Antimicrobial activity, phytochemical characterization and gas chromatography-mass spectrometry analysis of Aspilia pluriseta Schwein. extracts

Sospeter N. Njeru a,*,1, Jackson M. Muema b

a Department of Biochemistry, School of Health Sciences, Kisii University, PO Box 408-40200, Kisii, Kenya
b Department of Biochemistry, Jomo Kenyatta University of Agriculture and Technology (JKUAT), PO Box 62000-00200, Nairobi, Kenya

Abstract

Aspilia pluriseta is associated with various bioactivities, although with limited scientific justification. In this study, we evaluated the antimicrobial activity, and characterized the phytochemicals of root extracts of A. pluriseta aimed at validating its therapeutic potential. We used BACTEC MGIT™ 960 system to test for antitubercular activity, disc-diffusion together with the microdilution method to evaluate antimicrobial activities and qualitative phytochemical tests together with gas chromatography-mass spectrometry (GC-MS) analysis to determine the phytochemicals that associated with A. pluriseta extracts activity. We show that methanolic crude extract (at 1 g/mL) had high Mycobacterium tuberculosis (MTB) inhibitory activity (0 growth unit) and considerable potency against Escherichia coli (11.7 mm), Staphylococcus aureus (9.0 mm), and Candida albicans (7.7 mm). All the extract fractions exerted remarkable antmycobacterial activities with minimum inhibitory activity of between 6.26 – 25 μg/mL. The highest antimicrobial activity of petroleum ether and dichloromethane fraction was against E. coli at inhibition zone diameters of 8.3 mm, and 8.0 mm, respectively, while ethyl acetate fraction was against S. aureus with an inhibition zone of 8.7 mm. Methanolic fraction exhibited broad-spectrum activity against 87.5% of the tested microbes (inhibition zones 6.3–8.3 mm). Furthermore, we qualitatively detected terpenoids, alkaloids, and phenolics such as flavonoids, and anthraquinones in extract fractions. GC-MS analysis detected an abundance of fatty acid esters, 2-hydroxy-1-(hydroxymethyl) ethyl ester-hexadecanoic acid, and 2,3-dihydroxy propyl ester-acetate and methanolic fractions) have strong selective antitubercular activity, and thus, we scientifically validate the use of A. pluriseta as a potential source for the discovery of novel antitubercular agents.

1. Introduction

Infectious diseases are a primary cause of global human and animal mortality, which is further aggravated by frequent emergence and re-emergence of opportunistic infections [1]. However, one of the major global health challenges is attributed to tuberculosis. Tuberculosis (TB) is the leading cause of mortalities from a single infectious agent, which claimed the lives of over 1 million people, besides making an additional over 10 million people ill in 2018 [2,3]. In humans, TB is an airborne infection primarily caused by MTB [3, 4]. MTB thrives in the hostile environment of the human lungs, despite a sustained immunological onslaught of the host that prevents the growth of nearly all other bacteria [3, 5]. MTB effectively survives host defenses because of a highly impermeable cellular envelope that covers it. The mycobacterial cell envelope is a complex heteropolymer composed of peptidoglycan covalently attached to arabinogalactan terminated by mycolic acids, specific to mycobacteria. Also, MTB can manipulate the host immunological defense mechanisms to foster its survival in a harsh environment [5].

Effective management of MTB is hampered by a number of factors such as; (1) The widespread development of drug resistance (for example, multi-drug resistant TB (MDR-TB) which does not respond to isoniazid and rifampicin, and extensively drug-resistant TB (XDR-TB) which is resistant to isoniazid, rifampin, plus fluoroquinolones and one of the injectable second-line drugs such as amikacin, kanamycin, or

* Corresponding author.
1 Current address: Division of Immunology, Research group on T-Cell Homeostasis & Intercellular Competitiveness - Paul-Ehrlich-Institute, Federal Institute for Vaccines and Biomedicines, Paul-Ehrlich-Strasse 51–59, 63225 Langen (Hessen), Germany.

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resistant Pseudomonas aeruginosa. Such emerging drug-resistant pathogenic strains are usually not sensitive to
MRSA, among others [12]. This emerging drug-resistant pathogenic strains are usually not sensitive to
the first-line of antimicrobial therapy, thus forcing the use of a second- and third-line treatment option. Besides
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resistant to

Besides TB, there are other Gram-positive, Gram-negative, and fungal pathogens that have acquired noxious drug resistance. These
pathogens are often responsible for the hospital- and community-acquired infections including and not limited to methicillin-resistant
Staphylococcus aureus (MRSA), Klebsiella pneumoniae, fluoroquinolone-resistant Pseudomonas aeruginosa, Escherichia coli, among others [12].

