, corresponding to the total GC-MS running time.

2.12 Statistical Analysis

The data was analyzed using computer software called Microsoft Excel and GraphPad Prism 5 version 8.02. Statistical test, analysis of variance (ANOVA) was used to determine if there were any meaningful differences between the data points.

3. Results and Discussions

3.1 Fractionation of Methanolic Extracts

Forty fractions were collected from the column run and monitored on precoated TLC plates. The fractions with similar composition (components with the same or similar retardation factors (Rf)) were bulked together to obtain four bulked fractions A, B, C, and D. Fractions 1 to 6 were pooled together and called bulked fraction A. Likewise, fractions 7 to 9, 10 to 25, and 26 to 40 were respectively pooled to form the bulked fractions B, C, and D. Each bulked fraction when monitored on a pre-coated TLC plate gave more than one spot, indicating that they contained more than one compound, hence the bulked fractions were not isolated compounds but a mixture of compounds. The bulked fractions are subsequently referred to as fractions in the manuscript to avoid repeating the word 'bulked'.

3.2 Phytochemical Screening of the fractions of the methanol extract of *Aspilia silphioides*

Table 1 shows the results of the phytochemical screening for the fractions of the methanol extract of *Aspilia silphioides*.

Table 1: Results of the phytochemical screening of the fractions of the methanol extract of *Aspilia silphioides*.

Phytochemical	Fraction A	Fraction B	Fraction C	Fraction D
Phenols	-	-	+	-
Steroids	-	-	+	+
Tannins	+	+	+	+
Terpenoid	-	-	+	-
Flavonoids	+	+	+	-
Phytosterol	-	-	+	+
Polyphenols	-	-	+	-

Key: (+) = Presence of secondary metabolite; (-) = Absence of secondary metabolite or secondary metabolite not detected.

The phytochemical screening revealed the presence of phenols, steroids, tannins, terpenoids, flavonoids, phytosterols, and polyphenols in the various fractions of *Aspilia silphioides*. In fraction A, flavonoids and tannins were the only phytochemicals present. In fractions B, flavonoids and tannins were also present. The fractions C had the most phytocomponents including phenolic, steroids, tannins, flavonoids, phytosterol, and polyphenols. The fraction with the second higher number of phytochemicals was fraction D which contained steroids, tannins, and phytosterols.

3.3 GC-MS Analysis of the fractions of the methanol extract of *A silphioides*

3.3.1 GC-MS Analysis of fraction A of the methanolic extract of *Aspilia silphioides*

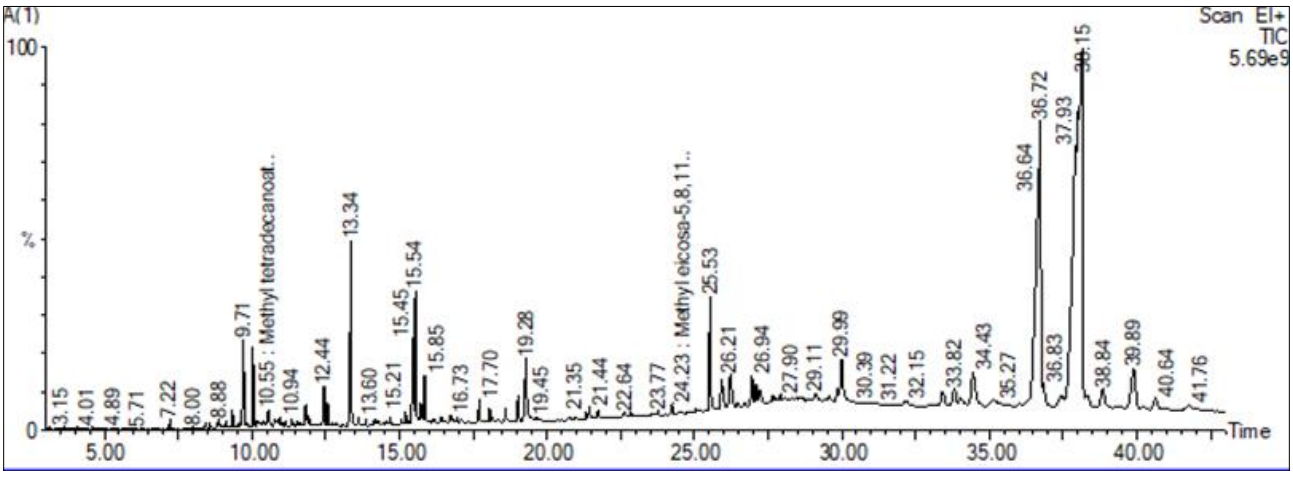

Fig 1: GC Chromatograph for A

Figure 1 depicts the GC chromatogram of the fraction A of the methanolic extract of *A. silphioides*. The Chromatogram of the GC-MS analysis of fraction A showed the presence of fourteen major peaks and the components corresponding to the peaks were determined and are reported in Table 2.

