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Purification and cytotoxic assays of four antimicrobial metabolites extracted from Actinomycetes of the soils of Menengai Crater, Kenya

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Abstract

The search for antibiotic metabolites is increasing due to drug resistance been witnessed today. This study was conceived to isolate, identify and compare the antimicrobial metabolites yield of four actinomycetes isolated from Menengai crater soil, Kenya. The actinomycetes were isolated using starch casein, Luria Bertani (M1) and starch nitrate agar. The antimicrobial metabolites were extracted using standard techniques followed by purification, test for antagonism against selected pathogenic microbes, and cytotoxicity assay using Brine shrimp lethality test. Totally, 138 actinomycetes isolates were obtained from all the soil samples. Four isolates showed the highest potent potential against selected bacterial and fungal pathogens. The selected actinomycetes were coded; PAN 30, PAN 37, PAN 41, and PAN 154, and preserved at 4°C for further analysis. The difference in yield of the antimicrobial metabolites between liquid and solid-state fermentation was statistically significant (P= 0.005). Zones of inhibition did not vary significantly (F = 6.6046, P = 0.001338). The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC) of the antimicrobial metabolites extracted from PAN 30, PAN 37, PAN 41 and PAN 154 isolates were equal. The Half Lethal Concentration (LC_{50}) in (µg/ ml) for the antimicrobial metabolites extracted from the 4 isolates were; PAN 30 (1.8168 ± 0.47), PAN 37 (3.4269 ± 0.56), PAN 41 (3.4269 ± 0.45), and PAN 154 (4.9397 ± 0.41). The four actinomycetes produced bioactive metabolites. Solid-state fermentation was superior to liquid-state fermentation in recovering the antimicrobial metabolites from the actinomycetes isolates. Molecular identification of the selected isolates needs to be carried out in a future study. Moreover, there is a need to determine the structure of the antimicrobials using nuclear magnetic resonance.

Keywords: Actinomycetes; Antimicrobial activities; Bio-autography; Cytotoxicity

1. Introduction

Actinomycetes are a class of aerobic, spore forming, Gram positive bacteria. They belong to the order Actinomycetales (Khouloud and Ehab, 2015). Actinomycetes exhibit both substrate and surface growth on the growth media. According to 16S ribosomal cataloguing and DNA: rRNA pairing, actinomycetes have a high GC ratio in their DNA (>55 mol %) (Ankita and Hotam, 2017).

Actinomycetes are one of the largest taxonomic units in the 18 major lineages within the bacterial domain (Gebreselema *et al*., 2014). They are the leading sources of antibiotics (Loganathan *et al*., 2014) but due to increased drug resistance, the need for novel drugs has gained a lot of prominence among researchers (Astalakshmi *et al*., 2014). Currently, antimicrobials from natural sources have been replaced by synthetic drugs (Masud and Anwar, 2017). Reports from different parts of the world have shown that, the use of synthetic drugs led to an increase in drug resistance. Anwarul *et al*., (2016) added that synthetic drugs have more side effects when taken orally than those obtained from natural sources.

Moreover, actinomycetes are the leading producers of antitumor agents, enzymes, immunosuppressive agents, antifungals, neurigenics, antioxidants, anti-helmitics, anti-malarials, anti-algals, and anti-inflammatory agents (Mangamuri *et al*., 2016). Currently, researchers are targeting the use of antibiotics from actinomycetes in other areas such as boosting immunity in AIDS and Alzheimer diseases, in addition to their use as ant-ageing agents (Bandari *et al*., 2017).

One of the methods used to test the toxicity of an antibiotic is the Brine shrimp (*Artemia salina*) lethality test (Ankita and Hotam, 2017). Brine shrimps are commercially used to feed fish. The shrimps' are useful in energy flow of the aquatic food chain (Aref *et al*., 2017). The brine shrimp bioassay determines the toxicity of antibiotics through determination of lethality concentration (LC_{50}) (Sudha, 2015). In addition, brine shrimp bioassay is used to detect anti-tumoral compounds, and their potential in treating cancer (Hashem *et al*., 2016). Previous study of Saranya *et al*., (2017) indicated positive correlation between brine shrimp toxicity and 9KB (human nasopharyngeal carcinoma) cytotoxicity (P= 0.036). As a result, brine shrimp lethality test is an internationally accepted bioassay for screening of antitumor compounds.

Manivasagan *et al*., (2014) pointed that the advantages of brine shrimp bioassay lie in the fact that it is rapid (24 h), inexpensive and simple in that no aseptic techniques are required. This method utilizes large number of shrimps thus validating the data obtained. The assay does not require specialized equipment. Cibi and Jayakumaran, (2016) added that the method does not require animal serum as is the case with other cytotoxic assays.

Besides, brine shrimp lethality assay has gained a lot of importance in analyzing pesticide residues in vegetables, stream pollutants, anesthetics, dino-flagellates toxins, toxicity of oil dispersants, and mycotoxins among others (Soccol *et al*., 2017). The aims of this study were to isolate actimicrobial producing actinomycetes from the soils of Menengai crater, extract antimicrobial metabolites from four isolates of actinomycetes (coded PAN 30, PAN 37, PAN 41 and PAN 154), using liquid and solid-state fermentation. Moreover, this study aimed to purify the metabolites using Thin layer chromatography (TLC) and column chromatography, and finally to test the cytotoxicity of the metabolites using brine shrimp lethality bioassay.

