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Research Article

Phytoconstituents and Antimycobacterial Activities of Root Extracts and Fractions from *Vernonia glabra*, (Steetz) Vatke

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Vernonia glabra is used by Malawian traditional healers to treat a myriad of ailments including shingles, diabetes, dysentery, diarrhoea, cough, wounds, gonorrhoea, and gastrointestinal problems. This study evaluated phytoconstituents and the *in vitro* antimycobacterial activities of the crude extracts and fractions from roots of *Vernonia glabra*. Agar disk diffusion and modified broth microdilution assays were used to determine the antimycobacterial activities of dichloromethane (DCM) and ethyl acetate (EtOAc) crude extracts and fractions from the roots. Gas chromatography-mass spectrometry (GC-MS) and Fourier-transform infrared spectroscopy (FTIR) analyses were used to characterise the major compounds in the crude extracts and fractions. The EtOAc fraction exhibited the highest antimycobacterial activity against *Mycobacterium tuberculosis* H37Rv. The EtOAc fraction achieved a minimum inhibitory concentration (MIC) of 4.88 μg/mL and a half-maximal inhibitory concentration (IC_{50}) value of 38.37 μg/mL, whereas gentamycin, a standard drug, achieved MIC and IC_{50} values of 3.13 μg/mL and 3.88 μg/mL, respectively. GC-MS and FTIR analyses of the EtOAc fractions revealed the presence of hexadecenoic acid, methyl ester (0.39%), diisooctyl phthalate (24.56%), oxalic acid, allyl undecyl ester (4.66%), *bis*(2-ethylhexylphthalate) (5.41%), octacosane (51.12%), tetradecane (12.63%), phenol, 3,5-*bis*(1,1-dimethylethyl) (0.07%), and 2-hydroxy-4-methylbenzaldehyde (6.47%). The identified compounds are reported in the literature to possess antimicrobial and anticancer activities. It was evident from the comparison of the hill slope (HS) values of the dose-response curves of the fraction (−1.005) and its crude extract (−1.322) that repeated fractionation improved the activity. Therefore, this study showed that the roots of *Vernonia glabra* present a potential source of new leads for antimycobacterial drugs.

1. Introduction

Vernonia glabra is a perennial glabrous herb that grows up to 1.2 metres high and is widespread in abandoned cultivation in Malawi. Various parts of the plant are reported to contain alkaloids, flavonoids, terpenoids, saponins, steroids and phytosterols, quinones, and sapogenins and have bioactivity against *Escherichia coli, Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, and *Aspergillus niger* [1, 2]. In ethnomedicine, *Vernonia glabra* has been used for the treatment of diabetes, wounds, gonorrhoea, dysentery, cough, diarrhoea, gastrointestinal problems, and HIV opportunistic ailments such as shingles in countries like Malawi and Kenya [2].

HIV infection weakens the body's immune system, increasing the risk of opportunistic infections [3, 4]. Tuberculosis (TB) is one of the common diseases among people living with HIV [3–5]. TB is caused by *Mycobacterium tuberculosis* and commonly attacks the lungs (pulmonary TB); however, other organs such as the brain, kidney, or spine may also be attacked (extrapulmonary TB). Globally, about 1.8 billion people are infected with TB *bacilli*, and one person dies from this disease every 18 seconds and nearly 1.5 million died in 2020 [5]. People living with HIV are more

susceptible to new TB infections and increased progression from latent TB infection to active TB disease [4]. For example, in Malawi, TB prevalence stands at 363 per 100,000 population, with 53% of the TB patients being HIV-positive [4]. In addition, Malawi is one of the countries that have registered multidrug-resistant TB (MDR-TB) among HIV-positive patients [4]. Further, the COVID-19 pandemic has exacerbated the death toll from TB (including HIV-positive population), thereby negating the progress made towards lowering the number of TB deaths worldwide [4, 6, 7]. In this context, the present study evaluated the antimycobacterial activities against *Mycobacterium tuberculosis* and characterised the phytoconstituents of crude extracts and fractions from roots of *Vernonia glabra*, which are used by Malawian traditional healers to treat HIV opportunistic infections. *Mycobacterium tuberculosis* was selected based on its public health relevance as ethnomedical data from the Malawian National AIDS Commission put it in the group of HIV/AIDS opportunistic infections, and it is one of the organisms to have drug-resistant strains [4, 8].

2. Materials and Methods

2.1. Chemicals and Reagents. Analytical grade chemicals were used in this study. Organic solvents: n-hexane, DCM, EtOAc, and MeOH and reagents: barium chloride dihydrate, sulphuric acid, and nutrient agar were all purchased from LAB ENTERPRISES Ltd, Blantyre, Malawi. Double distilled water was also used and was obtained from the Department of Chemistry of the University of Malawi.

2.2. Collection of Plant Materials and Preparation of Extracts. The fresh roots of the *Vernonia glabra* plant were collected on 27 October 2017, at the slopes of Zomba Mountain, 15°23.72′ S, 35°18.46′ E. The plant was identified by a taxonomist at the National Herbarium and Botanical Gardens of Malawi, and a voucher specimen of the plant was deposited at the institution for reference purposes (voucher specimen number 4693). The collected samples were cleaned and air-dried under shade for 10 days. The dried plant materials were then crushed using a mortar and pestle and ground into a fine powder using a blender and an electric miller. The powdered samples were kept in polyethylene bags for analysis.

