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RESEARCH ARTICLE

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Determination of *In Vitro* lethality of actinomycetes antibiotic extracts from Menengai crater geothermal vents, Kenya

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A R T I C L E I N F O

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A B S T R A C T

Introduction: Drug resistance is the biggest threat to human existence today. This study was conducted to investigate the sensitivity of selected pathogenic microorganisms to antibiotic extracts from soils of Menengai crater geothermal vents in Kenya.
Method: Antibiotic-producing actinomycetes were isolated from geothermal vents of the crater. The isolates were identified using colony characteristics and biochemical means. Sensitivity of the selected pathogenic microorganism was carried out using primary and secondary screening techniques. Four actinomycetes coded PAN 9, PAN 18, PAN 117, and PAN 138 were selected for further analysis based on the size of the zone of inhibition and the broad spectrum of activity. Extraction of antibiotics from the selected actinomycetes was carried out using ethyl acetate. A sensitivity test of the pathogens to the extracts was done using Karby Bauer disk diffusion technique. Cytotoxicity of the antibiotic extracts was carried out using Brine shrimp lethality test and sheep blood hemolytic bioassays. The isolates presented typical actinomycetes characteristics.
Results: A total of 20 actinomycete isolates showed antagonism against the test pathogenic microorganisms. The selected actinomycetes inhibited growth of Gram-positive and negative bacterial and fungal pathogens in both primary and secondary sensitivity tests. There was a significant difference in the diameters of zones of inhibition produced by the test pathogens when subjected to the antibiotic metabolites from the selected actinomycetes ($F = 6.6046$ $P = 0.001338$).
Discussion: The minimum inhibitory concentration and minimum bactericidal concentration/minimum fungicidal concentration did not vary significantly ($F=0.4503$, $P = 0.7187$). The LC_{50} values of the antibiotic extracts varied from 2.438±0.47µg in PAN 9 to 9.3455±0.41µg in PAN 138. PAN 18 and PAN 117 showed partial hemolysis while PAN 9 and PAN 138 exhibited complete hemolysis of sheep blood erythrocytes. Soils from Menengai crater geothermal vents have actinomycetes that produce antibiotics. The antibiotics are cytotoxic to brine shrimps. In addition, the antibiotics cause hemolysis of sheep blood erythrocytes. There is a need to purify the antibiotics and structure elucidate them. The active antibiotic extract needs to be evaluated to determine whether the hemolysis is due to the compound itself or some other chemical constituent.

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Introduction

Menengai crater is a product of volcanic eruption (Omenda *et al*., 2014). It has vents that produce geothermal power. Elevated temperatures, varying soil conditions, and strained nutrients are some of the conditions microorganisms must cope with in such conditions (Waithaka *et al*., 2020).

Actinomycetes are typically Gram-positive bacteria with a high G + C in their genome (Al-Ansan *et al*., 2020). Further, actinomycetes are characterized by the possession of LL-diaminopimelic acid and lack diagnostic sugars in the entire cell hydrolysates (Benhadi *et al*., 2019). They have diverse metabolic pathways which accord them the ability to produce a vast array of bioactive secondary metabolites (*Li et al*., 2016). Indeed, over 70 % of the antibiotics in the market today have been obtained from actinomycetes (Low *et al*., 2015).

The current increase in drug resistance demands new sources of antibiotics (Omeke *et al*., 2018). To increase the chances of getting novel antibiotics, exploration of antibiotics producing actinomycetes from unique environments is inevitable (Salim *et al.,* 2017). This inspired the choice of Menengai crater geothermal vents as a source of antibiotic-producing actinomycetes.

Pure and crude antibiotic extracts can be tested for bioactivity using brine shrimp lethality bioassay (Kekuda *et al.,* 2013). The shrimps are a convenient monitor for screening in the discovery of novel bioactive compounds (Maher, 2017). The bioassay is also used as an indicator of anticancer, antiviral, and cytotoxicity of a wide range of pharmaceutical compounds (Lertcanawanichakul *et al.,* 2021).