2. Materials and methods

2.1. The study area

This study was conducted in Menengai crater which is positioned at the boarder of Rongai and Nakuru North sub-counties, Kenya. Underground geothermal activities increase the temperatures to 82^{o}C (Omenda *et al*., 2014).

2.2. Soil sampling

Soil samples were collected from 32 randomly selected points within the study area. These samples were mixed to make a composite sample. The composite sample was placed in sterile khaki bags, and transported to Egerton University, Department of Biological Sciences laboratories. Air drying of the sample was carried out for one week to minimize the number of bacteria and mold fungi.

2.3. Isolation of the Actinomycetes and screening for antimicrobial activity

One gram of soil sample was placed in a test tube containing 9ml distilled water, then shaken vigorously using a vortex mixer (PV-1, V-32, rpm 200) for 10 min. Then serial 10 fold dilutions were prepared till 10-6 (Sujatha and Swethalatha, 2017). Isolation of actinomycetes was carried out using starch casein, Luria Bertani and starch nitrate agar media using spread plate technique. Screening for antagonism against the selected pathogens was carried out using the method of Pooja *et al*., (2015). Based on broad spectra of antimicrobial activity and size of inhibition zones, four actinomycetes coded PAN 30, PAN 37, PAN 41 and PAN 154 were selected for further analysis.

2.4. Characterization of the selected actinomycetes

2.4.1. Gram's staining reaction

Gram staining technique of the selected actinomycetes isolates was carried out according to Pooja *et al*., (2015).

2.4.2. Use of Analytical profile index (API) strips

Analytical profile index (API) strips are used in identifying microorganisms using biochemical means. The technique has the advantage of being rapid; easy to use and economical, compared to other microbial identification techniques that utilize separate tubes and use different reagents. Using a sterile wire loop, each isolate was inoculated into Luria Bertani broth, and then at 28^{o}C for 2 days. Incubation was carried out at 28^{o}C for 7 d after inoculation of the analytical profile index strips with each culture (Bi *et al*., 2017).

2.5. Extraction of the crude metabolites

2.5.1. Liquid state fermentation

Each of the selected isolates was inoculated into 3000 ml Luria Bertani broth medium, and incubated at 28°C on a shaker (200 rpm) for 7 days. Sterile Millipore filters were used to filter the broths, followed by storage at 4°C till further assays. Equal volumes (300 ml) of ethyl acetate which has high polarity and therefore high affinity to the antimicrobials were added to each filtrate followed by centrifugation at 5000 rpm for 10 min., to extract the antimicrobials (Noureen *et al*., 2016). The extracted antibiotics were concentrated by evaporating the solvent using a vacuum evaporator (HeidolphLaborota, 4001, Büchi Vacuum Controller, V-805), dried at 40± 2^{o}C, and then net weights were determined according to Bi *et al*., (2017).

2.5.2. Solid state fermentation

Briefly, each of the four selected isolates PAN 30, PAN 37, PAN 41 and PAN 154 was plated on Luria Bertani agar medium. The plates were incubated at 28°C for 7d. The agar medium containing the growing actinomycetes were cut into small pieces, and then separately placed into conical flasks (Sujatha and Swethalatha, 2017). About 3000 ml ethyl acetate was added. These flasks were placed on an orbital shaker for 2h at 200 rpm. Ethyl acetate phase was evaporated and the metabolites were concentrated using a vacuum evaporator at 40 ± 2^{o}C, and then weights were determined according to Pooja *et al*., (2015).

2.6. Antagonistic potency of the antimicrobials

According to Jayshree *et al*., (2017), filter paper disks 8mm in diameter were dipped into the extracted metabolites, and then air dried. These disks were aseptically placed on Mueller-Hinton agar plates (MHA) seeded with *Staphylococcus aureus*, *Bacillus subtilis*, *Enterococcus faecalis*, *Escherichia coli*,

Klebsiella pneumoniae, *Salmonella typhi* and *Xanthomonas campestris*, and on sabouraud dextrose agar (SDA) seeded with *Candida albicans*, *Alternaria citri*, and *Fusarium oxysporum* obtained from Kenya Medical Research Institute. Plates of MHA were incubated at 37°C for 48h; whereas, those of SDA were incubated at 28°C for 4 days. After incubation, zones of growth inhibition were measured using a ruler in millimeters.

2.7. Purification of the antimicrobials by column chromatography

Silica gel column chromatography (SRL, Mumbai) was used to purify of the antimicrobial metabolites. The column (35 × 10 mm) was rinsed with acetone and then allowed to dry. Silica gel was packed in the column using ethyl acetate: ethanol: water (4:4:2) as solvent system. The antimicrobial metabolites from the four actinomycetes were separately loaded at the top of the column and eluted using ethyl acetate: ethanol: water (4:4:2). Thirty fractions were recovered at 20 min. interval. These fractions were tested for antimicrobial activities using the agar well diffusion technique (Jayshree *et al*., 2017). The fractions showing antimicrobial activities were confirmed using TLC and bio-autography.