The fine powder was then subjected to successive solvent extraction for 48 hours for each solvent using a 1 : 5 w/v ratio. Five different solvent systems, namely, normal hexane (n-hexane), dichloromethane (DCM), ethyl acetate (EtOAc), methanol (MeOH), and water were used in the increasing order of polarity (Figure 1). The crude organic extracts were filtered using the Whatman No.1 filter paper and concentrated by evaporation under reduced pressure using a rotary evaporator at a temperature of 40°C, while aqueous extracts were freeze-dried. The residue was weighed using an analytical balance (Sartorius) and stored at −10°C.

2.3. Bioactivity Assays. The *Mycobacterium tuberculosis* H37Rv strain used in this study was purchased from

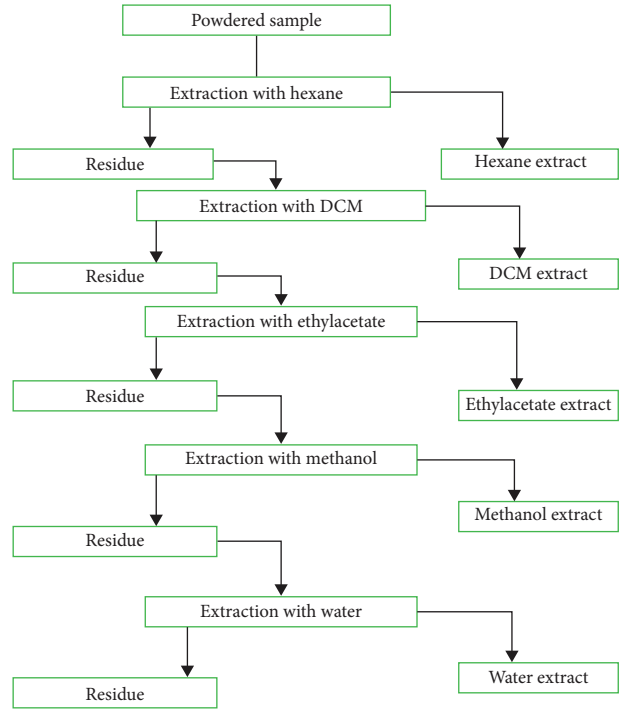


Figure 1: Flow diagram showing the extraction procedure.

American Type Culture Collection (Manassas, Virginia). The concentration of *M. tuberculosis* suspension was adjusted to 550,000 colony forming units (CFU) m·L^{-1}. This was performed with reference to a 0.5 McFarland turbidity standard that was prepared by the addition of barium chloride dihydrate (1.175% w/w, 0.05 mL) to sulphuric acid (1% v/v, 9.95 mL) and analysed using an Oakton turbidity meter. The *M. tuberculosis* strain from the stock was streaked onto nutrient agar plates, and the inoculated plates were incubated overnight at 37°C. A sterile loop was used to transfer a small portion of the subculture into a test tube containing nutrient broth and incubated at 37°C until the growth reached the log phase. Nutrient agar media seeded with standard inoculum suspension were poured into sterile Petri dishes and allowed to solidify [9].

The antimycobacterial assay was performed in triplicates using a modified agar disc diffusion method described by Ahmed et al. [9]. Sterile Petri dishes containing solidified media supportive of *Tuberculosis bacillus* were inoculated with microbial suspension (50 μL). Sterile paper discs (5.9 mm diameter) made from the filter paper (Whatman No. 5) previously soaked in each extract or fraction (1.25 mg/mL) were placed on the media surface. The concentration (1.25 mg/mL) of extracts or fractions was selected after a series of trials to determine a concentration of the crude extracts that would show significant potency. Discs impregnated with gentamycin (1 mg/mL) and pure solvents were used as positive and negative controls, respectively. The Petri dishes were then incubated at 37°C for 72 hours. The first observation was carried out 12 hours after incubation. The growth inhibition zone diameter was measured at 12, 24, 48, and 72 hours of incubation.

The values of minimum concentration required to inhibit the growth of *Tuberculosis bacillus*, minimum inhibitory concentration (MIC), and the half-maximal inhibitory concentration (IC_{50}) for each plant extract and fraction that showed antimycobacterial activity were determined using the spectrophotometric broth microdilution method [10, 11], with some modifications. There is no specific MIC value reported as the reference point for analysing antimicrobial activity because MIC values are not transferable among test chemicals even for the same susceptible microbe [11]. For this study, the Clinical and Laboratory Standards Institute (CLSI) and World Health Organization (WHO) criteria were used for interpretation. Gentamycin, one of the drugs used in the treatment of Gram-negative infections [12], was used as a reference drug in this study because *M. tuberculosis* is more related to Gram-negative bacteria [13].

A loopful of the *M. tuberculosis* culture was inoculated in nutrient broth and incubated at 37°C ± 1°C for 24 hours. Each of the crude extracts and fractions was dissolved in double distilled water and then diluted with the same to prepare a solution of 10 mg of extract/1 mL of water. Then, each extract (500 μL) was diluted with sterile nutrient broth (500 μL) in an Eppendorf tube making an extract dilution of 5 mg/mL. Then, each crude extract (125 μL) was diluted with sterile nutrient broth (125 μL) in an Eppendorf tube making an extract dilution of 2.5 mg/mL. This extract dilution (100 μL) was serially diluted in two folds with the sterile nutrient broth from a concentration of 1.25 mg/mL to 0.078125 mg/mL for DCM crude extract and one EtOAc root fraction. The same extract concentration was serially diluted with the sterile nutrient broth up to a concentration of 0.004882 mg/mL for crude EtOAc root extract and another EtOAc root fraction, and these were each placed in wells of 96-well ELISA trays.

