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Exploring Secondary Metabolites of *Hyptis spicigera* Using Integrated Chromatography and Spectroscopy

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ABSTRACT
It is fashionable to produce insect-resistant plants and use environmentally friendly gardening practices to invite nature into our yards. *Hyptis spicigera* is a Nigerian medicinal plant (NMP) employed to treat headaches and colds, as well as to repel insects. The current study aims to profile bioactive chemicals present in crude extract and fractions using thin layer chromatography (TLC), UV-Vis, FTIR, and GC-MS spectroscopic methods for analysis. The TLC results indicate that the extract and its fractions contain a mixture of phytochemicals with varying Rf values (0.35, 0.4, 0.43, 0.61, and 0.70). The spectroscopic analysis supports the TLC findings by showing that the composition of the plant's aerial parts includes hydrocarbons (alkanes and alkenes), phenols, steroids, alkaloids, glycosides, saponins, flavonoids, and fatty acids. The plants previously reported to have repellent properties may be attributed to the secondary metabolites; therefore, further research on the isolation of pure compounds and toxicological studies could be beneficial in determining the medicinal properties of the plant.

Keywords: TLC, GC-MS, UV-VIS, FTIR, Extracts and Fractions.

INTRODUCTION
Traditional medicine is commonly utilized to treat a variety of ailments. Medicinal herbs are thought to be significantly safer and more effective in the treatment of a variety of diseases. They play an important role in providing primary health care to individuals and communities in many underdeveloped nations. Because of its effectiveness, availability, low cost, and non-toxicity, it is becoming increasingly popular in international trade (Ashis *et al.*, 2003). As a result, analyzing the phytochemical constituents would aid in determining the biological activities of plants.

The purpose of this study was to assess the bio-efficacy and chemical characterization of the repellent capability of crude extract and its fractions. The purpose of this study was to analyze the extracts and fractions using UV-VIS, FTIR spectroscopy, and GC-MS to evaluate the phytoconstituents of the plant that are responsible for its repellency potentials, as it has traditionally been employed as a mosquito repellent (Muhammad *et al.*, 2024).

MATERIALS AND METHODS
Preparation of Plant Sample
The plant material (aerial part) was collected from its natural habitat in the Gumbi area, Wamakko Local Government, Sokoto, Nigeria, and identified at the Herbarium Section, Department of Biological Sciences, Faculty of Chemical and Life Sciences, Usmanu Danfodiyo University, Sokoto. The specimen was prepared and deposited with voucher number A/V ABU 0503. The samples were shade-dried, pulverized into powder, and stored until used.

Extraction of Plant Materials
The pulverized plant material (950 g) was cold macerated with methanol for (time spent for maceration). The solvent was evaporated *in vacuo* using a rotary evaporator at 40 °C to afford a crude methanol extract coded ME. The percentage recovery yield was calculated using the following formula:

$$Percentage\ yield = \frac{weight\ of\ extract}{weight\ of\ plant\ material} \times 100 \ldots\ldots\ldots\ldots\ldots$$

Equation 1

Liquid-liquid fractionation of ME
Crude methanol extract (80.0 g) was dissolved in distilled water (400 cm3) and fractionated with nonpolar (hexane) and polar (n-butanol) to afford HF and BF, respectively. The same procedures were employed for *in vacuo* evaporation of solvents at 400 °C (Kalsi and Jagtap, 2013).

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Thin Layer Chromatography (TLC) Analysis of ME and Its Fractions

Thin layer chromatography analysis of ME and its fractions was conducted using TLC pre-coated plates (Silica gel 60F254) by one-way ascending methods. Spotting was done manually using capillary tubes and developed (Hexane: Chlorophone 1:1) in an airtight chromatographic tank at room temperature with a mobile phase. The chromatographs were air dried and viewed under normal daylight, ultraviolet light (254 and 366 nm), and by spraying with 10% sulfuric acid followed by heating in an oven at 105 °C for 10 minutes.

UV-VIS spectroscopic analysis of ME

The ME was centrifuged at 3000 rpm for 10 minutes and filtered using filter paper. The sample was diluted 1:10 with the same solvent. The extract was scanned from 190 to 1100nm wavelength using a UV-visible spectrophotometer (Shimadzu UV-1800), and the distinctive peaks were discovered and recorded (Dhivya *et al.*, 2017).