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pharmacological activity against medically important bacteria, especially MTB, is at its early stages. As part of our continuous research
efforts to discover novel, potent, antitubercular agents from Kenyans ethnobotanicals, in this study we describe the antitubercular activity of
A. pluriseta solvent extract fractions. Our findings demonstrate that
A. pluriseta extract fractions (especially ethyl acetate and methanolic fractions) have remarkable selective antitubercular activity, which is
partly if not exclusively associated with phytochemicals such as terpenoids, phenolics, alkaloids, fatty acid alkyl esters detected in the extract
fractions.

2. Methods

2.1. Plant material collection

We used the ethnopharmacological approach to identify the plant
used in this study. The information on its herbal use and preparation
among the Mbeere community of Embu County, Kenya, was gleaned from community herbal practitioners and further confirmed from document-
tation by Riley and Brokensha (1988) in The Mbeere in Kenya (ii), botanical identity and use [52]. We collected the plant root materials in an
open community field. The plant is not among the endangered species, and therefore no prior permission was sought before sample collection.
The sampling was carried out within 0°46’27.0’S 37°40’54.9’E; -0.774156, 37.681908 of GPS co-ordinates. A botanist authenticated
plant sample identity at Egerton University, Kenya, where voucher specimen number NSN2 was given and deposited.

2.2. Extraction and fractionation of active ingredients

The root samples were mechanically size-reduced, air-dried in the
dark at 23 ± 2°C to a constant weight, then ground into a fine powder
using an electric miller (Retsch SR 200, Haan, Germany). In order to
mimic the traditional preparation method, a portion of the sample
powder (50 g) was subjected to cold extraction in distilled water with
occasional shaking, after which the extract was lyophilized. A similar
portion was macerated twice in 200 mL methanol (Sigma Aldrich, St.
Louis, USA) for 48 h, pooled and filtered using Whatmann 1 filter paper. Excess methanol was evaporated from the filtrate using a rotovapora-
tor (Labotora 4000 efficient, Heidelberg, Germany) and the extract
stored at -20°C until use. Fractionation of the A. pluriseta extract was
performed using organic solvents of increasing polarity. Fifty grams of
root powder was macerated in 200 mL of petrol ether with intermittent
shaking for 48 h. Subsequently, the material was filtered through Whatman number-1 filter paper. The residue was additionally re-
extracted using the same fresh solvent for 48 h, and after that, the
two filtrates pooled together. The resulting marc was air-dried and further extracted with dichloromethane solvent followed by ethyl ace-
tate, and methanol solvent, using the same procedure carried out for
petroleum ether. Organic crude extract and solvent fractions were
concentrated and reconstituted into appropriate stock solution with
100% dimethyl sulfoxide (DMSO) but diluted appropriately so that the
diluted so that the final DMSO in the test sample is 1% DMSO. Water crude extract was
reconstituted in physiological saline, which served as its negative
control.
2.3. Antimicrobial activity

2.3.1. Test microorganisms

All the test microorganisms were sourced from Kenya Medical Research Institute (KEMRI), Nairobi. These included; one acid-fast Mycobacterium tuberculosis strain H37Rv (ATCC 27294), one Gram-positive; Staphylococcus aureus (ATCC 25923) strain and Methicillin-resistant Staphylococcus aureus strain (clinical isolate), five Gram-negative bacteria; Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 27853), Klebsiella pneumoniae (clinical isolate), Salmonella typhi (clinical isolate) and Shigella sonnei (clinical isolate), and two fungi; Candida albicans (ATCC 90028), Cryptococcus neoformans (ATCC 66031).