2.8. Testing of the components of crude metabolites using TLC

TLC silica gel 60 F254 aluminum sheets (Merck) were used to test the number of components in the antimicrobial metabolites. The crude antimicrobial extracts were diluted to 1 mg/ ml and then 5 µl of each antimicrobial extract was separately spotted on the TLC sheets in 5 replicates using capillary glass tube. The TLC development jars were saturated with the mobile phase composed of ethyl acetate, ethanol and water (4:4:2), then the TLC sheets were placed in these jars. After the final solvent fronts were marked, the TLC sheets were allowed to dry. These TLC sheets were observed under UV light (254 nm, and 366 nm). The retention factor (R_f) of the spot was calculated using the following formula (Bi *et al*., 2017):

$$Rf = \frac{\text{Distance moved by the spot}}{\text{Distance moved by the solvent front}}$$

2.9. Bio-autography of the extracts

The developed TLC sheets loaded with the crude extracts before and after column chromatography were dried. These sheets were placed in previously sterilized Petri dishes. About 20 cm^3 sterile MHA was separately placed in 4 test tubes. These test tubes were seeded with 1 ml of standard *S. aureus* whose growth concentration had been previously standardized to (6×10^6 cells/ ml) using McFarland standard. The inoculum and media were mixed and then separately poured into the Petri dishes with the developed TLC plates. The Petri dishes were incubated at 37°C for 24 h after solidification of the media. These plates were checked for development of antagonistic inhibition zone against the tested pathogen as stated by Murukesan *et al*., (2015).

2.10. Determination of the Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of the pure antimicrobial metabolites was determined by Broth tube dilution procedure using two-fold dilution in nutrient broth (NB) and sabouraud dextrose broth (SDB) for bacteria and fungi, respectively, according to (Jayshree *et al*., 2017). In this method bacterial pathogens; *S. aureus*, *B. subtilis*, *E. faecalis*, *E. coli*, *K. pneumoniae*, *S. typhi* and *X. campestris*, and fungal species; *Candida albicans*, *Alternaria citri* and *F. oxysporum* were used. For each bacterium, 12 sterile screw capped test tubes were used. In each tube, a volume of 1 ml NB was dispensed into test tubes from (1-10), and 2 ml into test tube no. (11) (broth control). On the other hand, 1 ml of each crude antimicrobial extract solution was added into test tubes no. 1, and 2 ml to test tube no. (12) (crude extract control). One ml of well mixed solution was transferred from test tube 1

- 2, and this process was continued serially up to the test tube no. (10) through mixing. Finally, 1 ml was discarded from test tube no. (10). 0.1 ml of standardized inoculum of each bacterial pathogen was added separately into test tubes no. (1-10). This was repeated for the fungal pathogens using SDB. These tubes were incubated at 37°C for 24 h for bacteria, and 7 d for fungi at 28-30°C. After incubation, MIC values were determined by observing the least concentration of the antimicrobial metabolites showing growth of the tested microorganisms in the test tubes.

2.11. Determination of microbicidal and microbiostatic activities of the antimicrobials

The antimicrobial metabolites were separately used to prepare stock solutions using ethyl acetate, filtered and sterilized through 0.2 mm filter (Sartorius, India) to avoid contamination. The appropriate concentration of the antimicrobials was made by serial dilution up to 10^{-10} in test tubes. The test tubes were inoculated with 0.1 ml of the test pathogens, and then incubated at 37°C for 24 h for bacteria and 7 d at 28°C for fungi. These tubes were examined for development of turbidity after the incubation period. If turbidity occurred, these test microorganisms were resistant to the antibiotics; however if no turbidity occurred, thus it was considered sensitive. The Minimum bactericidal concentration (MBC) and Minimum fungicidal concentration (MFC) values of the antimicrobials were determined by sub-culturing 50 µl from tubes showing no visible turbidity; and then inoculating them onto MHA and SDA, for bacteria and fungi, respectively. The MBC and MFC values were determined as the lowest concentration of the antimicrobial metabolite that prevented growth of pathogens, after sub-culturing of 50 µl of suspension from tubes showing no visible turbidity in MIC assay on growth media (Prashith *et al*., 2017).

2.12. Determination of cytotoxic activity of the antimicrobial metabolites

The cytotoxic activity of the antimicrobial extracts was evaluated using the Brine shrimp lethality test, to determine the nature of these crude extracts according to Soccol *et al*., (2017). The brine shrimp (*Artemia salina*) eggs (Nihon Animal Pharmaceutical Inc., Tokyo, Japan) were hatched in a container filled with air bubbled artificial sea water, prepared from 10 g of a commercial salt mixture (Gex Inc., Osaka, Japan) and 500 ml of dist. water. The phototrophic shrimps were collected after 48 h using a pipette for the bioassay. 1 ml of different concentrations of each antimicrobial extract (0.5-1000 µg/ ml) were added into vials containing 5 ml of concentrated sea salt (brine) and 10 shrimps counted using a dissecting microscope. These vials were incubated at 25°C, and then surviving shrimps were counted after 24 h. The half lethal concentration (LC_{50}) value was calculated using regression and probit analysis (Noureen *et al*., (2016).

2.13. Statistical analysis

SPSS version 2017 was used for data analysis. The antimicrobial yields from the 4 selected actinomycetes isolates; and comparison of Rf's obtained from TLC and bio-autography, were determined using the t-test. Analysis of data from MIC, MBC and MFC was carried out using single factor ANOVA. Data on brine shrimp lethality test was analyzed using regression and probit analysis.

3. Results

3.1. Population of the Actinomycetes in Menengai crater soils

The number of actinomycetes isolated using starch casein (SC), Luria Bertani (M1) and starch nitrate (SN) agar media did not vary significantly ($F = 3.622$; $P = 0.52$). Totally, 138 actinomycetes isolates were recovered from the soils samples of the studied area. Their count ranged from 2 ± 0.3 - 9 ± 0.2 on starch casein agar (SC), Luria Bertani agar (M1) (5 ± 0.3 - 14 ± 0.3), and starch nitrate (SN) (4 ± 0.3 - 14 ± 0.2) (Table 1).