Erythrocytes are the most abundant cells in all mammals owing to their own replicative biological and morphological characteristics (Almuhayawi *et al.,* 2021). The hemoglobins and polyunsaturated fatty acids (PUFA) target the erythrocytes due to their redox-active oxygen transportation feature (Mogrovojo *et al.,* 2020). This predisposes erythrocytes to hemolysis (Berezin *et al*., 2019). Hemolysis is accompanied by several factors such as deficiencies in erythrocyte antioxidant coordination, radiation, high quantity of transition metals, oxidative drugs, and hemoglobinopathies (Ser *et al.,* 2016). The rate of hemolysis is higher when erythrocytes are exposed to toxins like hydrogen peroxide (Lin *et al*., 2020).

The use of hemolytic bioassay has proved to be an alternative screening method for simple toxicity (Wei *et al*., 2012). The bioassay is fast, reproducible, and inexpensive in testing hemolytic activity against concentrations of crude antibiotic extracts. This makes it possible to reduce the use of laboratory animals for *in vivo* tests (Warrand *et al.,* 2020).

This study aimed at isolating antibiotic-producing actinomycetes from Menengai crater, determining the sensitivity of the selected pathogens to antibiotic crude extracts from the actinomycetes isolates, and evaluating the cytotoxicity of the antibiotic extracts.

Materials and methods

Description of the study area

Soil sampling was carried out in the Menengai crater which lies North of Lake Nakuru, Kenya (Figure 1). The crater rises to a height of 2,278m above sea level and is a product of a volcanic eruption (Omenda *et al*., 2014). The crater is dormant with a high-temperature geothermal resource exhibited by steaming grounds at a temperature of 88°C (Omenda *et al*., 2014). After the eruption, the sides of the volcanic crater collapsed inwards forming a caldera. The caldera occupies an area of 90km^{2}. Menengai crater presents the second largest caldera in Africa after Ngorongoro in Tanzania (Wanjohi and Kwasira, 2016). The crater lies between Rongai and Nakuru North Sub-counties at 35° 28', 35° 36'E, and 0° 13', 1° 10'S (Waithaka *et al*., 2015).

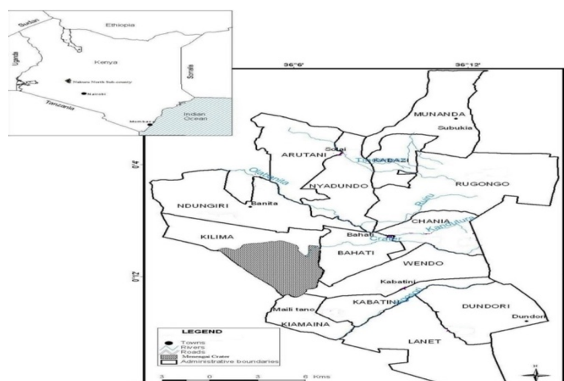

Figure 1: Map of Kenya showing the location of Nakuru North Sub-county and Menengai crater. Source; Waithaka *et al*., 2015.

Collection and processing of soil samples

Briefly 200 g of soil samples were collected from each the geothermal vents using a soil auger from the top 5 cm. The soil samples were mixed to make a composite sample (Chen *et al*., 2022). The composite sample was packed in new polythene bags and immediately carried to the laboratory. The sample was placed in sterile khaki bags and stored at 4 °C awaiting further processing and isolation of actinomycetes (Wekesa *et al*., 2016).

Isolation of actinomycetes

About 10 g of the composite soil sample was placed on aluminium foil and heat treated at 100 °C for 1 h in a hot air oven (Heidolph Laorota, 4001, Buchi Vacuum Conroller V-805). One gram of the composite soil sample was sprinkled on sterile Luria Bertani (M1) agar in Petri dishes (Ahmed, 2017). The plates were incubated at 28 ± 2 °C for seven days. The number of actinomycetes colonies per gram of soil was determined using a colony counter. Colonies showing typical actinomycetes characteristics were sub-cultured on M1 medium. Pure cultures were preserved on M1 medium slants and 50 % glycerol at - 4 °C (Lewin *et al*., 2016). The isolates were characterized using morphological and biochemical means.