After incubation for 24 hours at 37°C ± 1°C, *M. tuberculosis* culture (550,000 CFU/mL, 50 μL) was added to each well containing the serial dilutions. The absorbance of each well was determined using an ELISA tray reader adjusted to 540 nm (Multiskan FC). All plates were incubated at 37°C, and growth was estimated spectrophotometrically after 24 hours; the absorbance was read again. The difference between the absorbance before and after incubation was calculated, and microscopic visualisation of the wells was also carried out. GraphPad Prism v.8.00 software was used to obtain the IC_{50} values from the dose-response curves, using nonlinear dose-response curve fitting analyses [14].

2.4. Chromatographic Fractionation. The EtOAc crude extract was chosen for fractionation using column chromatography because it exhibited the highest bioactivity against *M. tuberculosis*. Wet packing of silica gel 60 (Merck) (130 g) in a 40.0 cm × 4.0 cm column was performed using a solvent mixture of n-hexane and EtOAc in a 6 : 4 ratio as eluent. The column was equilibrated before packing the sample by passing the same solvent system. Crude ethyl acetate extract (5.641 g) was dissolved in a small volume of EtOAc and then adsorbed onto silica gel 60 (12.0 g), dried, and loaded on top of the column. Gradient elution was used starting with n-hexane and increasing the polarity up to 100% EtOAc. Fractions of 20 mL were collected into test tubes placed in a rack. Thin-layer chromatography (TLC) was used to combine fractions with the same retardation factor (R_f) values, and the solvent was evaporated using a rotary evaporator. The fractions obtained were kept for the antimicrobial test.

2.5. Chemical Analysis of Active Crude Extracts and Fractions. Identification of functional groups and compounds available in the active fractions was performed using Fourier-transform infrared spectroscopy and gas chromatography-mass spectroscopy, respectively. FTIR (IR Prestige-2, Fourier-transform infrared spectrophotometer, Shimadzu) was used to identify bond types and the presence of functional groups of the compounds present in the active crude extracts and the two fractions. The sample discs were impregnated with each of the crude extracts (0.5 mL) and fractions (0.5 mL) for FTIR analysis. The scan range was 450–4500 cm^{-1}.

Hyphenated gas chromatography-mass spectroscopy was used to identify and quantify the compounds present in active fractions. The mass spectra of the compounds were matched with the spectra of the National Institute of Standard and Technology (NIST) library database using MS search v2.3 and AMDIS. EtOAc solvent blank (1 μL) was first injected into the GC-MS machine before sample injection was performed. After analysis of the blank sample, each of the fractions (1 μL) was separately analysed in the same manner. The analyses were carried out using a GC-MS model (Agilent Technologies 5975C inert XL EI/CI MSD with Triple-Axis Detector, 7890A GC system). The column type was HP-5MS 5% Phenyl Methyl Silox with measurements 30 m length × 250 μM diameter × 0.25 μM film thickness. It operated with an ionization energy of 70 eV, and the carrier gas was helium (99.999%) with a flow rate of 0.98736 mL/min; a split ratio of 10 : 1 was used. An injector temperature of 250°C and an ion-source temperature of 280°C were used. The oven temperature was set from 110°C (isothermal for 4 minutes), with an increase of 10°C/minute, to 220°C, then isothermal for 10 minutes, and raised to 300°C at 4°C/minute, ending with isothermal at 300°C for 9 minutes. The total run time was 54 minutes with a scan interval of 0.5 s, fragments from 50 to 550 amu. GC-MS match factors were used to identify the compounds available in the fractions using the NIST library of the GC-MS model. According to the NIST guidelines, a match factor of 900 and above is excellent, 800–900 is good, 700–800 is fair, and lastly <600 is poor [15]. ChemDraw software was used to draw the structures of compounds.

2.6. Statistical Analysis. The mean and percent yields of the crude extracts were computed using GraphPad Prism v.8.00 software. GraphPad Prism was also used to calculate averages of the zone of inhibition diameters and to determine the MIC and IC_{50} values of the crude extracts and fractions, using descriptive statistics. Each parameter was tested in triplicate, and the results were expressed as the mean ± standard error of the mean. Origin2019 software was used to plot FTIR and GC-MS data.

Table 1: Extraction yields of *V. glabra* roots.

Solvent	Average yield (g)	% Yield
Hexane	0.718 ± 0.0096	0.48
Dichloromethane (DCM)	9.297 ± 0.0044	6.20
Ethyl acetate (EtOAc)	19.30 ± 0.0018	12.87
Methanol (MeOH)	16.40 ± 0.00088	10.93
Water	13.98 ± 0.042	9.32