3.2. Characteristics of the selected actinomycetes isolates using API strips

All the selected isolates tested positive for Gram stain, catalase, oxidase, urea hydrolysis and gelatin liquefaction (Table 2). They were all negative for deaminase, Indole production, β-galactosidase and lysine decarboxylase. Isolates PAN 30 and 154 tested positive for ornithine decarboxylase and citrate utilization while PAN 37 and 41 were negative. In addition, PAN 37, PAN 41 and PAN 154 tested positive for hydrogen sulphide production while PAN 30 tested negative.

Table 1: Total count of Actinomycetes isolated from soils of the studied area

Dilution factor	**Count of Actinomycetes on three cultivation media**		
	Starch casein (SC)	Luria Bertani (M1)	Starch nitrate (SN)
10^{-3}	9± 0.2	14± 0.3	14± 0.2
10^{-4}	6± 0.2	12± 0.2	10± 0.2
10^{-5}	4± 0.3	10± 0.2	7± 0.2
10^{-6}	3± 0.2	8± 0.3	8± 0.3
10^{-7}	3± 0.3	7± 0.2	5± 0.2
10^{-8}	2± 0.3	5± 0.3	4± 0.3
Mean	**4.5± 0.3**	**9.3± 0.2**	**8.0± 0.2**

Where; values after ± represent the standard deviation of the independent experiments

Table 2: Biochemical characteristics of the selected actinomycetes

Isolate	**Biochemical test**											
	GS	ONPG	CAT	GLU	LDC	ODC	CIT	H_2S	URE	TDA	IND	GL
PAN 30	+	-	+	+	-	+	+	-	+	-	-	+
PAN 37	+	-	+	+	-	-	-	+	+	-	-	+
PAN 41	+	-	+	+	-	-	-	+	+	-	-	+
PAN 154	+	-	+	+	-	+	+	+	+	-	-	+

Where; GS: Gram stain, ONPG: beta-galactosidase, CAT: catalase test, GLU: oxidase, LDC: lysine decarboxylase, ODC: ornithine decarboxylase, CIT: citrate utilization, H2S: Hydrogen sulphide production, URE: urea hydrolysis, TDA: deaminase, IND: Indole production, GL: Gelatin liquefaction.

3.3. Yield of the crude antimicrobial metabolites

The crude antimicrobial yield between liquid and solid-state fermentation varied significantly (P= 0.005). These were evaluated according to the net weight of the dried antimicrobials, after extraction and evaporation. In liquid state fermentation, PAN 154 isolate was the highest producer (259.0 mg) of the antimicrobials, followed by PAN 41 (258.0 mg), PAN 30 (2536 mg), and PAN 37 (2314 mg). The antimicrobials produced had different colors after re-suspension in ethyl acetate solvent (Fig. 1). However; in solid state fermentation, the highest yield was recorded from the 4 selected isolates in a descending order; PAN 154 (753.0 mg), PAN 41 (726.0 mg), PAN 30 (700.0 mg), and lastly PAN 37 (680.0 mg) as clear in Fig. (2).

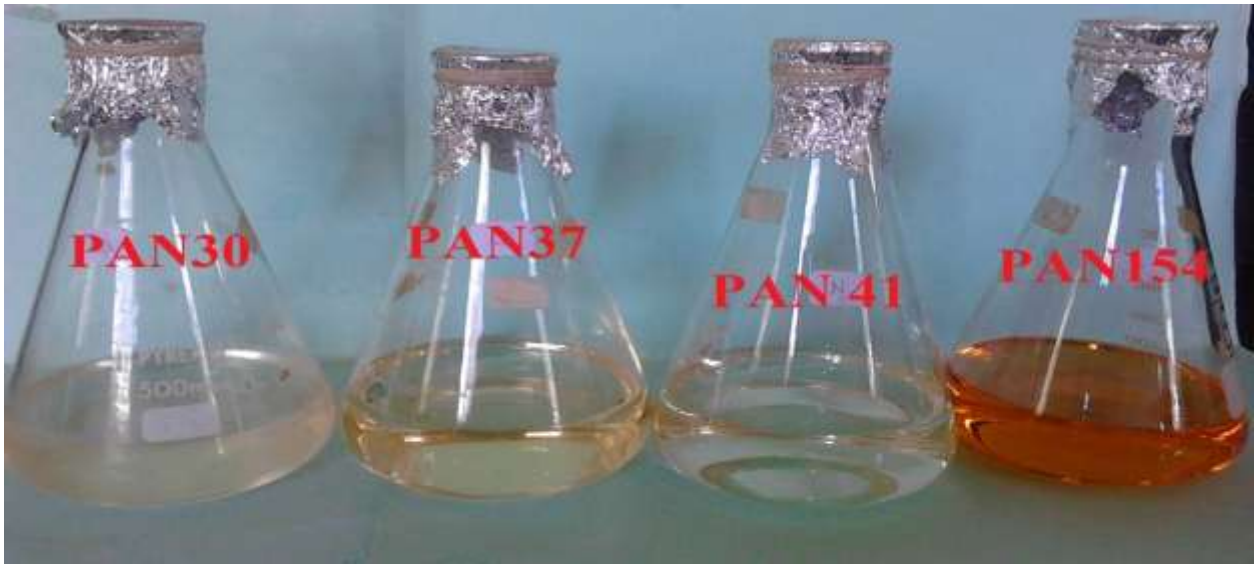

Fig. 1: Antimicrobials crude extracts from the 4 selected actinomycetes isolates; PAN 30, PAN 37, PAN 41, and PAN 154, suspended in ethyl acetate extracting solvent