Test pathogenic microorganisms

Methicillin-resistant *Staphylococcus aureus* (ATCC 25923), *Streptococcus pneumoniae* (ATCC 49617), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Proteus vulgaris* (ATCC 49990) were obtained from Kenya Medical Research Institute (KEMRI). *Alternaria citri* (ATCC 1015), *Candida albicans* (ATCC 10231), *Fusarium oxysporum* (ATCC 16608), and *Ustilago maydis* (ATCC 14826) were acquired from the Culture Collection Centre of the Department of Crop Science and Crop Protection, University of Nairobi.

Primary screening of actinomycetes for antagonism to test microorganisms

The antagonistic activity of the isolated actinomycetes was determined by the cross streak method (Mohammadipanah and Wink, 2015). Mueller-Hinton agar plates and sabouraud dextrose agar (SDA) were prepared and inoculated with isolated actinomycete cultures by a single streak. Incubation was carried out at 28 °C for 5 d. The Mueller-Hinton plates were inoculated with the bacterial test microorganisms while potato dextrose agar plates were seeded with the fungal pathogens by a single streak perpendicular to the actinomycete strains. As a positive control, vancomycin (30 µg/mL) was steaked at the center of Mueller- Hinton agar for bacterial pathogens and clotrimazole (1 % topical solution) for fungi. The pathogens were streaked perpendicularly to the positive control. Plane Petri dishes were used as negative controls. Antagonism was measured by the determination of the size of the inhibition zone in millimeters following incubation of bacteria cultures at 37 ± 2 °C for 24 h and fungal pathogens at 28 ± 2 °C for seven days (Elbendary *et al*., 2018).

Secondary screening of actinomycete isolates for antibiotic production

About 250 ml of M1 broth in conical flasks were prepared and separately inoculated with isolated actinomycete cultures using a sterile wire loop. The flasks were incubated at 28 ± 2 °C for seven days in an orbital shaker. The broth cultures were centrifuged for 10 mins at 600 rpm. Antibiotic activity against the selected test pathogens was determined using Kirby Bauer disk diffusion technique (Jayshree *et al.,* 2016). Four actinomycetes strain with the largest inhibition zones against Gram-positive bacteria, Gram-negative bacteria, filamentous fungi, and yeast and recorded the largest diameter of clear zones of inhibition coded PAN 9, PAN 18, PAN 117, and PAN 138 were selected for further studies (Jodi *et al*., 2017).

Extraction of the antibiotics from the selected actinomycetes

About 2 mL starter cultures of isolates PAN 9, 18, 117, and 138 were separately inoculated in three liters of sterilized M1 broth, in conical flasks and incubated at 28 °C in an orbital shaker (Gallenkamp, Model 10 X 400) (200 rpm) for seven days. Equal volumes of ethyl acetate were added and the mixture spun at 200rpm for 1h in a vortex mixer (PV-1, V-32, rpm 200) (Padma *et al.,* 2018). The antibiotics obtained from each solvent were tested for activity against the test pathogens using Karby Bauer disk diffusion techniques. (Elbendary *et al*., 2018).

Determination of Minimum Inhibitory Concentrations of the antibiotic metabolites

The minimum inhibitory concentration (MIC) of the antibiotic metabolites from isolates PAN 9, 18, 117, and 138 were determined using the broth tube dilution procedure in two-fold dilution of nutrient broth and sabouraud dextrose broth for bacterial and fungal pathogens respectively. For each pathogen, 12 sterile screw-capped test tubes were used. In each case, a volume of 1mL nutrient broth for bacteria and 1 mL of sabouraund dextrose broth was separately dispensed into test tubes 1-10 and 2 mL into test tube 11 (broth control). On the other hand, 1mL of the crude extract of antibiotic metabolites was added into test tubes 1 and 2 and 2mL to test tube 12 (crude extract control). One milliliter of the well-mixed solution was transferred from test tube 2 to 3 and this process was continued serially up to test tube 10 by mixing and changing the micropipette tips at each dilution. Finally, 1mL was discarded from test tube 10 and 0.1 mL of standardized inocula were added into test tubes 1-10 and incubated at 37 °C for 24h for bacteria and 28 °C for seven days for fungi. This was repeated for the four antibiotic

extracts. The tubes were examined for the development of turbidity. Minimum inhibitory concentration (MIC) was determined as the lowest concentration of the antibiotics that inhibited the growth of the test pathogen (Hathaway *et al*., 2014).