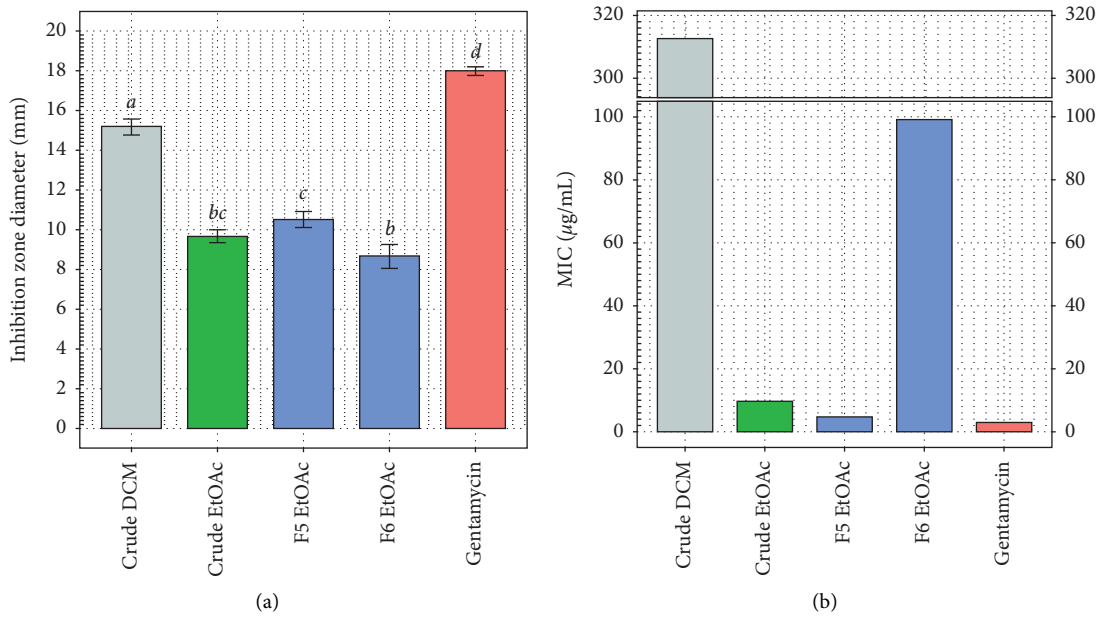


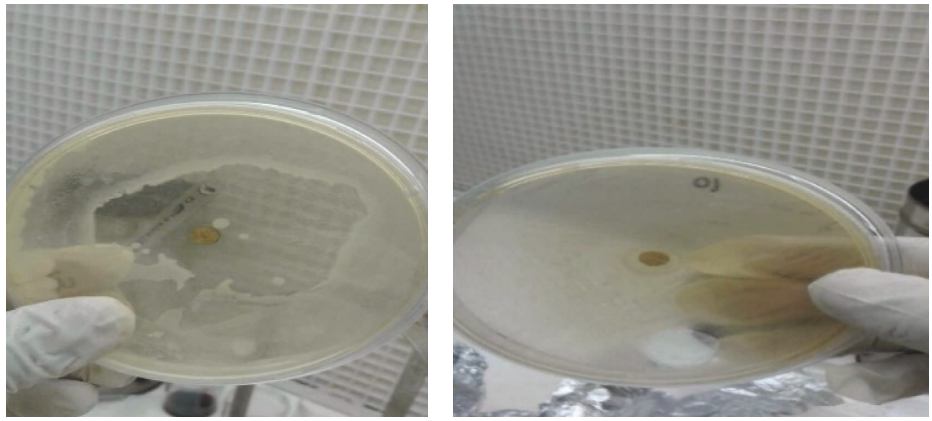
Figure 2: (a) Mean inhibition zone diameter of the bioactive crude extracts and EtOAc fractions against *M. tuberculosis* at a single concentration of 1.25 mg/mL. In each plot, means of test samples with the same letter have insignificant differences ($p = 0.05$). (b) Minimum inhibitory concentration (MIC) of bioactive crude extracts and fractions.

3. Results and Discussion

3.1. Extract Yield and Antimicrobial Activities of Crude Extracts and Fractions. The highest extraction yield from the roots of 12.87%, based on the weight of the dry-powdered sample, was obtained using EtOAc. Very low extract yields were achieved using hexane, indicating low concentrations of nonpolar compounds in the roots (Table 1).

All the five crude extracts (Figure 1) were initially screened to assess their activity against *Mycobacterium tuberculosis*. Only the DCM and EtOAc crude extracts portrayed significant activity against *Mycobacterium tuberculosis*. The inhibition zone of the EtOAc crude extract was lower than that of the DCM crude extract, but both crude extracts achieved lower inhibition zone diameters than gentamycin ($p < 0.05$; Figure2(a)). The EtOAc crude extract developed a clear inhibition zone with an average diameter of 9.67 mm, without any visible growth, whereas the DCM crude extract had a very high average inhibition zone of 15.20 mm; however, it had some colonies growing within the inhibition zone (Figure 3). In addition, the DCM crude extract required a much higher minimum inhibitory concentration (MIC) than the EtOAc crude extract (Figure 2(b)). The MIC provided a quantitative way of assessing the susceptibility of the microbe in question to the drug and the strength of the drug [16].

Therefore, the crude EtOAc extract was fractionated using column chromatography resulting in eleven fractions. The retardation factors (R_f) of spots on a TLC plate of the crude extract and the fractions obtained from the column chromatographic fractionation are shown in (Figure 4). Antimicrobial screening of fractions showed that only fractions F5 and F6 maintained the activity of the crude EtOAc extract against *M. tuberculosis*, with inhibition zone diameters of 10.53 mm and 8.67 mm, respectively (Figure 2(a)). There were no significant differences in inhibition zone diameters between the crude EtOAc extract and fractions F5 and F6. However, F5 achieved a significantly ($p < 0.05$) higher zone of inhibition diameter than F6 (Figure 2(a)). In addition, fraction F5 exhibited a lower MIC (4.88 μg/mL) than the crude EtOAc extract (9.77 μg/mL), which was within the range of the MIC value for gentamycin of 3.13 μg/mL (Figure 2(b)). Furthermore, the results from this study were compared against the standard critical concentration of ethambutol, one of the first-line drugs of multidrug-resistant *tuberculosis* isolates in the market. An extract/fraction is said to be effective if the MIC value is ≤ one step (two-fold) dilution above the critical



(a) (b)

Figure 3: Antimycobacterial activity at 72 hours for (a) the DCM crude extract and (b) the EtOAc crude extract in vitro.