Fourier Transform Infrared Spectroscopic Analysis of ME and Its Fractions

The crude ME's dried powder and its fractions (HE and BF) were subjected to FTIR analysis. To create the sample disc, 0.1g of dry ME and its fractions (HE and BF) were premixed with a potassium bromide (KBr) pellet and pressed into an FTIR spectrophotometer (Agilent) disk. The IR radiations were allowed to pass through the disk and scanned between 4000 and 600 cm^{-1}. Each sample was averaged from 200 scans with a spectral resolution of 4 cm^{-1}. It took three minutes to record. Then, for the specified materials, a final average spectrum was computed (Kumar and Ramaswamy, 2014).

GC–MS Analysis of ME and Its Fractions

The dried ME and Butanol fractions were dissolved in Methanol, while HE was dissolved Haxane and analyzed using a GC-MS model QP2010 plus SHIMADZU with a detector and slit injection system. The temperature was initially set to 60 °C for 3 minutes before progressively increasing to 250 °C. For analysis, 1.6µL of solution was injected at a temperature of 250 °C during the experiment. Hellium gas (99.999%) was used as the carrier gas, with a constant flow rate of 1ml/min and an injection volume of 2l (split ratio of 10:1); injector temperature 2500 °C, ion-source temperature 2800 °C. The oven temperature was programmed from 1100 °C (isothermal for 2 minutes) with an increase of 10 °C/min at 2000oC, then 50 °C/min at 2800 °C, and ending with a 9-minute isothermal at 2800 °C. Mass spectra were taken at 70 eV, with a scan interval of 0.5 seconds and fragments ranging from 45 to 450 Da. The total GC running time was 36 min. Each component's relative percentage amount was computed by dividing its average peak area by the overall area. The program used to handle mass spectra and chromatograms was Turbo Mass. The identification of components was based on comparison of their mass spectra with those present in the National Institute of Standards and Technology computer data bank (NIST 2009 Library)

RESULTS AND DISCUSSION

The results of TLC analysis of ME and its fractions at different solvent systems, separating the compounds at different spots (Rf) values and absorbance (Figure 3 and Table 1).

Table 1: Summary of the TLC analysis of ME and its fractions

Fractions	Mobile phase	Ratio	Major sports
Hexane	Hex: Chlo	3:1	5
n-butanol	But:Ac:H_2O	4:1:5	4
Crude Extract	Ch:EA	1:1	7

The findings of this study provide significant support for preliminary phytochemical screening of the fractions and crude extract. The appearance of the various colored spots on the TLC chromatogram suggests that the fractions (Hexane and n-Butanol) and crude extract of *Hyptis spicigera* contain a variety of components, and therapeutic uses may be due to the secondary metabolites present. Plant biological activity has been attributed to bioactive components such as alkaloids, saponins, tannins, flavonoids, steroids, and so on (Odugbemi 2006).

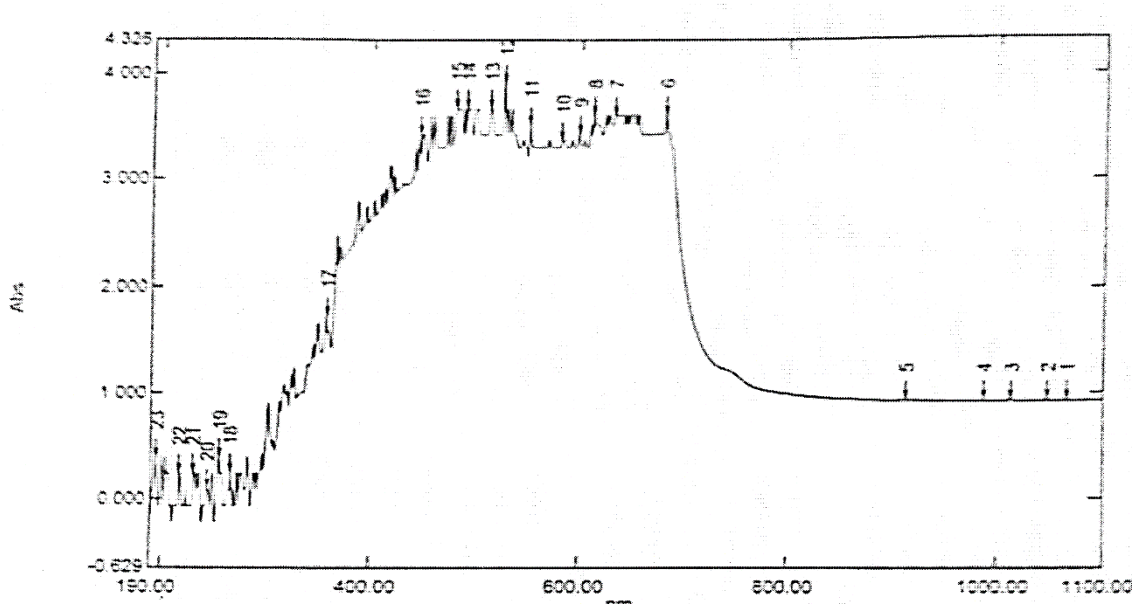

Figure 1: UV/Vis spectrum of methanol extract

The UV-VIS spectrum for methanol extract shows twenty-four (24) peaks at different wavelengths (nm) with corresponding absorption values as presented in Table 1