Determination of Microbicidal and Microbistatic properties of the antibiotic metabolites

The minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) values of the antibiotics were determined by subculturing 50µL from tubes not visibly turbid and spot inoculating onto Muller-Hinton agar. The MBC and MFC were determined as the lowest concentration that prevented the growth of the test pathogens (Saranya *et al*., 2017).

Hatching of brine shrimps and lethality bioassay

Brine shrimp lethality bioassay was used to evaluate the bioactivity of the antibiotics. Artificial seawater was prepared by dissolving sea salt (Gex Inc., Osaka, Japan) in distilled water in an incubation tank measuring 25 cm in diameter and 3.5 cm in height (Abraham, 2016). The tank was divided into two compartments separated by a perforated polystyrene material. Brine shrimp eggs (*Artemia salina*) (Nihon Animal Pharmaceutical Inc., Tokyo, Japan), weighing 0.5 g were placed on one part which was darkened by covering with a hard paper. The other compartment was illuminated using an electric bulb. After 48 h, the brine shrimps were collected using a pipette (Tan *et al*., 2017). Briefly, 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 ^{o}C, and then surviving shrimps were counted after 24 h. The half lethal concentration (LC_{50}) value was calculated using regression and probit analysis.

Determination of cytotoxicity of sheep erythrocyte

Briefly, a ten-fold dilution of each antibiotic extract was made in phosphate-buffered saline. For each antibiotic extract, 0.8 mL was placed in an Eppendorf tube (Ser *et al.,* 2016). Citrated sheep blood was centrifuged at 1500 rpm for 10 min at 4 °C to obtain the erythrocytes. The erythrocytes were washed three times with phosphate-buffered saline and re-suspended using the same buffer (Kumar *et al.,* 2014). The erythrocytes were added to the Eppendorf tubes having the antibiotic extracts to give a final volume of 1 mL and incubated at 37 °C for 30 min. Normal saline served as the negative control while 0.4mL of Triton X-100 was a positive control. All tubes were centrifuged for 5 min and the type of hemolysis was determined (Janardhan *et al.,* 2014).

Data analysis

All data were presented as mean ± standard deviation. Each value represented the mean value (± SD) of five independent replicates. The results were subjected to a one-way analysis of variance (ANOVA), followed by Tukey's HSD (Honestly Significant Difference) test in the case of null hypothesis rejection. The significance was defined at $p < 0.05$.

Results

Antagonism to selected pathogenic microorganisms

A total of 20 actinomycete isolates showed antagonism against the test pathogenic microorganisms. Four actinomycetes with the biggest zone of inhibition and showing a broad spectrum of activity were selected and coded PAN 9, PAN 18, PAN 138, and PAN 138. There was no significant difference in the zones of inhibition among the isolates (F = 0.242, P = 0.938). The mean diameter of the zone of inhibition varied from 11.32 ± 0.3 mm in isolate PAN 117 to14.67 ± 0.3 mm in isolate PAN 9 and PAN 138 (Table 1) in bacterial pathogens while in fungal pathogens, the zones of inhibition ranged from 10.25 ± 0.3 mm in PAN 117 to 14.25 ± 0.3 in PAN 138 (Table 2).

Table 1: Growth inhibition zones (mm) of test pathogenic bacteria in primary screening of the selected actinomycete isolates from Menengai crater.