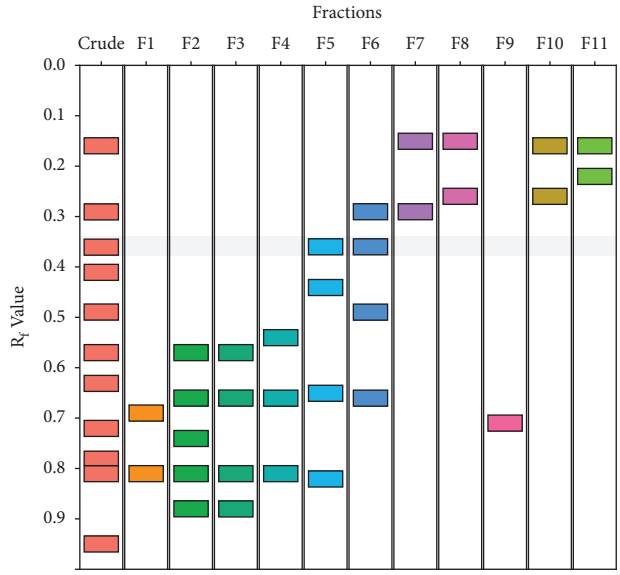


Figure 4: Retardation factors (R_f) from thin-layer chromatography (TLC) of crude EtOAc extracts and fractions of the crude extract obtained using column chromatography.

concentration, which is 5 μg/mL for ethambutol [8, 17]. Therefore, the activity of the crude EtOAc extract and fraction F5 may be said to be effective against *M. tuberculosis*. The bioactivity of the fractions F5 and F6 may be attributed to the retardation of compounds with an R_f of 0.36 from the crude extract, absent in the other fractions (Figure 4).

IC_{50}, obtained from dose-response curves (Figure 5), is the concentration of a drug that inhibits a biological or a biochemical activity by 50% [18]. An *in vitro* IC_{50} is a basic starting point in determining the potential efficacy of a drug. It is a guide for lead optimization and hence important in drug discovery [14, 19]. Both the crude EtOAc extract (47.18 μg/mL) and fraction F5 (38.37 μg/mL) had an order of magnitude higher values of the half-maximal inhibitory concentration (IC_{50}) than gentamycin (3.88 μg/mL). A dose-response curve with a small IC_{50} and a Hill slope (HS) of >1 implies that the drug is more potent and that less drug is needed to achieve the desired effect, hence less likely to cause off-target effects [20]. Despite fraction F5 having the lowest IC_{50} value of the test samples (Figure 5(b)), it exhibited a shallow dose-response curve (HS = −1.005) indicating that there may not be a substantial increase in the therapeutic benefit as the maximum tolerable dose is approached [20]. However, it is evident from the comparison of the HS of fraction F5 with the HS for the crude EtOAc extract (HS = −1.322) that the HS can increase with further fractionation steps. This proves the possibility of TB drug development from ethyl acetate root extracts of *Vernonia glabra*.

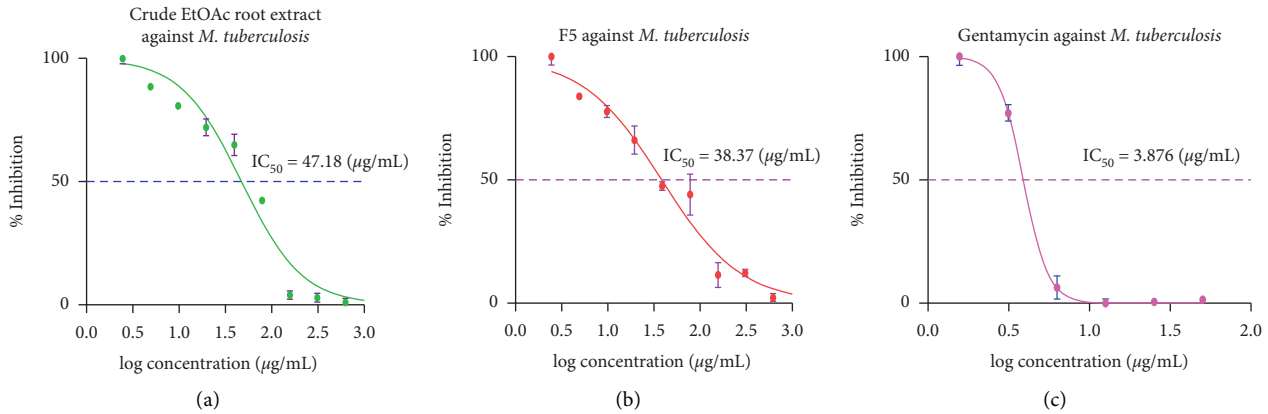


Figure 5: Dose-response curves for (a) crude EtOAc extract, (b) EtOAc fraction F5, and (c) Gentamycin against *M. tuberculosis*.