Table 2: The compounds present in fraction A

Compounds	Retention time (minutes)	Similarity Index (%)
Caryophyllene oxide	9.709	98.7
1,5,5,8-tetramethyl-12-oxabicyclo [9.1] dodeca-3,7-diene	10.039	93.5
Methylesterhexadecanoic acid	13.340	99.8
9,12-octadecadienioc acid (2,2) methyl ester	15.448	99.8
9- Octadecenamide	19.280	98.1
Squalene	25.533	99.8
2-[(1,1-dimethyletheroxycarbonyl)]-a-nitro-2,6-bis(1,1-dimethyl)-4,1-pyrrolidine butadioc acid	25.936	76.4
Cholestane-3,6,7-triol	26.211	98.0
Tocopherol quinone	29.988	93.8
Amyrin	34.425	97.4
12-oleanen-3-yl acetate	36.717	99.8
Lup-20-en-3-ol acetate	38.147	100
Friedelan-3-one	38.844	98.4
Phytol acetate	39.889	94.4

The results of the GC-MS analysis of fraction a are presented in Table 2.

The compounds were identified by comparing their mass and fragmentation patterns with those of authentic standards in the National Institute of Standards and Technology (NIST) library. Several components identified through GC-MS are associated with various pharmacological activities. For instance, caryophyllene oxide, an oxygenated terpenoid, is commonly used as a preservative in food, pharmaceuticals, and cosmetics and has demonstrated antifungal activity against dermatophytes *in vitro* (Yang *et al*., 2000) [27]. According to Idan *et al*. (2015) [13], 9-Octadecenamide exhibits anti-inflammatory and antibacterial properties. Alpha-tocopherol is recognized for its antioxidant effects. Squalene is primarily used as an adjunctive therapy in various cancers. Additionally, amyrin is noted for its anti-inflammatory activity.

3.3.2 GC-MS Analysis of Fraction B of Methanol Extract of *Aspilia silphioides*

Figure 2 depicts the GC chromatogram for fraction B, of the methanol extract of *A. silphioides*. The chromatogram of the GC-MS analysis of fraction B showed the presence of twelve major peaks and the components corresponding to the peaks were determined using the fragmentation patterns in the mass spectra by comparing them to the fragmentation patterns of authentic standards in the NIST library.

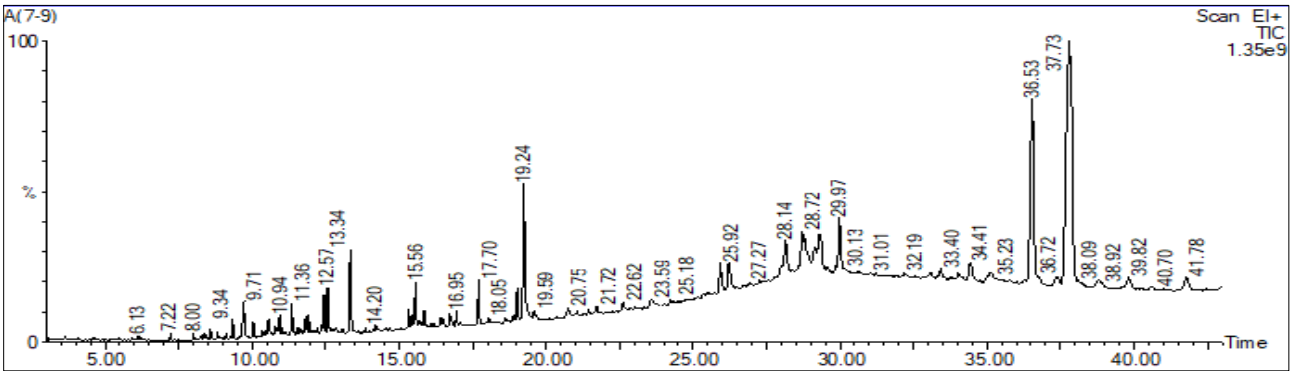

Fig 2: GC Chromatogram of fraction B of methanol extract of *A. silphioides*

Table 3: The compounds identified in fraction B of the methanol extract of *A. silphioides*