2.3.2. Antimycobacterial activity

MTB was revived in Lowenstein Jensen slants under previously adopted standard conditions [53, 54] and later subjected to BACTEC MGIT 960 system (BD Biosciences, New York, USA) for antitubercular activity assays [55, 56]. BACTEC MGIT 960 system is a fully automated, high volume, a non-radiometric instrument that undertakes continuous monitoring of culture growth. Growth supplement (0.8 mL) containing a combination of oleic acid, dextrose, bovine albumin, and catalase was added to five 7 mL BBL™ MGIT™ tubes labeled GC (growth control), STR (streptomycin), INH (isoniazid), RIF (rifampicin), ETH (ethambutol) to provide essential substrates for the rapid growth of MTB. MTB suspension in 0.1 mL Middlebrook 7H9 broth adjusted to 0.5 McFarland standard with 10 mL sterile physiological saline was aseptically transferred into each BBL™ MGIT™ tube and incubated at 37 °C. One hundred microliters of BBL™ MGIT™ SIRE (streptomycin, isoniazid, rifampicin, ethambutol) prepared aseptically following the manufacturers’ instructions were added into corresponding labeled BBL™ MGIT™ tube followed by addition of 0.5 mL of 1% MTB suspension. Streptomycin at 1.0 μg/mL, rifampicin at 1.0 μg/mL, ethambutol at 5.0 μg/mL, and isoniazid at 0.1 μg/mL were used as the positive controls whereas 1% DMSO (for solvent extract, and solvent extract fractions), and sterile physiological saline (for water extract) were used as negative controls. The protocol was repeated using crude extracts at 1.0 g/mL (for screening purposes) in place of SIRE and solvent fractions tested at concentrations ranging from 50 to 6.25 μg/mL for petroleum ether, dichloromethane, and methanol, and 25 to 3.125 μg/mL for ethyl acetate to determine the MTB minimum inhibitory concentration (MIC).

2.3.3. Disc diffusion test

To evaluate the general antimicrobial activity of A. pluriseta crude and solvent fractions extracts at various specified concentrations, we used the modified disc diffusion method [57, 58, 59, 60, 61]. A fresh microbial inoculum was made by suspending activated colonies in physiological saline. The bacterial and fungal suspensions were adjusted to 1.5 × 10^8 CFU/mL using 0.5 McFarland turbidity standard and aseptically inoculated onto Muller Hinton agar (MHA) and Sabouraud Dextrose agar (SDA) plates, respectively. Sterilized Whatman 1 filter paper discs (diameter 6 mm) were impregnated with 10 μL of stock extract solutions (1.0 g/mL crude methanol and water extracts, 500 μg/mL for petroleum ether, dichloromethane, methanol, and 250 μg/mL for ethyl acetate fraction). Three standard drugs were used as antibiotics positive controls; Oxacillin at 10 μg/disc (Oxoid Ltd, Tokyo, Japan) for Gram-positive bacteria, Gentamycin at 10 μg/disc (Oxoid Ltd, Tokyo, Japan) for Gram-negative bacteria, and Nystatin at 100 μg/disc (Oxoid Ltd, Tokyo, Japan) for fungi. Whatman filter paper discs loaded with 10 μL of 1% DMSO (for solvent extract, and solvent extract fractions), and 10 μL of sterile physiological saline (for water extract) served as negative controls. Air-dried discs were carefully placed on the agar plates at equidistance points using sterile forceps, including both positive antibiotic control and negative control discs into each plate. Subsequently, the plates were initially incubated at 4 °C for 2 h to allow pre-diffusion of extracts into media and incubated at 37 °C for 24 h. Antimicrobial activity was assessed in triplicates by measuring the size of the inhibition zone to the nearest mm. Fractions exhibiting strong antimicrobial inhibitory potential were considered for further MIC and minimum microbicidal concentration analysis (MMC) determination [62].

2.3.4. Determination of MIC and MMC

The MIC and MMC of A. pluriseta solvent fractions were analyzed as previously described [36, 57, 60, 61, 63]. Briefly, 50 μL of varying fraction concentrations (3.9–500 μg/mL petroleum ether, dichloromethane, and methanol; 1.95–250 μg/mL ethyl acetate) were added into 100 μL of nutrient broth held in a sterile 96-well plate followed by addition of 50 μL test organisms adjusted to 0.5 McFarland standard. All concentrations were tested in triplicates at 37 °C for 24 h. A negative control containing 1% DMSO in nutrient broth was included in column 11, while column 12 checked the capacity of the media to support the growth of the test organism. In order to evaluate the microbial growth in each well, 40 μL of 0.2 mg/mL p-iodonitrotetrazolium chloride (INT, Sigma) were added and incubated for 30 min. Formation of a pink-red color depicted growth while persistent clear coloration denoted growth inhibition. The lowest solvent fraction concentration that exhibited color change was considered as the MIC. MMC was determined by aseptically streaking a loopful of broth from wells that exhibited no color change onto sterile nutrient agar and SDA for bacteria and fungi, respectively, and thereafter incubated at 37 °C for 24 h. The lowest concentration that exhibited no growth was considered as the MMC [64].