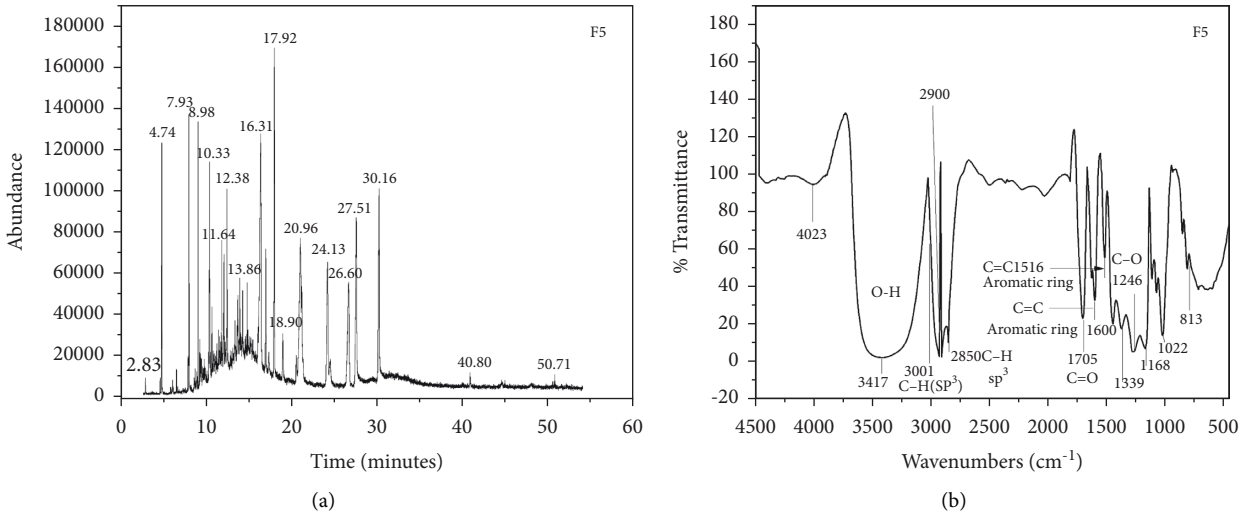


Figure 6: GC-MS chromatogram (a) and FTIR spectrum (b) for fraction F5.

3.2. Chemical Profile of Bioactive Ethyl Acetate Fractions. The results of the GC-MS and FTIR analysis identified hexadecenoic acid, methyl ester, octacosane, diisooctyl phthalate, and tetradecane as the major phytoconstituents of the bioactive fraction of the ethyl acetate extract. Using GC-MS, octacosane 51.12% and diisooctyl phthalate (24.56%) were the most abundant compounds and with high NIST library match factors of 912 and 947, respectively, followed by hexadecenoic acid, methyl ester (12.63%) and tetradecane (0.39%). Hexadecanoic acid, methyl ester ($t_R = 11.642$; Figure 6(a)) was identified from its mass spectrum, which gave a molecular ion peak [M^+] at 270, corresponding to a molecular formula, $C_{17}H_{34}O_2$ (Figure 7(a)). It also had a good NIST library match factor of F/R = 755/769 with the reference spectrum (Figure 7(b)). Ion mass peak 74 (Figure 7(a)) is a -$C_3H_6O_2$ acid moiety that resulted from the McLafferty rearrangement [21] as shown in Figure 8(b).

Correspondingly, the FTIR peak at 1705 cm^{-1} accounts for C=O stretch in the esters, hexadecenoic acid, methyl ester and diisooctyl phthalate. There were also two other ester bonds shown by FTIR that were C-C-O stretch to the left at 1246 cm^{-1} and O-C-C stretch to the right at 1022 cm^{-1}. FTIR also showed the presence of O-H at 3417 cm^{-1}, corresponding to 0.07% of phenol, 3,5 *bis*(1,1-dimethylethyl) in the GC-MS analysis ($t_R = 6.62$ min). It is evident from the FTIR spectrum of the EtOAc crude extract (Figure 11(b)) that the observed functional groups present in the fraction originated from the crude extract, and the GC-MS analysis results of the blank (Figure 11(a)) confirmed that the identified compounds were from the fraction itself. The identified compounds have previously reported antimicrobial potential. Hexadecenoic acid methyl ester and diisooctyl phthalate were all reported to possess antimicrobial activity [22–24]. Hexadecenoic acid methyl ester also showed antifungal and antioxidant activities, which reduce the levels of tumour necrosis factor-alpha, prostaglandin, and interleukin-10 without affecting ATP levels [23, 25]. Therefore, the antimycobacterial activity portrayed by the fraction could be attributed to the synergistic effect of these compounds.

The compound that gave rise to a peak at $t_R = 17.919$ (Figure 6(a)) was identified as diisooctyl phthalate. Diisooctyl phthalate fragmentation and loss of -C_8H_{15} moiety by transfer

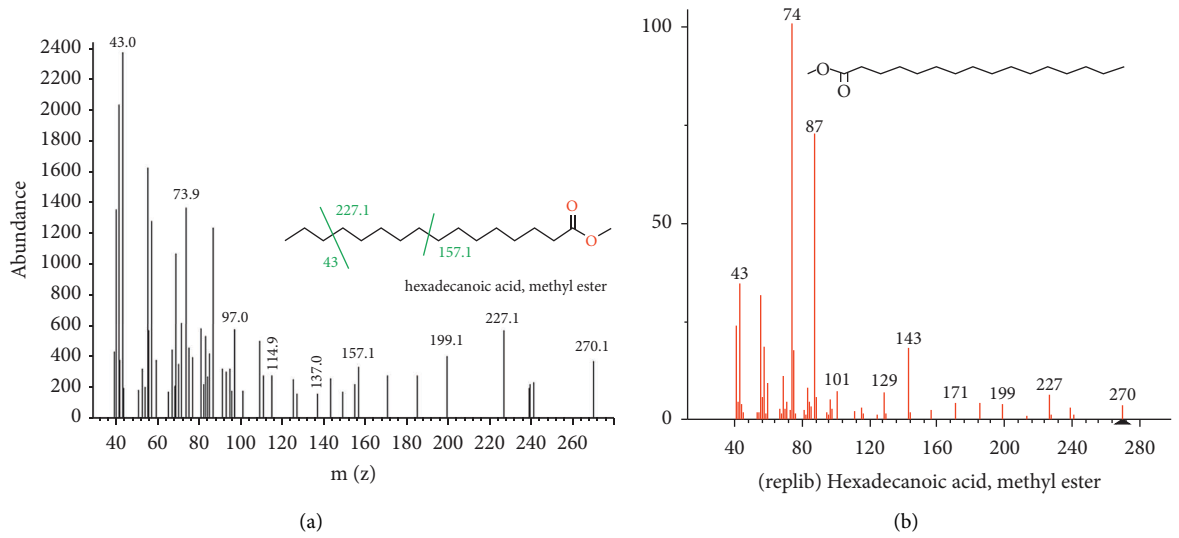


Figure 7: MS spectrum and the fragmentation pattern of hexadecanoic acid methyl ester (a) and the NIST library reference spectrum (b).