S. No.	Isolate	*S. aur*	*P.aeru*	*E. fae*	*E. col*	*S. pne*	*P.vul*	Mean
1	PAN 9	15±0.3	14±0.3	17±0.2	17±0.3	10±0.2	15±0.3	14.67±0.3
2	PAN 18	11±0.3	17±0.2	10±0.3	12±0.3	9±0.2	12±0.2	11.83±0.2
3	PAN 117	12±0.3	11±0.2	10±0.3	12±0.3	13±0.3	10±0.2	11.32±0.3
4	PAN 138	14±0.3	13±0.2	14±0.3	14±0.2	16±0.3	17±0.3	14.67±0.3

Each value represents the mean value (± SD) of five independent replicates, *S. aur; Staphylococcus aureus, P. aeru; Pseudomonas aeruginosa, E. fae; Enterococcus faecalis, E. col; Escherichia coli, S. pne; Streptococcus pneumonia* and *P. vul; Proteus vulgaris,*

Table 2: Growth inhibition zones (mm) of test pathogenic fungi in primary screening of the selected actinomycete isolates from Menengai crater.

S. No.	*Isolate*	*C.albicans*	*U.maydis*	*A. citri*	*F. oxysporum*	Mean
1	PAN 9	10±0.2	12±0.3	10±0.3	11±0.3	10.75±0.3
2	PAN 18	11±0.3	11±0.2	10±0.3	11±0.2	10.75±0.3
3	PAN 117	11±0.2	9±0.3	11±0.2	10±0.2	10.25±0.3
4	PAN 138	13±0.2	15±0.2	15±0.3	14±0.2	14.25±0.3

Each value represents the mean value (± SD) of five independent replicates, *C. albicans; Candida albicans,U. maydis; Ustilago maydis, A. citri; Alternaria citri* and *F. oxysporum; Fusarium oxysporum.*

Antibiotic activity of metabolites produced by actinomycetes isolates

The diameters of zones of inhibition indicated by extracted actinomycete metabolites when tested against the test pathogenic microorganisms varied significantly (F = 2.4473 P = 0.0089). Among the bacteria, the mean diameter of the zone of inhibition varied from 10.17 ± 0.3 mm in isolate PAN 138 to 16.00 ± 0.3 mm in isolate PAN 9 (Table 3) while in fungi, the zones of inhibition ranged from10.00 ± 0.2 mm in PAN 138 to 13.00 ± 0.2 mm in PAN 117 (Table 4).

Table 3: Growth inhibition zones (mm) of test pathogenic bacteria in secondary screening of the selected actinomycete isolates from Menengai crater soils.

S. No.	Isolate	*S. aur*	*P.aeru*	*E. fae*	*E. col*	*S. pne*	*P.vul*	Mean
1	PAN 9	17±0.3	15±0.3	19±0.2	18±0.3	11±0.2	16±0.3	16.00±0.3
2	PAN 18	14±0.3	19±0.2	11±0.3	13±0.3	10±0.2	13±0.2	13.33±0.2
3	PAN 117	12±0.3	11±0.2	12±0.3	14±0.3	15±0.3	12±0.2	12.67±0.3
4	PAN 138	12±0.3	9±0.2	10±0.3	11±0.2	8±0.3	11±0.3	10.17±0.3

Each value represents the mean value (± SD) of five independent replicates, *S. aur; Staphylococcus aureus, P.aeru; Pseudomonas aeruginosa, E. fae; Escherichia faecalis, E. col; Escherichia coli, S. pne; Streptococcus pneuminiae* and *P.vul; Proteus vulgaris.*

Table 4: Growth inhibition zones (mm) of test pathogenic fungi in the secondary screening of the selected actinomycete isolates from Menengai crater soils.

S. No	*Isolate*	*C. alb*	*U.may*	*A. cit*	*F. oxy*	Mean
1	PAN 9	13±0.2	14±0.3	11±0.3	10±0.3	12.00±0.3
2	PAN 18	12±0.3	13±0.2	11±0.3	13±0.2	12.25±0.3
3	PAN 117	15±0.2	12±0.2	13±0.2	12±0.2	13.00±0.2
4	PAN 138	10±0.2	9±0.2	11±0.3	10±0.2	10.00±0.2

Each value represents the mean value (± SD) of five independent replicates, *C. alb; Candida albicans, U. may; Ustilago maydis, A. cit; Alternaria citri* and *F. oxy; Fusarium oxysporum.*