Compounds	Retention time (minutes)	Similarity Index (%)
9-octadecenamide	19.243	95.3
1-pyrrolidine butanoic acid	25.918	74.8
3-[3-bromophnyl]-7-chloro-3,4-dihydro-1,9-(2H, 10H) acridine Dione	26.211	95.8
isopropyl linoleate	28.136	88.7
i-propyl-9,12,15-octadecatrienoate	28.723	91.8
Phytol acetate	29.291	89.1
a-tocopherol quinone	29.970	93.0
10,12,14-nanocosatrienoic acid	32.188	93.6
Rhodopin	33.087	99.6
4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one.	34.443	96.7
a-Amyrin	35.122	90.7
12-oleanen-3-yl acetate	36.534	99.7

The results of the GC-MS analysis of fraction B are presented in Table 3. The compounds ranged from 9-octadecenamide (Rt=19.24 min) to 12-oleanen-3-yl acetate (Rt =36.53 min). Compounds like 9-Octadecenamide for example was present in both fractions A and B. Idan *et al*. (2015) [13] reported that 9-Octadecenamide is known to have anti-inflammatory and antibacterial activity. Some of the compounds were common to both fractions A and B, and these include 9-Octadecenamide, Phytol acetate, 12-oleanen-3-yl acetate, and tocopherol quinone. These and many other compounds could be contributing to the antimicrobial activity of fraction A. Also a-tocopherol is known for antioxidant activity.

3.3.3 GC-MS Analysis of fraction C of methanolic extract of *Aspilia silphioides*

Figure 3 depicts the GC chromatogram for the fraction C of the methanol extract of *A. silphioides*. The Chromatogram of the GC-MS analysis of fraction C showed the presence of twelve major peaks and the components corresponding to the peaks were determined using a comparison of the fragmentation patterns of the compounds and those of authentic standards in the NIST library.

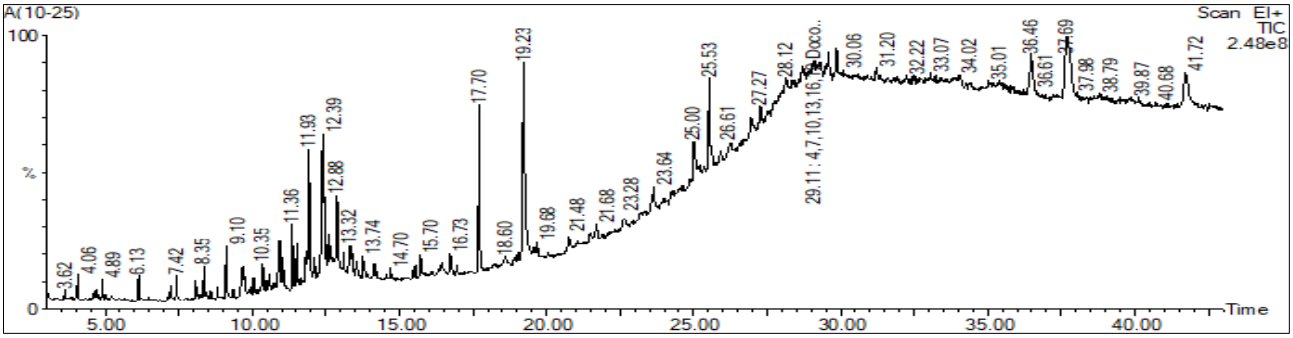

Fig 3: GC chromatogram for fraction C of the methanol extract of *A. silphiodes*

Table 4: The compounds identified in fraction C of the methanol extract of *A. silphiodes*