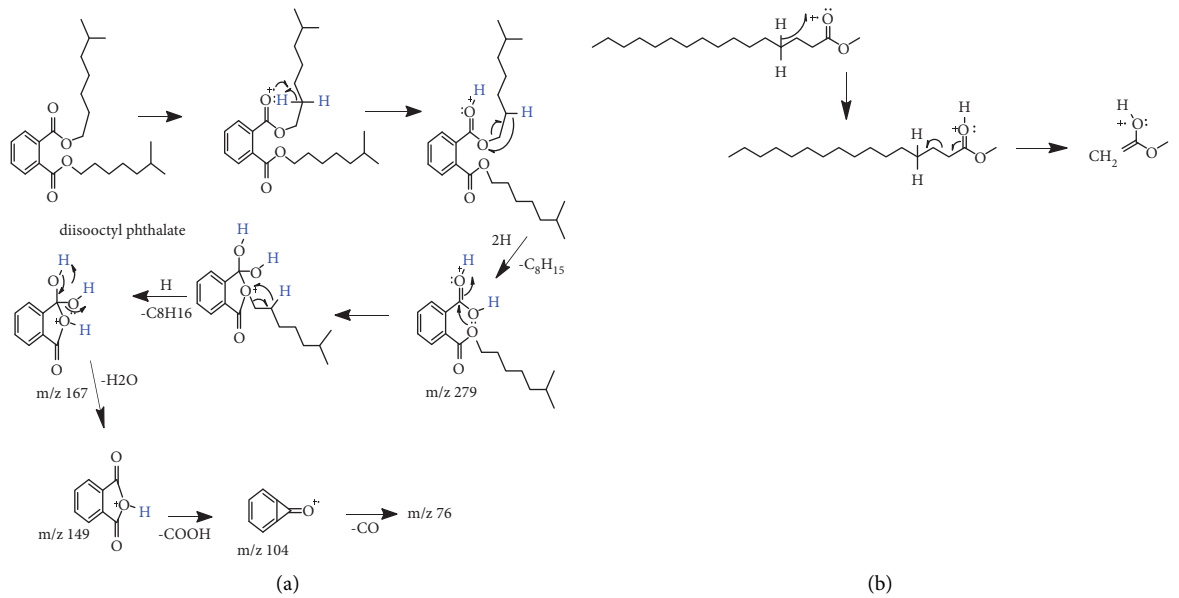


Figure 8: MS fragmentation of diisooctyl phthalate (a) and the McLafferty rearrangement in hexadecenoic acid methyl ester (b).

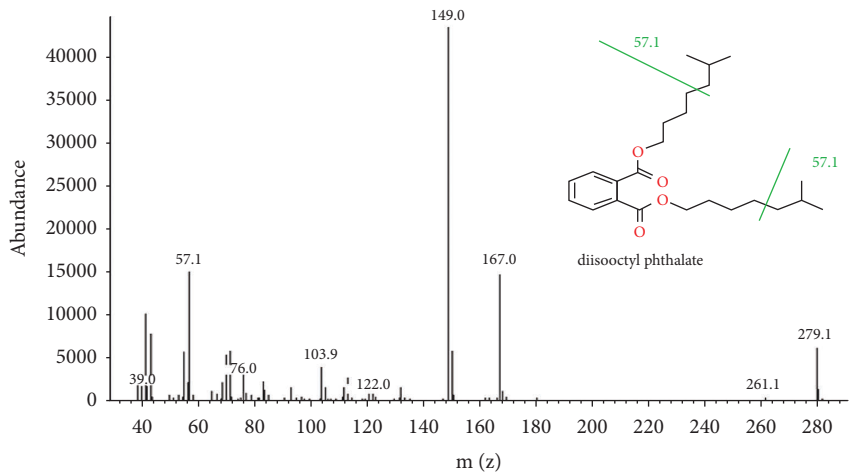


Figure 9: MS spectrum and the fragmentation pattern of diisooctyl phthalate.