Antibiotic properties of the ethyl acetate extracted metabolites

There was a significant difference in the diameters of zones of inhibition produced by the test pathogens when subjected to the antibiotic metabolites from the selected actinomycetes (F = 6.6046 P = 0.001338). Among the bacteria, the mean diameter of the zone of inhibition varied from 22.67 ± 0.2 mm in isolate PAN 9 to 39.83 ± 0.2 mm in isolate PAN 117 (Table 5) while in fungi, the zones of inhibition ranged from 27.00 ± 0.2 mm in PAN 9 to 30.75 ± 0.2 mm in PAN 118 (Table 6).

Table 5: Mean diameters of zones (mm) of inhibition of test pathogenic bacteria by ethyl acetate extracts from the selected actinomycete isolates.

Isolate	*S. aur*	*S. pne*	*E. fae*	*E. col*	*P. aeru*	*P. vul*	Mean
PAN 9	24±0.2	22±0.3	23±0.2	21±0.2	18±0.3	26±0.2	22.67±0.2
PAN 18	33±0.2	26±0.2	39±0.3	45±0.3	40±0.1	35±0.3	36.33±0.2
PAN 117	37±0.1	33±0.2	43±0.3	47±0.3	38±0.1	41±0.3	39.83±0.2
PAN 138	30±0.1	23±0.2	36±0.2	45±0.1	39±0.2	34±0.3	34.50±0.2
Vancomycin	30±0.1	27±0.2	25±0.2	18±0.1	15±0.2	17±0.1	22.00±0.2

Each value represents the mean value (±SD) of five independent replicates. *S. aur; Staphylococcus aureus, S. pne; Streptococcus pneuminiae, E. fae; Enterococcus faecalis, E. col; Escherichia coli, P.aeru; Pseudomonas aeruginosa,* and *P. vul; Proteus vulgaris.*

Table 6: Mean diameters of zones (mm) of inhibition of test pathogenic fungi by ethyl acetate extracts from the selected actinomycete isolates.

	C.alb	*U.may*	*A. citri*	*F. oxy*	Mean
PAN 9	25±0.2	26±0.2	29±0.2	28±0.1	27.00±0.2
PAN 18	38±0.1	28±0.3	27±0.1	30±0.2	30.75±0.2
PAN 117	35±0.3	23±0.2	28±0.3	31±0.1	29.25±0.2
PAN 138	39±0.2	28±0.1	25±0.2	24±0.3	29.00±0.2
Clotrimazole	19±0.1	12±0.2	11±0.1	13±0.2	13.75±0.2

Each value represents the mean value (±SD) of five independent replicates, *C. alb; Candida albicans, U.may; Ustilago maydis, A. citri; Alternaria citri, F. oxy; Fusarium oxysporum.*

Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), and Minimum Fungicidal Concentration (MFC) of the antibiotic metabolites

There was no significant difference between the minimum inhibitory concentration and minimum bactericidal concentration /minimum fungicidal concentration (F = 0.4503, P = 0.7187) of the antibiotic metabolites produced by isolating PAN 9, PAN 18, PAN 117, and PAN 138 against the test pathogens. Among the Gram-positive bacteria, the MICS ranged from 0.06 ± 0.1 to 0.13±0.2mg/mL (Table 7). In Gram-negative bacteria, the MICS varied from 0.06 ± 0.1 to 0.25±0.2mg/mL. However, among the fungal pathogens, the MICS ranged from 0.06 ± 0.1 to 0.25±0.2mg/mL. The MIC values were equal to MBC and MFC values.

Table 7: Minimum Inhibitory Concentration, Minimum Bactericidal Concentration, and Minimum Fungicidal Concentration of the ethyl acetate crude antibiotic metabolites on selected test pathogens.