2.4. Phytochemical analysis using GC-MS

We performed a preliminary screening for the presence of various phytochemicals such as alkaoids, terpenoids, phenolics such as flavonoids, and anthraquinones qualitatively as previously reported by us and others [4, 20, 36, 65]. Additionally, we undertook a GC-MS analysis of the methanolic extract fraction since it exhibited a broad-spectrum activity. An aliquot of the methanolic fraction (1.3 mg) was dissolved in 1 mL dichloromethane and analyzed with an Agilent Technologies 7900A gas chromatography coupled with a 5975C mass spectrometer in full scan mode (EI, 70 eV, Agilent, Palo Alto, CA). The system was equipped with an HP-5 MS low bleed capillary column (30 m × 0.25 mm i.d. 0.25 μm film thickness (J&W, Folsom, CA, USA). An injection volume of 1 μL was subjected to a splitless mode during analysis, with helium used as the carrier medium at a constant flow rate of 1.25 mL/min. The oven temperature was maintained at 35 °C for 5 min, then programmed to increase at 10 °C/min to 280 °C and held at this temperature for 10.5 min. The obtained compound profiles were identified by comparing the corresponding reference retention indices and mass spectral in databases (NIST 05, NIST 08, Adams, and chemical).

2.5. Data analyses

The data was analyzed using Analysis of variance (ANOVA) using R (version 3.5.1) with Tukey HSD post-hoc. A p-value of less than 0.05 was considered statistically significant. Values were expressed as mean ± SEM of experimental replicates.

3. Results

3.1. Screening for general antimicrobial activities of A. pluriseta crude extracts

In order to mimic the traditional preparation of A. pluriseta herbal medicine, we initially assayed for the general bioactivity of the crude water and methanol extracts. Using a BACTEC MGIT™ 960 system (BD, New York, USA) to assay for antimycobacterial activity, we found that the water crude extract had no antituberculous activity (400 growth unit (GU)), equivalent to GU of the negative control. Interestingly, the
methanol crude extract exhibited high inhibitory activity against MTB similar to SIRE positive control (0 GU; Table 1).

Further, we screened for general antimicrobial activity by disc diffusion method against representative Gram-positive bacteria (*S. aureus*), Gram-negative bacteria (*E. coli*), and fungi (*C. albicans*). Our results demonstrated a significant difference in antimicrobial activities of tested extracts against test microbes relative to controls (ANOVA, *S. aureus*; F(3,8) = 160.1, *p* < 0.001; *E. coli*, F(3,8) = 53.67, *p* < 0.001; *C. albicans*, F(3,8) = 72.67, *p* < 0.001). The water extract exhibited low general antimycobacterial activity in all tested cases (zone of inhibition <10 mm), while the methanolic crude extract gave moderate but broad-spectrum activity, with the highest inhibition of 11.7 mm against *E. coli* (Table 2).

Even though the crude extract concentrations were in the range of 10^4 times higher than the standard antibiotic controls, methanolic crude extract showed a remarkable antitubercular activity, that compared with the activity of the positive control. Since the active components in the crude extract could have comprised only a fraction of the total extract used, we reasoned that further purification would allow enrichment of active molecules in extract fractions. Therefore, we hypothesized that extract solvent fractionation would result in enhanced activity, and possibly at a lower concentration.

3.2. Antimycobacterial activities of *A. pluriseta* solvent extract fractions

To test whether fractionation of *A. pluriseta* could lead to improved antimycobacterial activity, as well as determine the MIC of different fractions, we used the BACTEC MGIT 960 system. If the GU of the extract fraction-containing tubes was greater than 100 when the GU of the growth control was 400, we defined the results as non-responsive. If GU values of the extract fraction-containing tubes were ≤100, the results were considered susceptible, and the concentration of that tube was used to define the MIC [55, 56]. Our results revealed the most robust activity against MTB by more polar ethyl acetate (EA) fraction (MIC 6.25 µg/mL) followed by methanolic (MeOH) fraction (12.5 µg/mL). The less polar dichloromethane (DCM) and petroleum ether (PE) fractions had a MIC of 25 µg/mL, with DCM fraction inhibiting MTB growth in a dose-dependent manner (Table 3). For EA and MeOH fractions, concentrations ≥6.25 and 12.5 µg/mL, respectively, completely inhibited MTB growth, an observation comparable to a positive control (SIRE). These results confirmed our hypothesis that *A. pluriseta* fractionation would lead to robust antitubercular activity, and at a lower concentration. Further, the results are in agreement with other studies that have reported polar solvent extract fractions usually have higher activity than less polar fractions [66, 67, 68].