Compounds	Retention time (minutes)	Similarity Index (%)
3,7,11,15-tetramethyl-2-hexadecen-1-ol	12.386	99.6
9-octadecenamide	19.225	96.9
3-acetoxy-7,8-epoxylanonstan-11-ol	28.118	99.1
N-[(3a,5a,7a,12a)-24-oxo-3,7,12-tris (trimethylsilyl)oxy] cholan-24-ylmethyl ether glycine	29.108	94.3
3-acetoxy-24-phenyl-4,4,14-trimethyl-25-norisopropyl-9,19-cyclolanostan-22-en-24-one	29.566	98.0
O-methyl- (+)-a-tocopherol	29.860	86.2
Octadecamethyl cyclononasiloxane	31.235	73.7
D-galactopyranoside methyl-2,6-bis-O-(trimethylsilyl) cyclic butyl boronate	35.397	80.7
a-Amyrin	36.460	92.2
Urs-12-en-3-ol acetate	37.689	93.5
1,3-bis[(2,2-isopropyl-3,3-dimethyloxiran-2-yl) ethenyl]-2,4-diacetylcyclobutane	38.844	89.8
7,9-ditert-butyl-1,1-oxapiro (4,5) deca-6,9-diene-2,8-dione	41.723	79.3

The results of the GC-MS analysis of fraction C are presented in Table 4. The compounds ranged from 3,7,11,15-tetramethyl-2-hexadecen-1-ol (Rt=12.39 min) to 7,9-ditert-butyl-1,1-oxapiro (4,5) deca-6,9-diene-2,8-dione (Rt = 41.72 min). Some of the compounds present in fraction B were also present in fraction C, and this was expected because both fractions contained components of similar Rf when monitored on TLC plates. A compound like 9-Octadecenamide for example was present in both fraction B and C. Idan *et al*. 2015 [13] reported that, a-tocopherol is also known for antioxidant activity. These and many other compounds contributed to the antimicrobial activity of fraction C. Again 9-Octadecenamide is known to have anti-inflammatory and antibacterial activity.

3.3.4 GC-MS Analysis of fractions D of *Aspilia silphioides*

The GC-TIC chromatogram of fraction D is depicted in figure 4. The Chromatogram of the GC-MS analysis of fraction D showed the presence of twelve major peaks and the components identified are presented in Table 5.

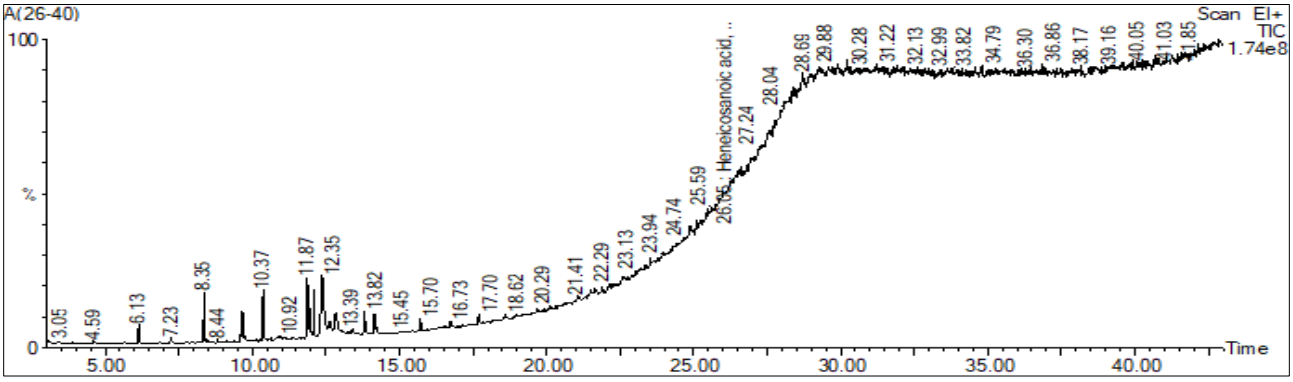

Fig 4: GC chromatogram for fraction D of the methanol extract of *A. silphioides*

Table 5: The compounds present in fraction D of the methanol extract of *A. silphioides*