Table 2: UV – VIS Spectrum Peak Values of Methanol Crude Extract

S/No	Wavelength (nm)	Absorption value
1.	1055.00	0.922
2.	1045.00	0.921
3.	1022.00	0.913
4.	1014.00	0.929
5.	999.00	0.919
6.	914.00	0.924
7.	682.00	3.512
8.	632.00	3.512
9.	612.00	3.512
10.	597.00	3.435
11.	551.00	3.324
12.	550.00	3.524
13.	525.00	3.913
14.	511.00	3.515
15.	490.00	3.575
16.	479.00	3.675
17.	444.00	3.236
18.	355.00	3.710
19.	258.00	0.235
20.	257.00	0.355
21.	245.00	0.088
22.	232.00	0.236
23.	216.00	0.235
24.	195.00	0.355

The qualitative UV-VIS spectrum profile of the methanol extract of *Hyptis spicigera* was chosen at a wavelength of 190 to 1100nm due to the sharpness of the peaks and the correct baseline. The UV-VIS spectrum of this plant methanol extract showed peaks at 216, 355, 444, 479, and 490nm, with absorption values of 0.235, 3.710, 3.436, 3.675, and 3.573, respectively. According

to prior research, these absorption bands are associated with phytochemicals such as alkaloids, saponins, terpenoids, flavonoids, and phenols. Our findings are likewise consistent with the aforementioned investigations. Kalaichelvi and Dhivya (2017) discovered two prominent absorption peaks in the UV-VIS spectra of terpenoids, flavonoids, and related glycosides at 230-290nm (band I) and 400-550nm (band II). Chlorophyll spectra typically consist of two absorption maxima in the 600-700nm region, which is consistent with our results given in Table 1. Characterization of secondary metabolite fingerprints by chromatography and spectroscopy provides useful information about qualitative and quantitative formulation of the plant species and their pattern of recognition by chemometry. Table 3 shows the findings of Butanol extract UV-VIS analysis, which indicated a total of thirty-two (32) peaks at different wavelengths in the following ranges: 200-300nm, 400-600nm, 600-700nm, and 800-1100nm.