3.3. General antimicrobial activities, MIC and MMC of *A. pluriseta* extract fractions

With crude solvent extract having demonstrated moderate broad-spectrum antimicrobial activity, we reasoned that solvent fractionation might yield improved antimicrobial activity. However, fractionation of root extract with solvents of increasing polarities resulted in attenuated but broad-spectrum mild antimicrobial activity, with all fractions giving inhibitory zones of less than 10 mm (Table 4). The best activity (though still a weak one) by petroleum ether fraction was against *E. coli* (zone of inhibition 8.3 mm), dichloromethane against *E. coli* (8.0 mm), ethyl acetate against *S. aureus* (8.7 mm) and methanolic against *S. aureus* (8.3 mm), *C. albicans* (8.3 mm) and against *E. coli* (8.0 mm) (Table 4). EA fraction showed a weak growth inhibition against *C. neoformans* (zone of inhibition 6.7 mm), while MeOH fraction weakly inhibited *K. pneumonia* (zone of inhibition 7.0 mm). Generally, the methanolic extract fraction exhibited significant broad spectrum activity against seven of the eight tested microorganisms (ANOVA; *S. aureus*, F(5,12) = 2267, *p* < 0.001; MRSA, F(4,10) = 129.7, *p* < 0.001; PA, F(5,12) = 2464, *p* < 0.001; *E. coli*, F(5,12) = 248.3, *p* < 0.001; KP, F(5,12) = 922.1, *p* < 0.001; SS, F(5,12) = 507, *p* < 0.001; ST, F(5,12) = 143.7, *p* < 0.001; CN, F(3,8) = 1849, *p* < 0.001; CA, F(5,12) = 302.7, *p* < 0.001). Equally, relative to the negative controls, all the extract fractions significantly inhibited the growth of tested microorganisms (*p* < 0.001) but in a manner less comparable to the positive control.

Further, we tested for the MIC and MMC concentrations of the solvent extract fractions (Table 5), and we established that petroleum ether fraction against *E. coli* had a MIC of 250 µg/mL while methanolic fraction against *S. aureus* had a MIC of 125 µg/mL. In all other cases, the MIC was greater than 500 µg/mL, indicating that the extract fractions were bacteriostatic in action.

Therefore, the general antimicrobial activity results were counterintuitive, considering the fractions yielded robust antimycobacterial activity. This would suggest that the active principles in the extract fractions are, to some extent, specific to acid-fast bacteria. This would be very important for the discovery of selective antitubercular leads, as previously reported [69, 70].

3.4. Phytochemical analysis

The preliminary examination of *A. pluriseta* solvent extract fractions pointed to the presence of different phytoconstituents such as terpenoids, alkaloids, and phenolics such as flavonoids, and anthraquinones (Table 6). We speculate that these are the bioactive constituents that may be responsible for the bioactivity demonstrated by these extract fractions.

Methanolic extract fraction exhibited broad spectrum activity (inhibiting the growth of Gram-negative, Gram-positive, acid-fast bacteria, and fungi (Tables 3 and 4)). Additionally, it was easier to get more materials from this extract fraction for further analysis. Therefore, we subjected this fraction to GC-MS analysis to identify the specific bioactive compounds that could be partly associated with the activity of this fraction (Table 7; Figures 1 and 2). We detected six compounds from the extract fraction, four alkanes, and two fatty acid esters. Based on the mass spectral library databases, these compounds were tentatively identified as hexadecane, octadecane, eicosane, 2-hydroxy-1-(hydroxymethyl) ethyl ester-hexadecanoic acid, tetracosane, and 2,3-dihydroxy propyl ester-octadecanoic acid (Table 7; Figure 2). The fatty acid esters represented by peaks at retention times 28.61 min (2-hydroxy-1-(hydroxymethyl) ethyl ester-hexadecanoic acid) and 30.23 min (2,3-dihydroxy

### Table 1. Screening for the antimycobacterial activity of *A. pluriseta* crude extracts.

| Sample  | Solvent | GU   | NR/S |
|---------|---------|------|------|
| *A. pluriseta* |         |      |      |
| Water   |         | 400  | NR   |
| Methanol|         | 0    | S    |
| SIRE    |         | 0    | S    |
| GC      |         | 400  | NR   |

Water and methanol crude extract at 1 g/mL; SIRE: Positive control of streptomycin at 1.0 µg/mL, isoniazid at 0.5 µg/mL, rifampicin at 1.0 µg/mL and ethambutol at 5.0 µg/mL; GC: Growth control acting as a negative control of media treated with 1% DMSO (methanolic crude extract) or physiological saline (water crude extract); NR: Non-responsive; S: Sensitive.
### Table 2. Screening for general antimicrobial activity of *A. pluriseta* crude extracts.