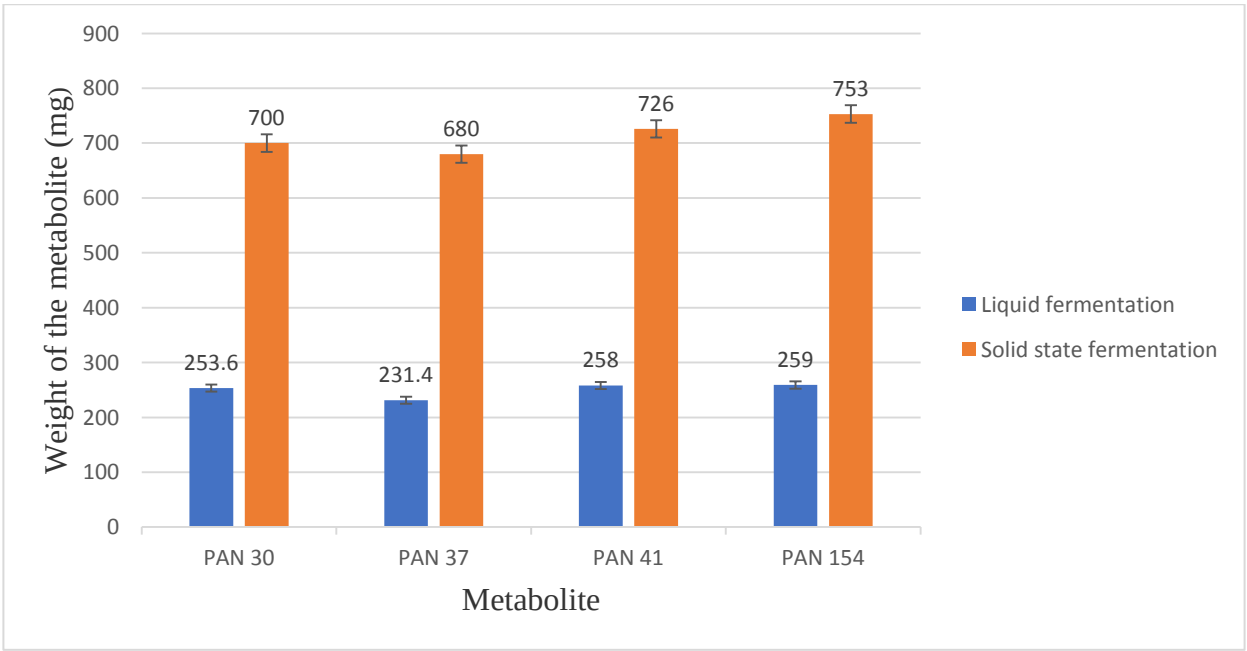

Fig. 2: Weight of the antimicrobial metabolites (mg) extracted from the 4 selected actinomycetes isolates; PAN 30, PAN 37, PAN 41 and PAN 154, using liquid and solid-state fermentations

3.4. In vitro antimicrobial potential of the extracted metabolites against the tested microbial strains

The zones of inhibition produced by the antimicrobial extracts varied significantly (F = 6.6046, P = 0.001338). The highest diameter of inhibition zone was recorded by PAN 41 (49 ± 0.3 mm). This was followed by; PAN 30 (35.36 ± 0.2 mm), PAN 37 (34.09 ± 0.2 mm), and 154 (26.27 ± 0.2 mm), respectively (Table 3a, b). The highest sensitivity was recorded by *E. coli* (49 ± 0.3mm) against antimicrobials of PAN 41 while the lowest was recorded by *B. subtilis* (20 ± 0.3mm) exhibited by PAN 154 metabolites.

3.5. Components of the antimicrobial metabolites crude extracts

The Rf's produced by column chromatography and TLC did not vary significantly (P= 0.3143). In column chromatography, the most active fractions against the test pathogens ranged from 5 to 10. The Rf's produced by TLC were PAN 30 (0.87), PAN 37 (0.83), PAN 41 (0.86), and PAN 154 (0.82), while those given by bio-autography were PAN 30 (0.87), PAN 37 (0.85), PAN 41 (0.86), and PAN 154 (0.83) (Fig. 3). The bio-autography bioassay indicated that all the extracts produced a single active spot after using ethyl acetate, ethanol and water (4:4:2) as the solvent system (Fig. 4).

Table 3a: Diameters of zones of inhibition (mm) of the selected pathogenic strains produced by ethyl acetate extracts of the 4 selected actinomycetes isolates

Extracts	Zones of inhibition (mm)					
	S. aur	*B. sub*	*E. faec*	*E. col*	*K. pne*	*S. typ*
PAN 30	35± 0.2	42± 0.1	41± 0.3	47± 0.3	28± 0.2	37± 0.3
PAN 37	32± 0.1	41± 0.2	38± 0.2	47± 0.1	25± 0.2	36± 0.3
PAN 41	39± 0.1	40± 0.1	45± 0.3	49± 0.3	35± 0.2	43± 0.3
PAN 154	26± 0.2	20± 0.3	25± 0.2	24± 0.2	25± 0.3	28± 0.2

-Values after ± represent the standard deviation of the independent experiments. Where; *S. aur*; *Staphylococcus aureus*, *B. sub*; *Bacillus subtilis*, E. fae; *Enterococcus faecalis*, *E. col*; *Escherichia coli*, K. pne; *Klebsiella pneuminiae*, S. typ; *Salmonella typhi*.