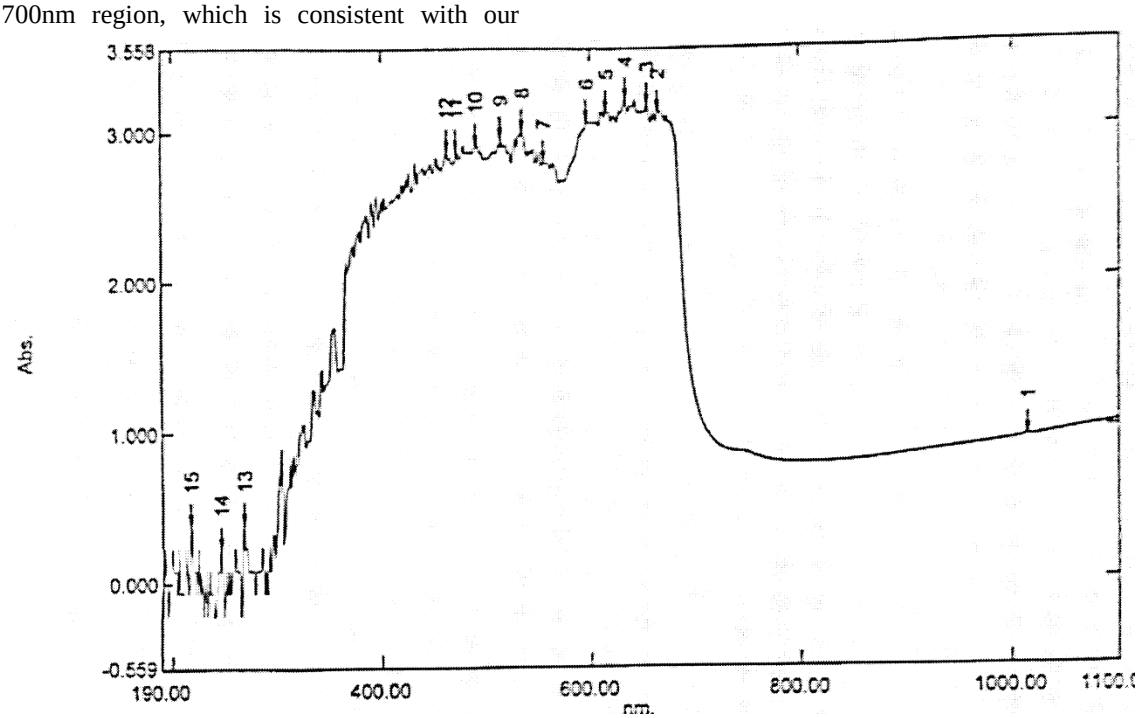

Figure 2: UV/Vis Spectrum of Butanol fraction

Table 3: UV-VIS Spectrum Peaks Values of Butanol Fraction.

S/No	Wavelength (nm)	Absorption value
1.	1020.00	0.941
2.	1016.00	0.945
3.	805.00	0.779
4.	665	3.135
5.	658	3.085
6.	654	3.175
7.	645	3.135
8.	633	3.125
9.	625	3.065
10.	615	3.135
11.	608	3.039
12.	596	0.068
13.	569	2.799
14.	554	2.783
15.	552	2.783
16.	534	3.010
17.	524	2.799
18.	514	2.960
19.	496	2.834

20.	489	2.914
21.	473	2.834
22.	471	2.874
23.	466	2.791
24.	462	2.872
25.	288	0.066
26.	273	0.055
27.	269	0.356
28.	250	0.216
29.	245	0.235
30.	240	0.215
31.	215	0.356
32.	204	0.065

Table 2 shows the results of the butanol fraction obtained after dividing the methanol extract. The fraction's UV-VIS spectrum consists of four wavelength ranges: 200-300nm, 400-550nm, 600-700nm, and 800-1100nm. Similar phytochemicals were found in Butanol extract, as reported by Joshi et al (2012), as flavonoid and related glycosides are mostly observed in the 230-290nm absorption maxima. Mamta and Jyoti (2012) also reported similar observations. Our findings in the second range of 400-550nm were consistent with those published by Renuka et al. (2016) and Neha and Jyoti (2013). Appearance of peaks at 600-700nm absorption maxima was attributed to chlorophyll in the plant extract, which is also in line with the results reported by Neha and Jyoji (2013), Renuka *et al.* (2016). A total of seventeen (17) peaks were recorded for UV-VIS spectra of the Hexane fraction that correspond to different wavelengths, which indicate that the fraction contains a mixture of different phytoconstituents.