| Sample         | Extract    | The diameter of zone of inhibition (mm) |
|----------------|------------|----------------------------------------|
|                |            | *S. aureus* | *E. coli* | *C. albicans* |
| *A. pluriseta* | Water      | 6.7 ± 0.3ab | 7 ± 0.6 ab | 6.0 ± 0.0b   |
|                | Methanol   | 9.0 ± 0.6ab | 11.7 ± 0.3b| 7.7 ± 0.3ab  |
|                | Positive control | 24.0 ± 1.3 | 22.0 ± 0 | 16.3 ± 0.9  |
|                | Negative control | 0        | 0     | 0           |

Water and methanol crude extract at 10 × 10^4 μg; Positive control (Oxacillin 10 μg/disc for Gram-positive, Gentamycin 10 μg/disc for Gram-negative bacteria and Nystatin 100 μg/disc for fungi); Negative control (Discs loaded with 10 μL of 1% DMSO (for methanol extract) or physiological saline (for water extract)); n = 3; Values = Mean ± SEM. Values followed by similar superscript letters are not significantly different from each other (ANOVA - Tukey’s post-hoc multiple comparisons, P < 0.05).

### Table 3. Antimycobacterial activity of *A. pluriseta* solvent extract fractions.

| Plant         | Fraction | Concentration μg/mL | GU  | NR/S | MIC (μg/mL) |
|---------------|----------|---------------------|-----|------|-------------|
| *A. pluriseta*| PE       | 50                  | 0   | S    | 25          |
|               |          | 25                  | 0   | S    |             |
|               |          | 12.5                | 0   | S    |             |
|               |          | GC                  | 400 | NR   |             |
|               |          | SIRE                | 0   | S    |             |
| DCM           | 50       | 0                   | S   |      | 25          |
|               | 25       | 0                   | S   |      |             |
|               | 12.5     | 128                 | S   |      |             |
|               | GC       | 400                 | NR  |      |             |
|               | SIRE     | 0                   | S   |      |             |
| EA            | 25       | 0                   | S   |      | 6.25        |
|               | 12.5     | 0                   | S   |      |             |
|               | 6.25     | 0                   | S   |      |             |
|               | GC       | 400                 | NR  |      |             |
|               | SIRE     | 0                   | S   |      |             |
| MeOH          | 50       | 0                   | S   |      | 12.5        |
|               | 25       | 0                   | S   |      |             |
|               | 12.5     | 0                   | S   |      |             |
|               | GC       | 400                 | NR  |      |             |
|               | SIRE     | 0                   | S   |      |             |

PE: Petroleum ether fraction; DCM: Dichloromethane fraction; EA: Ethyl acetate fraction; MeOH: Methanol fraction; SIRE: Positive control of streptomycin at 1.0 μg/mL, isoniazid at 0.5 μg/mL, rifampicin at 1.0 μg/mL and ethambutol at 5.0 μg/mL; GU: Growth unit; GC: Growth control as the negative control of media treated with 1% DMSO; NR: Non-responsive; S: Sensitive.

### Table 4. Antimicrobial activity of *A. pluriseta* solvent extract fractions.

| Fractions | The diameter of zone of inhibition (mm) |
|-----------|----------------------------------------|
|           | Gram-positive | Gram-negative | Fungi |
|           | SA | MRSA | PA | EC | KP | SS | ST | CA | CN |
| PE        | 7.7 ± 0.3ab | 0± 0 | 8.3 ± 0.3ab | 0± 0 | 0± 0 | 7.7 ± 0.3ab | NT |
| DCM       | 7.7 ± 0.3ab | 0± 0 | 8.0± 0.3ab | 0± 0 | 7.7 ± 0.3ab | 0± 0 | 7.3 ± 0.3ab | 0± 0 |
| EA        | 8.7 ± 0.3ab | 0± 0 | 8.0± 0.3ab | 0± 0 | 7.7 ± 0.3ab | 0± 0 | 6.7 ± 0.3ab | 0± 0 |
| MeOH      | 8.3 ± 0.3ab | 6.3± 0.3ab | 8.0± 0.3ab | 7.0± 0.3ab | 8.0± 0.3ab | 6.7 ± 0.3ab | 8.3 ± 0.3ab | NT |
| PC        | 33.7 ± 0.3 | 24.3± 0.3 | 23.7± 0.6 | 15± 0 | 15.7± 0.3 | 19.7± 0.6 | 21.3± 0.3 | 20.3± 0.3 |
| NC        | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