Table 3b: Diameters of zones of inhibition (mm) of the selected pathogenic strains produced by ethyl acetate extracts of the 4 selected actinomycetes isolates

Extracts	Zones of inhibition (mm)				
	X. cam	*C. alb*	*A. citri*	*F. oxy*	Mean
PAN 30	28± 0.2	30± 0.3	29± 0.1	32± 0.2	35.36± 0.2
PAN 37	32± 0.3	30± 0.1	27± 0.2	26± 0.3	34.09± 0.2
PAN 41	38± 0.2	25± 0.2	30± 0.3	33± 0.1	37.64± 0.2
PAN 154	25± 0.3	28± 0.2	31± 0.2	30± 0.1	26.27± 0.2

-Values after ± represent the standard deviation of the independent experiments. Where; *X. cam*; *Xanthomonas campestris*, *C. alb*; *Candida albicans*, *A. citri*; *Alternaria citri*; *F. oxy*; *Fusarium oxysporum*.

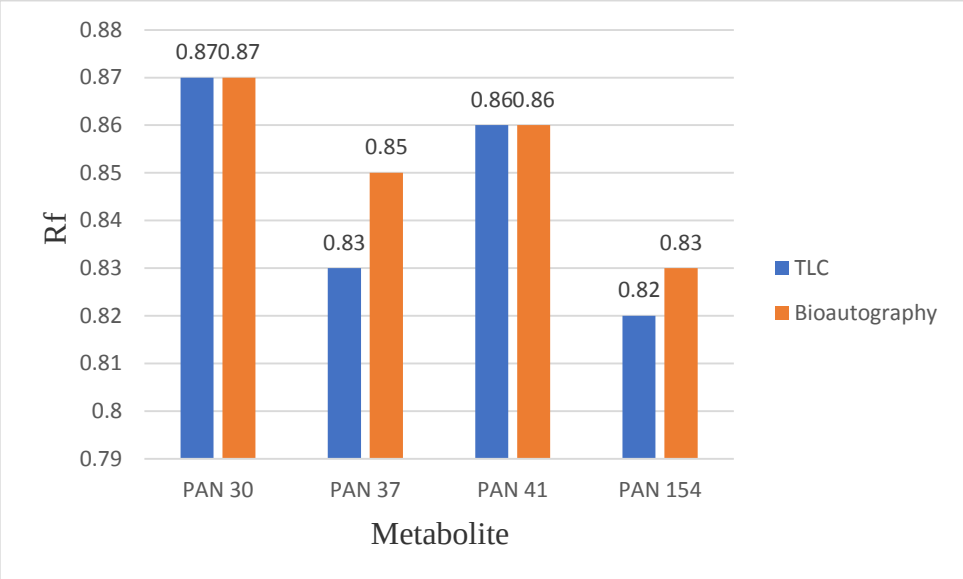

Fig. 3: Retention factor (Rf) values of the selected actinomycetes isolates; PAN 30, PAN 37, PAN 41 and PAN 154, based on TLC and Bio-autography

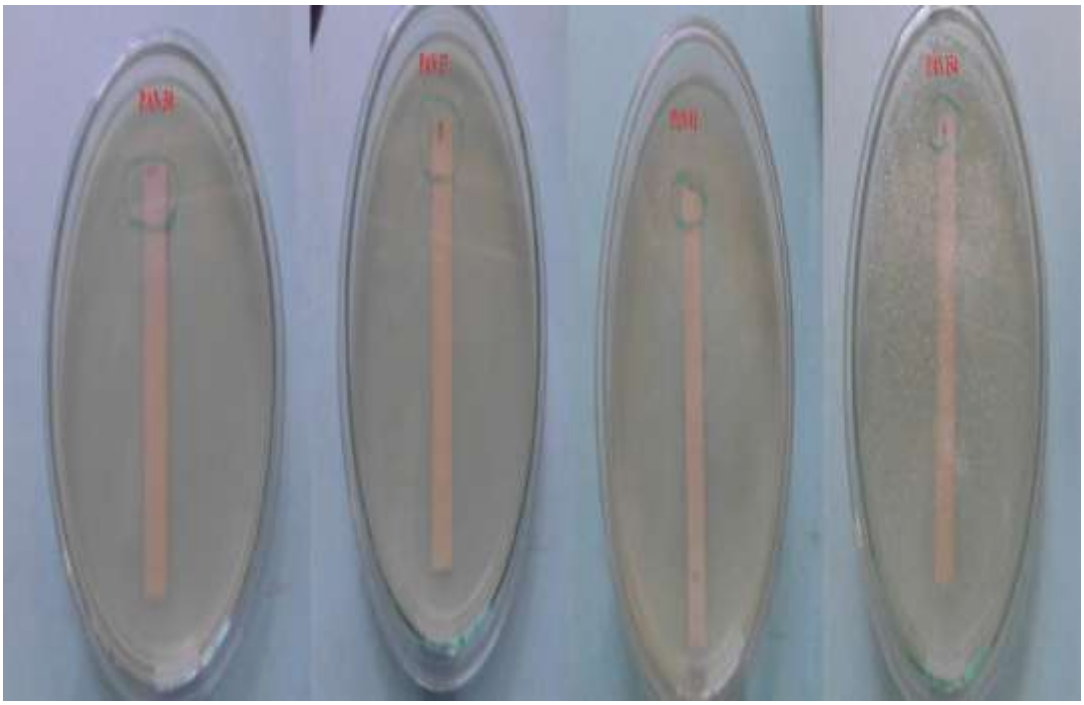

Fig. 4: Bio-autography of the 4 selected actinomycetes isolates; PAN 30, PAN 37, PAN 41, and PAN 154