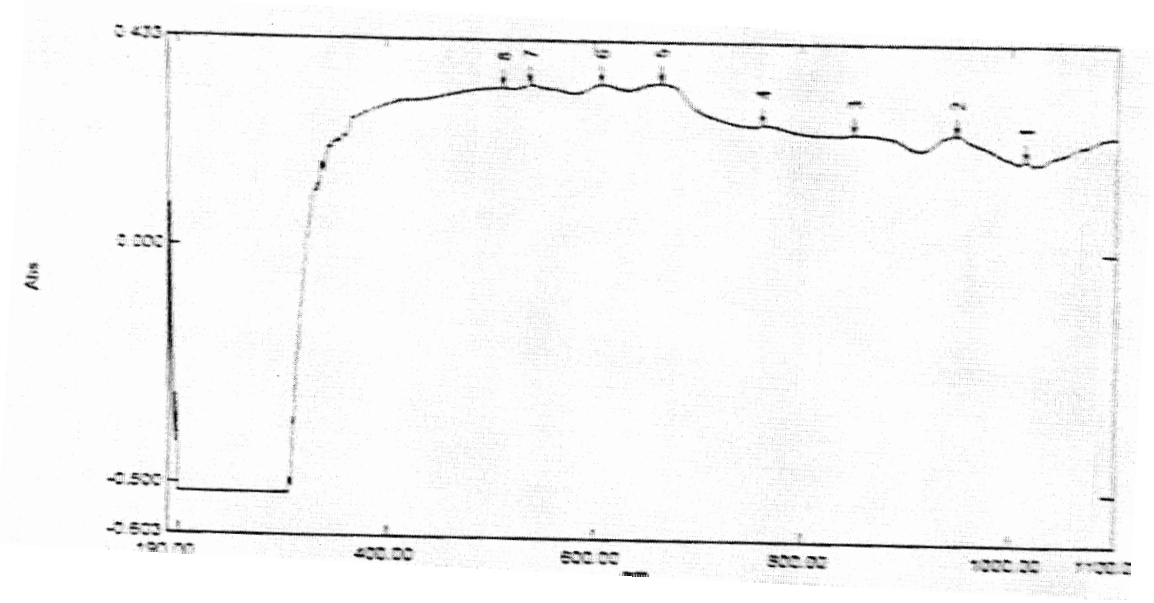

Figure 3: UV/Vis spectrum of Hexane fraction

Table 4: UV-VIS Spectrum peak values of Hexane Fraction

S/no	Wavelength (nm)	Absorption value
1.	1022.00	0.874
2.	1006.00	0.823
3.	814.00	0.214
4.	811.00	2.334
5.	782.00	7.288
6.	755.00	0.283
7.	654.00	2.347

8.	631.00	2.223
9.	607.00	2.321
10.	576.00	2.225
11.	551.00	2.257
12.	538.00	2.346
13.	527.00	2.532
14.	358.00	0.254
15.	324.00	0.244
16.	283.00	0.274
17.	220.00	0.246

The Hexane fraction UV-VIS spectrum profile revealed peaks at 283nm, 324nm, and 358nm, with absorption values 0.274, 0.244, and 0.254, respectively, corresponding to saponins and terpenoids. Other peaks were identified in the 500–1100 nm range, which could be attributed to alkaloids, phenols, and glycosides. Previous studies that used the UV-VIS technique to validate the presence of various phytoconstituents from different regions coincide with our findings because the peaks and absorption were in the same range as reported by Kalaichelvi and Dhivya (2017). Identification of different functional group in the methanol extracts has clearly revealed the presences of secondary metabolite in the aerial part of the plant under study as shown in Table 4.

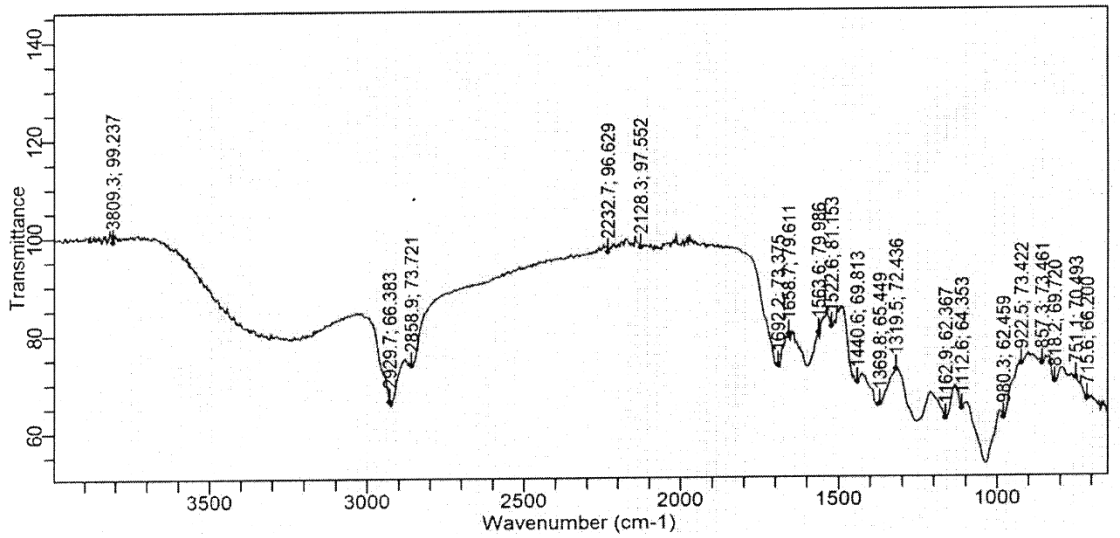

Figure 4: FTIR spectrum of methanol extract

Table 5: FTIR Results for Methanol Extracts (MET)

Frequency of literature (cm^{-1})	Frequency of sample (cm^{-1})	Functional group	Assignment
3000-3900	3809.3	Alcohol	OH Stretching
2990-2850	2929-7 & 2858.9	Alkane	C-H Stretching
2280-2200	2232.7	Nitriles	C=N Stretching
1700-1665	1692.2 & 1650.8	Amides	C=O Stretching
1570-1490	1563.6 & 1522.6	Alkene	C=C Stretching
1400-1450	1440.6	Aromatic compounds	C-C Stretching
1390-1300	1369.6	Nitro compounds	NO_2 Stretching
1400-1000	1162.9 & 1112.6	Alkyl & Aryl Halide	C-F Stretching
800-700	751.1 & 715.6	Aliphatic Organohalogen compound	C-CL Stretching