PE: Petroleum ether fraction at 5 μg/disc; DCM: Dichloromethane fraction at 5 μg/disc; EA: Ethyl acetate fraction at 2.5 μg/disc; MeOH: Methanol fraction at 5 μg/disc; PA: Pseudomonas aeruginosa; EC: Escherichia coli; SA: Staphylococcus aureus; KP: Klebsiella pneumoniae; MRSA: Methicillin Resistant Staphylococcus aureus; SS: Shigella sonnei; ST: Salmonella typhi; CA: Candida albicans; CN: Cryptococcus neoformans; PC: Positive control (Oxacillin 10 μg/disc and Gentamycin 10 μg/disc for Gram positive and Gram negative bacteria respectively. Nystatin 100 μg/disc for fungi); NC: Negative control (Discs loaded with 10 μL of 1% DMSO); n = 3; values = Mean ± SEM; Values followed by similar superscript letters are not significantly different from each other (ANOVA - Tukey's post-hoc multiple comparisons, P < 0.05).
propyl ester-octadecanoic acid) were the most abundant compounds detected (Figure 1), and it is highly possible that on their own or in combination with other secondary metabolites contributed wholly or in part to the bioactivity of this extract fraction.

### 4. Discussion

Increasing demand for effective antimicrobials to lessen antimicrobial drug resistance burden and accelerate prompt prevention and treatment of microbial infections necessitates the discovery of new pharmaceutical molecules. This calls for a collaborative approach involving the herbal practitioners and scientific community in search of pharmaceutical molecules from traditionally-claimed active plants and scientifically validating their bioactivity. This comes at a time when most pharmaceutical industries seem reluctant and/or slow to develop novel antimicrobial agents, prompting an overreliance on the limited available bioactive compounds enriched in various solvents, and that methanol is a better and more powerful extractant compared to water [66, 67, 68].

The current study was motivated by an ethno-based claim by the Ambeere residents and herbalists from Embu county - Kenya that, *A. pluriseta* root extracts are used in the management of ‘strong’ coughs and complicated respiratory tract infections. We hypothesized that the alluded to ‘strong’ cough and complicated respiratory tract infection represented TB. We therefore sought to investigate the antitubercular and general antimicrobial activities of this plant, as well as identify the bioactive compounds therein that could be responsible for the bioactivity.

The traditional-based approach of preparing the extracts using water yielded very low antitubercular and antimicrobial activities, while the crude methanolic extract exhibited a broad-spectrum antimicrobial activity against acid-fast, Gram-positive, Gram-negative, and fungi. This could point to a possible implication of differential solubility of various plant compounds in solvents of different polarities. It is established that solvent polarity affects the qualitative and quantitative composition of plant compounds in solvents of different polarities. It is established that solvent polarity affects the qualitative and quantitative composition of plant compounds in solvents of different polarities. It is established that solvent polarity affects the qualitative and quantitative composition of plant compounds in solvents of different polarities. It is established that solvent polarity affects the qualitative and quantitative composition of plant compounds in solvents of different polarities. It is established that solvent polarity affects the qualitative and quantitative composition of plant compounds in solvents of different polarities. It is established that solvent polarity affects the qualitative and quantitative composition of plant compounds in solvents of different polarities. It is established that solvent polarity affects the qualitative and quantitative composition of plant compounds in solvents of different polarities. It is established that solvent polarity affects the qualitative and quantitative composition of plant compounds in solvents of different polarities. It is established that solvent polarity affects the qualitative and quantitative composition of plant compounds in solvents of different polarities. It is established that solvent polarity affects the qualitative and quantitative composition of plant compounds in solvents of different polarities.
methanolic extract was \(10^4\)-fold higher. We reasoned that the active compound(s) in the crude methanolic extract comprised only a small fraction of the crude extract and if purified and isolated, might work at a lower concentration, or even more potently than the standard antibiotic used. To test this assumption, we fractionated the plant sample by organic solvents of increasing concentration. Interestingly, all solvent extract fractions had a strong antitubercular activity with MIC ranging between 25 and 6.25 \(\mu\)g/mL that yielded similar inhibitory capacity (0 GU) to the commercially available antimycobacterial drugs, streptomycin, isoniazid, rifampicin, and ethambutol (SIRE) (Table 3). These findings are consistent with other studies that have demonstrated that plants are an excellent potential source of active antitubercular compounds [18, 39, 51, 75]. However, solvent fractionation yielded relatively lower general antimicrobial activity against Gram-positive, Gram-negative, and fungi compared to the crude extract (Tables 2 and 4). This phenomenon suggests a possible synergistic and/or additive effect of the active molecules in the crude extract, an effect that was possibly lost by fractionation [76, 77, 78, 79]. Furthermore, the fact that fractions demonstrated a robust antitubercular activity, and low general activity against Gram-positive, Gram-negative, and fungi compared to the crude extract (Tables 2 and 4). This phenomenon suggests a possible synergistic and/or additive effect of the active molecules in the crude extract, an effect that was possibly lost by fractionation [76, 77, 78, 79].