3.6. MIC, MBC, and MFC, of the extracted antimicrobial metabolites

There were no significant differences among the MIC, MBC, and MFC (F = 0.4503, P = 0.7187). Among the Gram-positive bacteria; *Staph. aureus* had MIC ranged from 0.06 ± 0.2 in PAN 37, to 0.13 ± 0.2 in PAN 41; in *B. subtilis* the MIC's ranged from 0.06 ± 0.1 in PAN 37, to 0.13 ± 0.1 in PAN 41; whereas, in *E. faecalis* the range was from 0.06 ± 0.1 in PAN 30 and PAN 41; while being 0.13 ± 0.1 in PAN 30 and PAN I54 (Table 4). However, in Gram negative bacteria; *E. coli* MIC's varied from (0.06 ± 0.2 in PAN 37, 0.25± 0.1 in PAN 154); *K. pneumoniae* (0.13 ± 0.1 in PAN 30 and PAN 154; 0.25 ± 0.2 in PAN 37 and PAN 41), in *S. typhi* (0.06 ± 0.1 in PAN 37; 0.13 ± 0.2 in PAN 30 and PAN 41), and in *X. campestris* (0.13 ± 0.2 in PAN 41; 0.25± 0.2 in PAN 30, PAN 37, and PAN 154). In fungi, the MIC's in *C. albicans* ranged from (0.06 ± 0.1 in PAN 30; 0.13 ± 0.2 in PAN 37); *F. oxysporum* (0.13 ± 0.1 in PAN 37 and PAN 154; 0.25 ± 0.2 in PAN 41; *A. citri* (0.13 ± 0.1 in PAN 30 and PAN 41; 0.25 ±0.1 in PAN 154). The MIC values were equal to the MBC and MFC values.

3.7. Cytotoxic activity of the antimicrobial metabolites

The cytotoxic activity of antimicrobials extracts of the selected isolates was investigated against brine shrimp. In each extract, the antimicrobial concentration ranged from 0.5- 31µg (Table 5). The % of mortality of the shrimps varied among the extracts such as; PAN 30 (28 - 100%); PAN 37 (14 - 100%); PAN 41 (5 - 100%); and PAN 154 (9 - 100%). The LC_{50} values were; PAN 30 (1.8168 ± 0.47 µg); PAN 37 (3.4269 ± 0.56 µg); PAN 41 (3.4269 ± 0.45 µg); and PAN 154 (4.9397 ± 0.41 µg).

Test Pathogens	MIC's\ MBC\ MFC (mg/ml)			
	PAN30	PAN37	PAN41	PAN154
S. aureus	0.13± 0.1	0.06± 0.2	0.13± 0.2	0.13± 0.1
B. subtilis	0.06± 0.2	0.06± 0.1	0.13± 0.1	0.06± 0.2
E. faecalis	0.06± 0.1	0.13± 0.1	0.06± 0.1	0.13± 0.1
E. coli	0.13± 0.2	0.06± 0.2	0.13± 0.2	0.25± 0.1
K. pneumoniae	0.13± 0.1	0.25± 0.2	0.25± 0.1	0.13± 0.2
S. typhi	0.13± 0.2	0.06± 0.1	0.13± 0.2	0.13± 0.1
X. campestris	0.25± 0.2	0.25± 0.1	0.13± 0.2	0.25± 0.2
C. albicans	0.06± 0.1	0.13± 0.2	0.06± 0.2	0.06± 0.2
F. oxysporum	0.13± 0.2	0.13± 0.1	0.25± 0.2	0.13± 0.1
A. citri	0.13± 0.1	0.12± 0.2	0.13± 0.1	0.25± 0.1

-Each value (±) represents the standard deviation (SD) of the *in vitro* assays. Where; *S. aureus*; *Staphylococcus aureus*, *B. subtilis*; *Bacillus subtilis, E. faecalis*; *Enterococcus faecalis, E. coli*; *Escherichia coli, K. pneumoniae*; *Klebsiella pneuminiae, S. typhi*; *Salmonella typhi, X. campestris*; *Xanthomonas campestris, C. albicans*; *Candida albicans, F. oxysporum*; *Fusarium oxysporum; A. citri*; *Alternaria citri*. MBC; applies to bacterial pathogens; whereas, MFC applies to fungal pathogens.