UV/VIS and infrared spectroscopies have proven to be useful methods for characterizing and identifying chemicals, functional groups, and chemical bonds in an unknown combination of plant extracts. They are employed as qualitative instruments to identify and characterize molecular species or some of their qualities, such as molecular structure and characteristic absorption spectra, which serve as fingerprints for their unique characteristics. The spectral data of the methanol extracts indicate sharp absorption peaks at 2929.7 and 2858.9 cm-1, which are caused by symmetric stretching of saturated carbon (C-H) in aliphatic alkane molecules. The bands at 751.1 and 715.6 cm-1 correspond to aliphatic organohalogen chemicals (C-Cl). Similar peaks were found at 1162.9 and 1112.6 cm-1, corresponding to alkyl and aryl halides (C-F) stretching. Another unsaturated absorption peak at 1650.8 cm-1 represents a C=C stretching bond. According to the literature, a peak between 1563.6 and 1522 cm-1, which falls between 1700 and 1665 cm-1, corresponds to a C=O amide absorption band defining esters or carboxylic acids. Peaks at 1440.6, 1522.6, and 1563.6 cm-1 appear within a frequency range of 1570-1440 cm-1 due to C=C stretching vibrational bands for aromatic molecules. The methanol extracts were also discovered to include Nitro compounds (NO_2) with a vibrational frequency of 1369.8cm-1. Again, an absorption broad peak develops at 3809.3 cm-1, which is ascribed to OH stretching in the alcoholic compounds band.
The principal functional groups observed in butanol fraction spectra are C-H, C=C, C=O, C-N, C-F, and aromatic compounds, which could indicate the presence of alkaloids, saponins, flavonoids, and terpenoids.

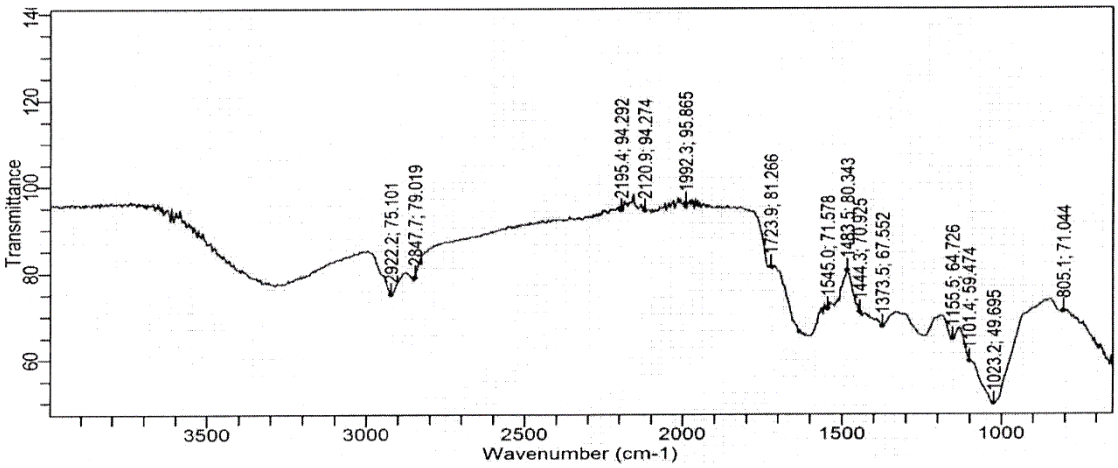

Figure 4: FTIR spectrum butanol fraction

Table 6: FTIR Results for Butanol Fraction (BF)

Frequency of literature (em^{-1})	Frequency of sample (cm^{-1})	Functional group	Assignment
2990-2850	2922.6	Alkane	C-H Stretching
2900-2800	2847.7	Alkane	C-H Stretching
1740-1720	1723.9	Aldehyde	C=O Stretching
1625-1440	1545.0	Aromatic compounds	C=C Stretching
1485-1445	1483.5	Alkane (CH_2)	C-H Bending
1470-1430	1444.3	Alkane (CH_3)	C-H Bending
1390-1300	1373.5	Alkyl group	C-H Medium
1190-1130	1155.5	Secondary amino	C-N stretching
1090-1020	1023.2	Amines	C-N stretching
1150-1000	1101.4	Aliphatic organohalogen compounds	C-F Stretching
900-680	805.1	Aromatics compounds	C-H Bending