Figure 1. GC-MS analysis spectrum of A. pluriseta methanolic extract fraction. The spectrum highlights the compound abundance and separation based on mass fragmentation and retention times.

Bioactivity of plant extracts is the work of secondary metabolites produced for purposes of normal plant defenses; to deter, stun, poison or kill threatening species, but inadvertently inhibiting various physiological targets/processes required for growth, biosynthesis of macromolecules, metabolism, and virulence of microbial systems [30, 80, 81, 82]. To characterize and identify the active compounds mediating activity against the tested organisms, we initially performed qualitative phytochemical screening in all extract solvent fractions (Table 6), and subsequent GC-MS analysis of the methanolic solvent extract fraction (Table 7 & Figures 1 and 2). We qualitatively identified terpenoids, phenolics such as flavonoids and anthraquinones, and alkaloids in A. pluriseta extracts, and thus speculated that they are the phytochemicals partly or wholly responsible of the antitubercular activity demonstrated by various extract fractions. Previous studies have also reported presence of terpenoids, alkaloid, flavonoids, anthraquinones and phenolic in A. pluriseta aqueous extract [40, 47, 83, 84]. Terpenes were enriched in all fractions tested, and a broad range of terpenes identified by various extract fractions. Previous studies have also reported presence of terpenoids, alkaloid, flavonoids, anthraquinones and phenolic in A. pluriseta aqueous extract [40, 47, 83, 84]. Terpenes were enriched in all fractions tested, and a broad range of terpenes identified by various extract fractions. Previous studies have also reported presence of terpenoids, alkaloid, flavonoids, anthraquinones and phenolic in A. pluriseta aqueous extract [40, 47, 83, 84]. Terpenes were enriched in all fractions tested, and a broad range of terpenes identified by various extract fractions. Previous studies have also reported presence of terpenoids, alkaloid, flavonoids, anthraquinones and phenolic in A. pluriseta aqueous extract [40, 47, 83, 84]. Terpenes were enriched in all fractions tested, and a broad range of terpenes identified by various extract fractions. Previous studies have also reported presence of terpenoids, alkaloid, flavonoids, anthraquinones and phenolic in A. pluriseta aqueous extract [40, 47, 83, 84]. Terpenes were enriched in all fractions tested, and a broad range of terpenes identified by various extract fractions. Previous studies have also reported presence of terpenoids, alkaloid, flavonoids, anthraquinones and phenolic in A. pluriseta aqueous extract [40, 47, 83, 84]. Terpenes were enriched in all fractions tested, and a broad range of terpenes identified by various extract fractions. Previous studies have also reported presence of terpenoids, alkaloid, flavonoids, anthraquinones and phenolic in A. pluriseta aqueous extract [40, 47, 83, 84].
receptors as well as inhibiting natural methods for MTB resistance development [87].

Additionally, using GC-MS, we identified two abundant fatty acid alkyl esters in methanolic solvent extract fraction; 2-hydroxy-1-(hydroxymethyl)ethyl ester-hexadecanoic acid, (5) Tetraicosane, and (6) 2,3-dihydroxy propyl ester-octadecanoic acid.

Figure 2. Compounds identified in A. pluriseta methanolic extract fraction using GC-MS. The chemical structures of the identified compounds (1) Hexadecane, (2) Octadecane, (3) Eicosane, (4) 2-hydroxy-1-(hydroxymethyl)ethyl ester-hexadecanoic acid, (5) Tetraicosane, and (6) 2,3-dihydroxypropyl ester-octadecanoic acid.

5. Conclusion

The findings from this work demonstrate that A. pluriseta root extract fractions have robust selective antitubercular activity. The extract fractions, especially the ethyl acetate and methanolic fraction, provide a potential source of novel, antitubercular lead candidate(s). GC-MS analysis revealed an abundance of fatty acid esters, which we strongly associated with demonstrated antitubercular activity. Further work is required to isolate pure compounds, test their specific molecular targets, with a view of deciphering the mode(s) of action.

Declarations

Author contribution statement

S.N. Njeru and J.M. Muema: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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