Compounds	Retention time (minutes)	Similarity Index (%)
cycloheptasiloxane tetradecamethyl	8.352	99.6
cyclooctasiloxane hexadecamethyl	10.369	94.2
3,7,11,15-tetramethyl-2-hexadecen-1-ol	12.349	96.6
3-hydroxy-3,5,14,20,22,25R-spirost -8-en-11-one	24.872	93.6
25-Norisopropyl-9,19-cyclolanostan-22-en-24-one	25.364	94.3
1,1,2,3,4,4,5,6,7,10,11,11,7-cyclopenta[a] cyclopropane[f] cyclo undecane-2,4,7,7,10,11-hexol	28.686	95.5
12-(acetyloxy)-3,8,14-trihydro-3,12,14-pregn-5-en-20-one	29.291	88.3
1,3,7-tris (acetyloxy)-14,15,21,2-diepoxy-4,4,8-trimethyl D-homo-24-nor-17-oxachola-20,22-dien-16-one	29.585	96.5
Trimethyl silylestrone	29.915	90.5
5H-cyclopropa [3,4] benz [1,2-e] anzlen-5-one	30.226	95.6
1,2,5,5,6,9,10-octahydro-5,5-dihydromethyl Dodecanoic acid	30.575	99.8
1,4,7-androstantrin-3,17-dione	31.216	84.1

The identified compounds ranged from cycloheptasiloxane tetradecamethyl (Rt =8.352 min) to 1,4,7-androstantrin-3,17-dione (Rt=31.22 min). Hosseinabadi et.al 2014 reported that, 1,4,7-androstantrin-3,17-dione and cycloheptasiloxane tetradecamethyl is known for antifungal activity. These and many other compounds may confer antimicrobial activity on fraction D.

3.4 *In Vitro* Antioxidant Capacity Determination of the Various Fractions

The antioxidant activity of various fractions of *Aspilia silphioides* was evaluated using DPPH radical scavenging activity.

3.4.1 DPPH radical scavenging activity of Fraction B

DPPH radical scavenging activity was used to determine the antioxidant activity for the fractions of the leaves of *Aspilia silphioides*, with ascorbic acid as the standard. Table 6 presents the results of the DPPH scavenging activity of the fraction B and the ascorbic acid.

Table 6: The DPPH scavenging activity for Fractions B, C, D and ascorbic acid

Conc. (µg/mL)	Percent Inhibition			
	Fraction B	Fraction C	Fraction D	Ascorbic acid
100	84.83	75	50.19	86.09
80	83.44	60	41.34	83.56
60	82.17	45	34.13	82.30
40	79.65	30	30.72	81.04
20	64.18	15	24.57	45.51

The antioxidant activity of fraction B was determined using DPPH-free radical scavenging assay described by Mahdi-Pour *et al*. (2012) [15] with slight modifications.

Table 7: The IC_{50} values for fractions B, C, D and ascorbic acid

Sample	IC_{50} (µg/mL)
Fraction B	15.47±0.01
Fraction C	28.16±0.01
Fraction D	19.62±0.01
Ascorbic acid	40.29±0.01

The DPPH scavenging activity of fraction B and ascorbic acid increases with increasing concentration. The percent inhibition increases with increasing absorbance for both fraction B and ascorbic acid. The IC_{50} for reference drugs (ascorbic acid) and that of fraction B were 40.29 µg /mL and 15.47 µg /mL, respectively (Table 7). The lower the IC_{50} the more the antioxidant activity of the drug. Hence fraction B has more antioxidant activity than ascorbic acid. The IC_{50} for fraction B was observed to be significantly higher (P< 0.0043) than the ascorbic acid.

3.2.2 DPPH radical scavenging activity of Fraction C

DPPH radical scavenging activity was used to determine the antioxidant activity for the fractions of the leaves of *Aspilia silphioides* in comparison to ascorbic acid. Table 6 presents the DPPH scavenging property of fraction C and the ascorbic acid.

The results obtained from the DPPH scavenging activity of both the ascorbic acid and the fraction C showed that the scavenging activity increases with increasing concentration. At higher concentrations such as 100 µg/mL, fraction C was able to mop up more free radicals. The IC_{50} of the reference drug and fraction C was 40.29 µg/mL and 28.35 µg/mL, respectively (Table 7). The fraction C has more antioxidant activity than ascorbic acid. This could be due to synergism exhibited among the phytochemicals present in fraction C. The IC_{50} for the fraction C was observed to be significantly higher (p<0.0001) than the ascorbic acid.

3.2.3 DPPH radical scavenging activity of Fraction D

DPPH radical scavenging activity was used to determine the antioxidant activity for the fractions of the leaves of *Aspilia silphioides* in comparison to ascorbic acid. Table 6 presents the scavenging activity of the fraction D and the ascorbic acid.