Table 6 shows the findings of the FTIR investigation, which revealed various absorption peaks. If the sample has a simple spectrum (less than five (5) absorption bands), the substances analyzed are simple organic compounds, tiny molecular weight, or inorganic compounds; nevertheless, if the FTIR spectrum has more than five (5) absorption bands, the sample may be a complex molecule. The greatest absorption band was found at 2922.6 cm^{-1}, representing C-H stretching for aliphatic molecules, whereas the peak at 2847.7 cm^{-1} (C-H) is for alkanes. Other peaks that followed include 1483.5 cm-1 and 1444.3 cm-1 for C-H. Aliphatic compounds' bending vibration, whereas aromatic compounds' (C-H) bending at 805.1 cm^{-1}. Another absorption vibration for aromatic compounds (C=C) was identified at 1545.0 cm^{-1}, whereas stretching C=O for aldehyde appeared at 1723.9 cm-1. The band 1373.5 cm^{-1} corresponds to a methyl group (CH3) with medium absorption intensity for a bending vibration. Absorptions described for C-N bond stretching may result in noticeable bands at 1155.5 and 1023.2 cm^{-1}. The signal at 1101.4 cm^{-1} indicates aliphatic fluorocarbon compounds (C-F) stretching. The existence of several secondary metabolites in the extract might be attributed to the distinct functional groups detected in the Hexane fraction, which may contribute to its insect repellent properties, as indicated in Table 6 below.

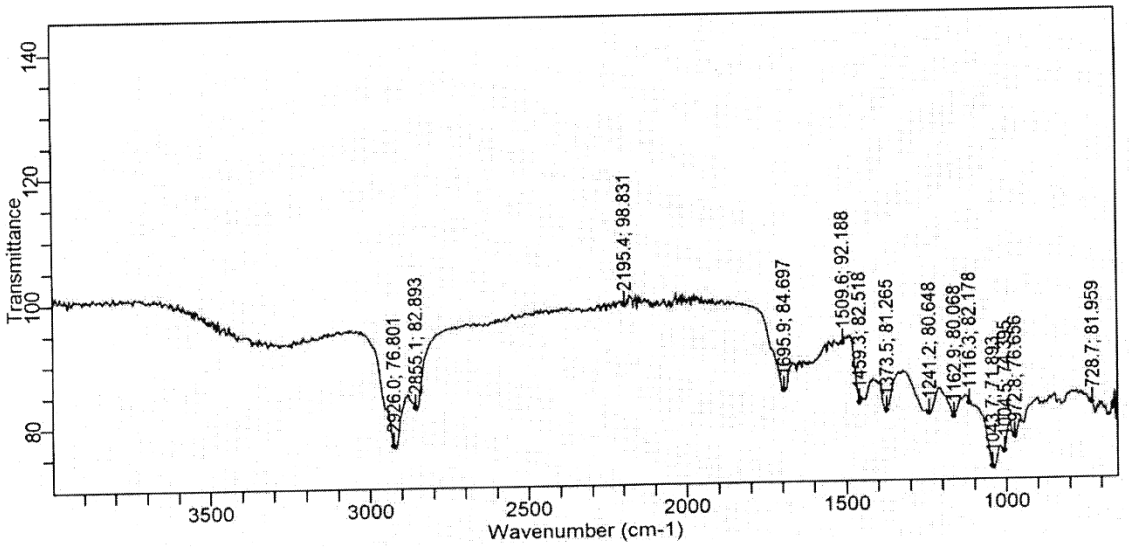

Figure 4: FTIR spectrum hexane fraction

Table 7: FTIR Results for Hexane Fraction (HF)

Frequency of literature (em^{-1})	Frequency of sample (cm^{-1})	Functional group	Assignment
2990-2850	2926.0 & 2855.1	Alkane	C-H Stretching
1700-1665	1695.9	Ketone	C=O
1625-1440	1509.6	Aromatic Compounds	C=C
1485-1445	1459.3	Alkane (CH2)	C-H
1380-1370	1373.6 & 1241.2	Aliphatic Alkane	C-H
1190-1130	1162.9	Secondary Amino	C-N
1150-1000	1116.3,1043.7 & 1004	Aliphatic Organohalogen Compound	C-F stretching
800-700	728.7	Aliphatic Organohalogen Compounds	C-cl Stretching

The FTIR results of the hexane fraction revealed the presence of various functional groups that are similar or nearly identical to those reported in methanol or butanol extracts. Similarly, all absorption frequencies are tightly connected.