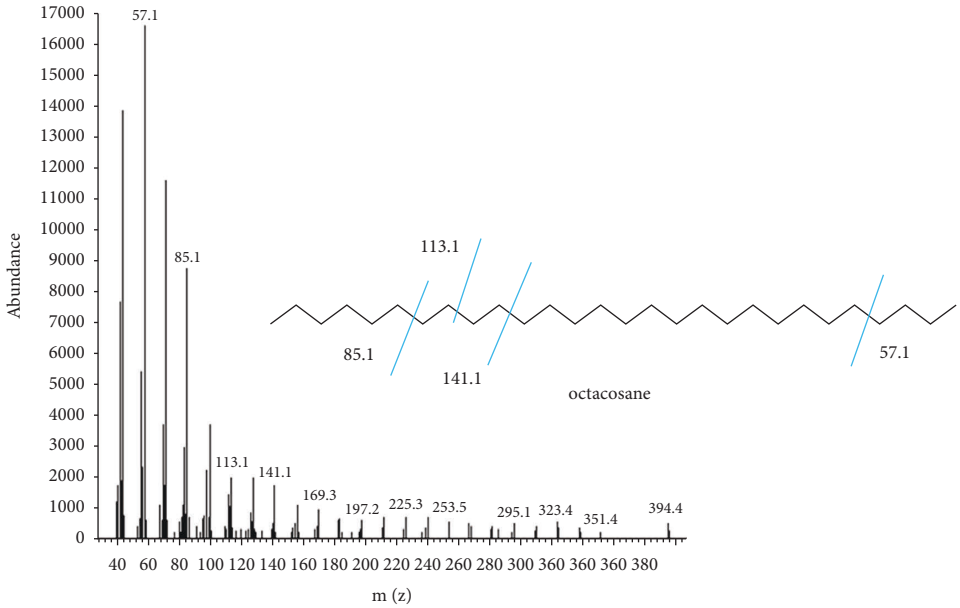


Figure 10: MS spectrum and the fragmentation pattern of octacosane.

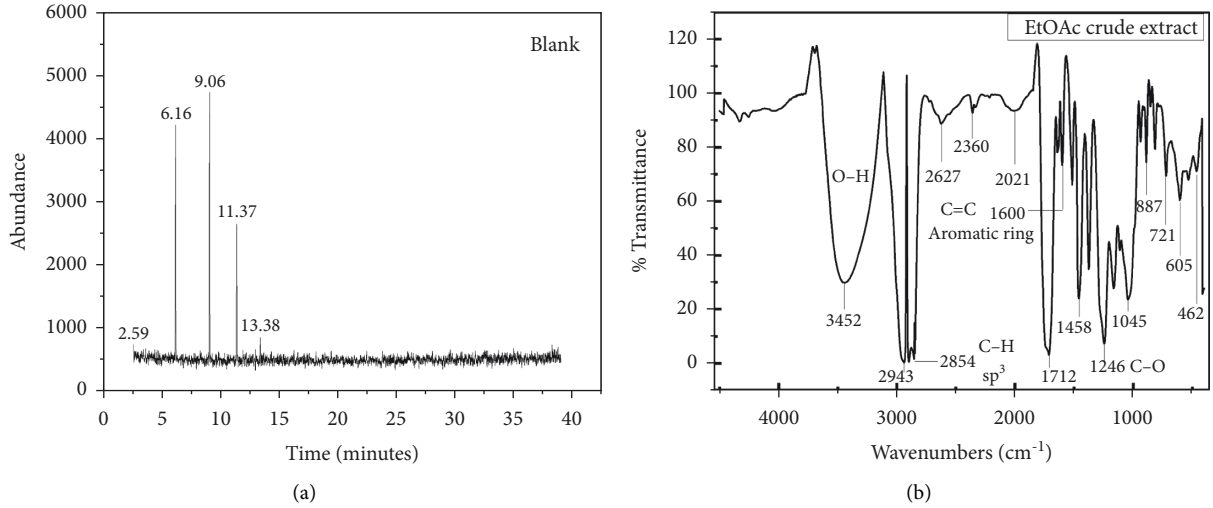


Figure 11: GC-MS chromatogram for the blank (a) and FTIR spectra for the EtOAc crude extract (b).

of two hydrogen atoms produced m/z of 279, with further removal of -C_8H_{16} leading to $m/z = 167$ (Figure 9). The base ion, protonated phthalic anhydride, ($m/z = 149$) was produced by loss of H_2O molecules from the product at $m/z = 167$. The fragmentation of the base ion ($m/z = 149$) resulted in the product at $m/z = 104$ by removal of a carboxyl group, -COOH. The product ($m/z = 104$) was further fragmented by the loss of -CO to produce the final product with $m/z = 76$ [21]. Octacosane was identified corresponding to a peak at $t_R = 16.311$ (Figure 6(a)) using its [M^+] ion (Figure 10).

4. Conclusions

This study reports on the *in vitro* antimycobacterial activities of crude extracts and fractions of roots of *Vernonia glabra* against *M. tuberculosis*. The ethyl acetate fraction showed the best activity with MIC and IC_{50} values of 4.88 μg/mL and 38.37 μg/mL, respectively. The MIC value was within the range achieved by gentamycin (3.13 μg/mL), but the IC_{50} value was one order of magnitude higher than that of gentamycin (3.88 μg/mL). However, the IC_{50} value was below the critical concentration of ethambutol, one of the first-line drugs used in the treatment of multidrug-resistant *M. tuberculosis*. GC-MS results revealed the presence of esters in the bioactive fraction such as hexadecenoic acid, methyl ester and diisooctyl phthalate. Alkanes such as octacosane and tetradecane and phenol, 3,5-*bis*(1,1-dimethylethyl) were also detected. The overall results from this study showed that *Vernonia glabra* is a potential new source of antimycobacterial drug lead. The good antimicrobial activities of the extracts and fractions support the medicinal use of *Vernonia glabra* by traditional healers.

Abbreviations

EtOAc: Ethyl acetate
DCM: Dichloromethane
MeOH: Methanol
FTIR: Fourier-transform infrared spectroscopy
GC-MS: Gas chromatography-mass spectroscopy
TLC: Thin-layer chromatography
CLSI: Clinical and Laboratory Standards Institute
HIV: Human immune virus
AIDS: Acquired immunodeficiency syndrome
t_R: Retention time.

Data Availability

The data used to support the findings of this study are included in the article. However, further data may be obtained from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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