The results obtained from the DPPH scavenging activity of the fraction D and ascorbic acid showed that the scavenging activity increases with increasing concentration. At a high concentration such as 80 µg/mL, the methanol extract was able to mop up more free radicals at an absorbance mean of 0.55. The DPPH scavenging activity (IC_{50}) of the reference drug and fraction D was 40.29 µg/mL and 19.62 µg/mL respectively (Table 7). Fraction D has higher antioxidant activity than the ascorbic acid. The IC_{50} for the fraction C was observed to be significantly higher (p<0.0083) than the ascorbic acid.

Comparing the antioxidant activity of the extract and fraction, with respect to DPPH scavenging activity, their IC_{50} values were employed. The IC_{50} values decreased in the order: methanol extract (47.66 µg/mL) > C (28.19 µg/mL) > D (19.63 µg/mL) > B (15.47 µg/mL). The lower the IC_{50} value, the higher the antioxidant activity hence, fraction B has the highest antioxidant activity with the methanol extract having the least activity. All the fractions displayed higher antioxidant activity than the methanol extract. The standard drug (ascorbic acid) had an IC_{50} value of 40.29 µg/mL,

meaning it has higher antioxidant activity than the methanol extract but lower antioxidant activity than the fractions. The higher activity of the fractions could be attributed to reduction in or removal of antagonism due to the separation of some of the active components that might have been antagonizing the components with antioxidant activities in the extract.

In plants, the main compounds with antioxidant activity are phenols, as they have an aromatic ring that allows the stabilization and relocation of the unpaired electrons of their structure, thus facilitating the donation of hydrogen atoms and electrons from their hydroxyl groups (Rice-Evans C, Miller N, Paganga G 1997) [22] and others such as steroids, flavonoids, and polyphenols.

3.3 *In vitro* anti-helminthic capacity determination of fraction C

The anti-helminthic activity of fractions C was determined using earthworm (Eudrilus eugeniae).

Table 8: The Mean Paralysis and Death Time of the Earthworm (Eudrilus eugeniae) for fraction C and Mebendazole standard drug

Treatment	Fraction C		Mebendazole	
Concentration (mg/mL)	Mean Paralysis Time(min) ±SEM	Mean Death Time(min) ±SEM	Mean Paralysis Time(min) ±SEM	Mean Death Time(min) ±SEM
20	19.45±0.15[a]	52.06±2.89[a]	31.60±0.18[b]	82.03±47.37[b]
10	22.45±0.18[a]	91.00±7.22[a]	37.70±0.21[b]	126.07±72.55[b]
5	30.45±0.24[a]	141.00±5.09[a]	26.02±0.15[b]	170.00±98.15[b]
2.5	32.45±0.30[a]	177.07±0.24[a]	63.37±0.36[b]	184.33±106.42[b]
1.25	39.45±0.38[a]	210.00±8.51[a]	74.40±0.42[b]	223.00±128.74[b]
Normal saline	-	-	-	-

The anti-helminthic assay was carried out using a modification of the standard methods by Ajaiyeoba *et al*., (2001). Anti-helminthic potency of the extracts was examined using Eudrilus eugeniae (Earthworms) owing to its anatomical and physiological resemblance with that of intestinal roundworm parasites of human beings as well as their ease of availability. Table 8 presents the mean time for the paralysis and death of the worm at various concentrations of the fraction C and mebendazole. As the concentration of both the fraction C and mebendazole increases, the death time and the time for paralysis of the worm reduces, indicating increasing anthelminthic activity. At all concentrations, except 5 mg/mL, the potential to paralyse the worm was higher for the fraction C than the mebendazole. The fraction C showed a greater ability to kill the worms at all concentrations than the standard drug as it recorded a shorter death time than the mebendazole. This shows that fraction C is more potent an anthelminthic than the standard drug (mebendazole).

As the concentration of the fraction C and mebendazole increases the death time for the worms reduces. This means that the higher the concentration, the lesser the death time of the worm, and the greater the anthelminthic activity. The anthelminthic activity of fraction C (in terms of death time) was greater than that of the mebendazole standard at all concentrations.