The functional groups found in crude methanol extracts include alcohol, alkane, alkene, amides, nitriles, nitrogen compounds, and aliphatic organohalogens. However, the Butanol fraction was discovered to contain Alkane, Aldehyde, Amino groups, Amines, and Aliphatic organohalagen Compounds. Similarly, Hexane has shown the presence of alkanes, ketones, amino groups, aromatic compounds, and aliphatic organohalogen compounds. All of these substances are plant secondary metabolites, as proven by an FTIR spectrophotometer analysis that predicted the existence of C-H, OH, C=O, C=C, C=C, C-F, Nitro groups, and nitrile stretching. Based on FTIR results obtained from methanol and its fractions, it has been determined that the extracts include complex compounds, as all spectra exhibit more than five peaks (Nandeyanto *et al.*, 2019). As reported in Lingegowda *et al.* (2012). Based on the observations, all of the spectra showed no absorption peaks between 2220-2260cm^{-1}, suggesting the lack of cyanide groups in the extract and fractions, revealing that the plant phytochemical ingredients contain no harmful compounds.

Table 8 shows the findings of GC-MS analysis of the *Hyptis spicigera* crude ME and its fractions. In methanol extracts, eight (8) chemical compositions were detected, whereas the n-butanol fraction included nine (9) compounds, and the hexane fraction had six.

Table 8: Results for GC-MS Analysis for Crude ME, BF, and HF Fractions.

Phytoconstituents	Crude ME (%)	BF (%)	HF (%)
Alcohol	7.21	1.06	2.88
Alkaloids	5.71	4.28	3.15
Alkane	7.18	8.98	9.30
Steroids	10.82	46.92	5.59
Ketone (flavonoids fragment)	9.05	0.99	14.87
Phenols	5.53	1.67	ND
Fatty acids	50.49	30.79	64.20
Glycosides	4.01	2.17	ND
Phosphine	ND	2.17	ND

ND = Not detected

The chemical composition of the methanol extracts, n-butanol, and hexane fractions was determined by GC-MS analysis. The methanol extract and Hexane fraction were found to contain 50.49 and 64.20% fatty acids, respectively, while the n-butanol fraction had 30.79% fatty acid as the major phytochemical molecule. The n-butanol fraction contained 46.92% steroids, making it the most abundant phytochemical, whereas methanol and hexane contained 10.82% and 5.59%, respectively. A fragment suspected of containing flavonoids was found to be 9.05, 0.99, and 14.87% in the methanol extract, n-butanol, and hexane fractions, respectively. This indicates that the hexane fraction has the highest flavonoid percentage fragment composition, followed by the methanol extracts. Other substances found in the extracts and fractions include alcohols, alkaloids, and alkanes.

However, phenols and glycosides were discovered in both the methanol and n-butanol fractions, while phosphine (2.17%) was exclusively found in the n-butanol fraction. The higher percentage fatty composition of crude extracts and fractions was consistent with earlier studies, indicating that the plant or extract had repellency potential, as reported by Haruna *et al.* (2019). Many plants include phytoconstituents such as alkaloids, flavonoids, phenolic compounds, and essential oil (fatty acid), which have insect repellent properties. This plant's secondary metabolites generated an unpleasant and irritating odor to insects. Which can deter insects from eating food. The plant extracts are used in crude and commercial formulation to repel, kill and disturb the development of insects. In line with forgoing plant extracts are considered as ecofriendly source they are relatively safe and inexpensive as well do not persist in the environment like synthetic pesticides, similarly the plant extracts have minimal effects on non-target organisms. Some non-toxic glycosides can be hydrolyzed into phenolic compounds that can repel insects; hence the repellent effect of this plant may be attributable to the presence of these secondary metabolites (glycosides).

CONCLUSION

In this study, methanol extracts and their fractions were analyzed using thin layer chromatography (TLC), UV-Vis, FTIR, and GC-MS spectroscopic methods. TLC results indicate how many phytoconstituents are present in plant materials. The methanol extracts include seven (7) spots with varying Rf values, displaying a combination of chemicals, whereas hexane and butanol yielded five (5) and six (6), respectively. UV-visible and FTIR spectroscopy revealed the presence of alkanes, alkenes, amides, alcohols, aliphatic organohalide compounds, aldehydes, amines, and ketones. Gas chromatography mass spectroscopy (GC-MS) is a useful method for safely identifying phytocompounds. The GC-MS results confirm the presence of Alcohol, Alkaloids, Alkane, Steroids, flavonoids, and fatty acids in the crude extracts and their fractions. Phenols and Glycosides were identified in methanol and Butanol extracts, while phosphine was only observed in Butanol fractions. It can be concluded that the phytoconstituent identified could be responsible for the repellency potentials of the plant materials' aerial parts.

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Declaration of Competing Interest

The authors are hereby declared no conflicting and financial problem that affect the quality of this work.

Author Contributions

All authors in this work contributed equally to this work and thus share the same credit of the publication

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