Table 5: LC_{50} values of antimicrobials crude extracts on brine shrimp lethality bioassay

Extract	Sample conc. (µg/ ml)	% Mortality	LC_{50} (µg/ ml)
PAN 30	0.5	28	1.8168±0.47
	1	31	
	2	50	
	4	59	
	8	64	
	16	100	
	31	100	
PAN 37	0.5	14	3.4269±0.56
	1	25	
	2	60	
	4	67	
	8	100	
	16	100	
	31	100	
PAN 41	0.5	5	3.4269±0.45
	1	15	
	2	26	
	4	33	
	8	84	
	16	92	
	31	100	
PAN 154	0.5	9	4.9397±0.41
	1	14	
	2	16	
	4	33	
	8	50	
	16	64	
	31	99	

Where; Conc.: concentration; LC_{50}: Half lethal concentration at 50 % mortality of brine shrimps

4. Discussion

In the current study, Starch casein, Luria Bertani (M1) and Starch nitrate agar media, were used for the isolation of actinomycetes. Luria Bertani agar was the best medium for recovering actinomycetes from the soils of Menengai crater based on the number of the actinonmycetes isolates recovered. This contradicted studies carried elsewhere in which starch casein agar was the best isolation medium of actinomycetes (Attimarad *et al*., 2012; Al-Hulu, 2013; Ahmed *et al*., 2016). This difference might be attributed to the variations in the environmental conditions of the studied areas. Menengai crater presents high temperatures with varying soil physico-chemical characteristics. This implied a possibility of variations in actinomycetes nutrients requirements, which determines the number of the recovered isolates (Deshmukh, and Vidhale, 2014).

Results for biochemical tests obtained in the present study disagreed with those of previous studies carried out in other regions (Astalakshmi *et al*., 2014; Kothagorla *et al*., (2017). Garima and Jugmendra, (2016) asserted that actinomycetes showed varying biochemical reactions which influenced their classification.

According to Gebreselema *et al*. (2014), solid state fermentation of actinomycetes was important for

the production of the antimicrobial metabolites. In addition, some actinomycetes do not produce antimicrobials in liquid media (Loganathan *et al*., 2014). The current study demonstrated a significant difference in the amount of antimicrobials produced in liquid, and in solid-state fermentation of the isolates. In addition, Deshmukh and Vidhale, (2014) asserted that agar has some nutritional properties that favor production of antimicrobials, which might have led to the observed results. Moreover, yield of the antimicrobial metabolites obtained in this study was higher than in previous ones (Khouloud and Ehab, 2015; Savi *et al*., 2017).

Zones of inhibition obtained in this study were bigger than those obtained in previous studies carried out in other regions (Polpass *et al*., 2013). Ilayaraja *et al*. (2014) reported that zones of inhibition exhibited by antimicrobials from actinomycetes were determined by the strains of the actinomycetes from which they were obtained. However, Mangamuri *et al*., (2016) pointed that the environment in which the actinomycetes grow determines their biochemical activities, a factor that influences the types of the produced antimicrobials.

A study carried out in India by Murukesan *et al*. (2015), obtained R_f values of 0.11 and 0.29. These results disagreed with those of the present study. The differences could be attributed to variations in mobility of the antimicrobial metabolites (Khattab *et al*., 2016). In addition, differences in the mobile phase used in running of TLC may be a contributing factor (Savi *et al*., 2017).

In the study of Ekundayo and Faniomi, (2017), results of MICs obtained for *E. faecalis* was (19.69 ± 0.01mg/ ml); E. coli (19.64 ± 0.02 mg/ ml); *Staph. aureus* (21.31 ± 0.01 mg/ ml); and *P. aeruginosa* (17.21 ± 0.02 mg/ ml). These MICs were higher than those of the present study. In addition; Gamal *et al*., (2017) obtained fungal MICS for *C. albicans* (23± 0.23 mm); *A. niger* (15± 0.29 mm); and *F. oxysporum* (20± 0.25 mm). These contradicted the results of the current study which might be due to differences in the environmental conditions of the studied areas, which led to production of different antimicrobials. The MICs obtained in this study were equal to the MBCs and MFCs. This suggested that antimicrobial metabolites from the 4 selected actinomycetes isolates (PAN 30, PAN 37, PAN 41 and PAN 154), had bactericidal and fungicidal potentials in accordance with Khattab *et al*., (2016).

The brine shrimp lethality test revealed that antimicrobial from the extracts of the 4 actinomycetes isolates were biologically active. The mortality of the brine shrimps increased with increasing concentration of the antimicrobial metabolites. This concurred with previous studies (Kumar et al., 2014; Madhusudhan *et al*., 2014; Haque *et al*., 2016). A previous study carried out by Haque *et al*., (2016) to evaluate the antifungal, hemolytic and cytotoxic potential of ethyl acetate extract of a new marine *Streptomyces* sp., recorded LC_{50} of 17.78 µg/ ml, which was higher than that of the current study. Kiruthika and Bhuminathan, (2015) obtained an LC_{50} of 0.15µg/ ml which did not coincide with the results of the present study. The differences might be attributed to variations in the extracted antimicrobials.

Conclusion

Soils samples collected from Menengai crater had actinomycetes, which produced broad spectrum antimicrobial metabolites. Solid state fermentation was the best technique for obtaining these antimicrobials from extracts of the 4 selected actinomycetes isolates. These antimicrobials exhibited a single spot with antimicrobial property on TLC sheets. The MIC, MBC and MFC concentrations were the same, thus these antimicrobial metabolites has microbicidal and fungicidal activities. Results of the brine shrimps toxicity assay suggested that the antimicrobials have the potential to exhibit antitumor and or anti-cancerous properties.

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Conflict of interest

No conflict of interests has been declared by the authors.

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