Comparing the anti-helminthic activity of the extract and fraction C, fraction C is more potent than the extract in paralyzing the worms as it took a shorter time in paralyzing the worms. Similarly, fraction C causes the death of the worms earlier than the extract, indicating that fraction C is more potent than the extract in killing the worms. The greater potency of the fraction could be attributed to antagonizing agents in the extract which have been reduced or removed during the fractionation process to obtain fraction C. It could also be due to enhanced synergy between the components that make up the fraction C. The methanol extract exhibited anthelminthic activity. The concentration for the fraction C was observed to be significantly higher ($P< 0.0003$ and 0.0002) than the mebendazole.

3.4 *In vitro* antimicrobial assessment of the various fraction:

In the determination of minimum inhibitory concentration (MIC), micro-well broth dilution method was used. The concentrations of the fraction range from 50 mg/mL to 1.25 mg/mL, with $5*10^{-3}$ mg/mL being that of gentamicin. The results of the antimicrobial activities of the fractions and standard gentamicin is presented in Table 9.

Table 9: The minimum inhibitory concentration and the minimum bactericidal concentration of fractions A, C, D and gentamicin standards.

Test organisms	MIC (mg/mL) ±SEM			MBC (mg/mL) ±SEM			Gentamicin (mg/mL) ±SEM
	Fractions			Fractions			
	A	C	D	A	C	D	
C. albicans	25±0.011	12.5±0.011	12.5±0.015	25±0.011	12.5±0.012	12.5±0.018	$5*10^{-3}$ ±0.011
E. coli	25±0.013	25±0.012	25±0.013	25±0.013	25±0.011	50±0.012	$5*10^{-3}$±0.012
S. aureus	25±0.012	12.5±0.014	25±0.017	25±0.012	12.5±0.013	25±0.012	$5*10^{-3}$±0.012
S. pyogenes	25±0.010	6.25±0.015	12.5±0.016	25±0.013	6.25±0.014	25±0.018	$5*10^{-3}$±0.011
S. typhi	25±0.012	25±0.016	12.5±0.017	25±0.016	25±0.015	25±0.019	$5*10^{-3}$±0.013
P. aeruginosa	50±0.015	No inhibition	25±0.014	50±0.015	No inhibition	50±0.018	$5*10^{-3}$±0.012

Fraction A exhibited antimicrobial activity against all the test organisms at different concentrations. It exhibited similar level of antimicrobial activity against all the organisms (MIC = 25 mg/mL) except *P. aeruginosa* that exhibited stronger resistance (MIC= 50 mg/mL). Fraction A of the plant can be used as a potential source of antimicrobial drug. The potency of the gentamicin is higher than that of fraction A. Fraction A may also show antimicrobial activity against other microbes that cause a variety of diseases.

Fraction C exhibited antimicrobial activity against all the microbes at different concentrations. The MICs ranged from 6.25 to 25 mg/mL, with S. pyogenes being the most susceptible and *P. aeruginosa* being the least susceptible (Table 9). In fact, fraction C did not show activity against *P. aeruginosa.* The fraction C showed less antimicrobial activity

than gentamycin at all levels, with gentamicin showing activity against *P. aeruginosa* that the fraction C could not inhibit.

Fraction D displays antimicrobial activity against all organisms at various concentrations. The MICs ranged from 12.5 to 25 mg/mL, with C. albicans, S. pyogenes, S. typhi being the most susceptible. The MBCs ranged from 12.5 to 50 mg/mL (Table 9). The MBC values were generally higher than those of the MICs for all organisms except C. Albicans.

Fraction D of the plant can be utilized as a possible source of antimicrobial medication. It might likewise show antimicrobial action on different organisms that cause an assortment of illnesses.

The fractions and the extract exhibited varying degrees of potency against the tested organism. The potency of the extract and the fractions were the same with respect to *E. coli*. With respect to *S. aureus*, fraction C and the extract had the same potency, and this was greater than the potency of fractions A and D which exhibited equal potency. For *S. pyogenes*, fraction C was the most potent, followed by the extract, fraction A, and fraction D, and the least potent being fraction C. The fraction D was more potent against *S. typhi* than the extract, and the extract was more potent than the fraction A and fraction C. Fraction D exhibited the highest potency against *P. aeruginosa*, *followed by* the extract and fraction A, then fraction D, and least was fraction C which no inhibition. Against *C. albicans*, fraction C and fraction D were the most potent, with the extract and fraction A having less potency. Phytochemicals such as tannins, steroids and flavonoids are known to exhibit antimicrobial property.

Table 10: The minimum inhibitory concentration and the minimum bactericidal concentration of fractions C.

Test organisms	MIC (mg/mL) ±SEM	MBC (mg/mL) ±SEM	Gentamycin (mg/mL) ±SEM
C. albicans	12.5±0.011	12.5±0.012	$5*10^{-3}$±0.011
E. coli	25±0.012	25±0.011	$5*10^{-3}$±0.013
S. aureus	12.5±0.014	12.5±0.013	$5*10^{-3}$±0.012
S. pyogenes	6.25±0.015	6.25±0.014	$5*10^{-3}$±0.014
S. typhi	25±0.016	25±0.015	$5*10^{-3}$±0.012
P. aeruginosa	No inhibition	No inhibition	$5*10^{-3}$±0.011

Table 11: The Minimum Inhibitory Concentration and the Minimum Bactericidal Concentration of fraction D.

Test organisms	MIC (mg/mL) ±SEM	MBC (mg/mL) ±SEM	Gentamicin (mg/mL) ±SEM
C. albicans	12.5±0.015	12.5±0.018	$5*10^{-3}$±0.018
E. coli	25±0.013	50±0.012	$5*10^{-3}$±0.015
S. aureus	25±0.017	25±0.012	$5*10^{-3}$±0.011
S. pyogenes	12.5±0.016	25±0.018	$5*10^{-3}$±0.013
S. typhi	12.5±0.017	25±0.019	$5*10^{-3}$±0.013
P. aeruginosa	25±0.014	50±0.018	$5*10^{-3}$±0.011

4. Conclusion

The pharmacological properties of the methanol extract of *A. silphioides* have been assessed, and its phytoconstituents have been screened. The extract was separated into four primary fractions A, B, C, and D using column chromatography in conjunction with qualitative and preparative thin layer chromatography. Bulked fractions A, C, and D also exhibited varying antimicrobial activities against the tested microbes, with bulked fraction C showing no activity against *P. aeruginosa*. Bulked fraction C exhibited a strong anti-helminthic activity against *Eudrilus eugeniae*. GC-MS analysis revealed that bulked fraction A contained 14 phytochemicals including Caryophyllene oxide, 1,5,5,8-tetramethyl-12-oxabicyclo [9.1] dodeca-3,7-diene, methylesterhexadecanoic acid, 9,12-octadecadienioc acid (2,2)-methyl ester, 9- Octadecenamide, squalene, 2-[(1,1-dimethyletheroxycarbonyl)]-a-nitro-2,6-bis(1,1-dimethyl)-4,1-pyrrolidine butadioc acid, cholestane-3,6,7-triol, tocopherol quinone, Amyrin, 12-oleanen-3-yl acetate, Lup-20-en-3-ol acetate, Friedelan-3-one and Phytol acetate. Bulked fraction B contained 13 Phytochemicals including 9-octadecenamide, 1-pyrrolidine butanoic acid, isopropyl linoleate, i-propyl-9,12,15-octadecatrienoate, phytol acetate, a-tocopherol quinone, 10,12,14-nanocosatriynoic acid, rhodopin, a-amyrin, and 12-oleanen-3-yl acetate. 9-octadecenamide was common to both A and B. Bulked fraction C contained 12 phytochemicals including 3,7,11,15-tetramethyl-2-hexadecen-1-ol, 9-octadecenamide, 3-acetoxy-7,8-epoxylanonstan-11-ol, O-methyl- (+)-a-tocopherol, D-galactopyranoside methyl-2,6-bis-O-(trimethylsilyl) cyclic butyl boronate, a-Amyrin, Urs-12-en-3-ol acetate, 7,9-ditert-butyl-1,1-oxapiro (4,5) deca-6,9-diene-2,8-dione. Bulked fraction D contained 11 phytochemicals including 3-hydroxy-3,5,14,20,22,25R-spirost-8-en-11-one, 25-Norisopropyl-9,19-cyclolanostan-22-en-24-one, Trimethyl silylestrone, 5H-cyclopropa [3,4] benz [1,2-e] anzlen-5-one, 1,2,5,5,6,9,10-octahydro-5,5-dihydromethyl Dodecanoic acid, 1,4,7-androstantrin-3,17-dione.

Data Availability

Data, graph, tables and other information is in the manuscript.

Conflict of Interest

The authors whatsoever have no conflict of interest.

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