Path	MICS (mg/mL)				MBC/MFC (mg/mL)			
	PAN 9	PAN 18	PAN 117	PAN 138	PAN 9	PAN 18	PAN 117	PAN 138
S. au	0.13±0.1	0.06±0.2	0.13±0.2	0.13±0.1	0.13±0.1	0.06±0.2	0.13±0.2	0.13±0.1
S. pn	0.06±0.2	0.06±0.1	0.13±0.1	0.06±0.2	0.06±0.2	0.06±0.1	0.13±0.1	0.06±0.2
E. fa	0.06±0.1	0.13±0.1	0.06±0.1	0.13±0.1	0.06±0.1	0.13±0.1	0.06±0.1	0.13±0.1
E. co	0.13±0.2	0.06±0.2	0.13±0.2	0.25±0.1	0.13±0.2	0.06±0.2	0.13±0.2	0.25±0.1
P. ae	0.13±0.1	0.25±0.2	0.25±0.1	0.13±0.2	0.13±0.1	0.25±0.2	0.25±0.1	0.13±0.2
P. vu	0.13±0.2	0.06±0.1	0.13±0.2	0.13±0.1	0.13±0.2	0.06±0.1	0.13±0.2	0.13±0.1
C. al	0.06±0.1	0.13±0.2	0.06±0.2	0.06±0.2	0.06±0.1	0.13±0.2	0.06±0.2	0.06±0.2
F. ox	0.13±0.2	0.13±0.1	0.25±0.2	0.13±0.1	0.13±0.2	0.13±0.1	0.25±0.2	0.13±0.1
U.ma	0.13±0.1	0.12±0.2	0.13±0.1	0.25±0.1	0.13±0.1	0.12±0.2	0.13±0.1	0.25±0.1
A. cit	0.25±0.2	0.25±0.1	0.13±0.2	0.25±0.2	0.25±0.2	0.25±0.1	0.13±0.2	0.25±0.2

Each value represents the mean value (±SD) of five independent replicates. S. au; *Staphylococcus aureus*, *S. pn; Streptococcus pneumoniae, E. fa; Enterococcus faecalis*, *E.co; Escherichia coli, P. ae; Pseudomonas aeruginosa, P. vu; Proteus vulgaris, C. al; Candida albicans, F. ox; Fusarium oxysporum, U. ma; Ustilago maydis* and *A. cit; Alternaria citri.*

brine shrimp (*Artemia salina*). The sample extract concentration ranged from 0.5 to 31 µg (Table 8). The mortality of the shrimps increased with an increase in the concentration of the antibiotic extract. The LC_{50} values varied from 2.438±0.47µg in PAN 9 to 9.3455±0.41µg in PAN 138.

Cytotoxic activity of the antibiotic metabolites

The cytotoxic activity of metabolites from isolates PAN 9, PAN 18, PAN 117, and PAN 138 was investigated against

Table 8: LC_{50} values of crude antibiotic metabolites on brine shrimp lethality bioassay.

Extract	Sample conc. (µg/ml)	Log C	% Mortality	Regression equation	LC_{50} (µg/ml)
PAN 9	0.5	0.301029996	26	Y=3.410x + 3.680	2.438±0.47
	1	0	29		
	2	0.301029996	48		
	4	0.602059991	57		
	8	0.903089987	62		
	16	1.204119983	100		
	31	1.491361694	100		
PAN 18	0.5	0.301029996	13	Y=2.882x +3.734	2.7498±0.56
	1	0	23		
	2	0.301029996	58		
	4	0.602059991	65		
	8	0.903089987	75		
	16	1.204119983	95		
	31	1.491361694	100		
PAN 117	0.5	0.301029996	3	Y=3510x +2.9367	4.1276±0.45
	1	0	12		
	2	0.301029996	23		
	4	0.602059991	30		
	8	0.903089987	80		
	16	1.204119983	88		
	31	1.491361694	100		
PAN 138	0.5	0.301029996	6	Y=1.9596x +3.3098	9.3455±0.41
	1	0	12		
	2	0.301029996	13		
	4	0.602059991	30		
	8	0.903089987	48		
	16	1.204119983	60		
	31	1.491361694	96		

Each value represents the mean value (±SD) of five independent replicates, Conc.; concentration.

Hemolysis of sheep blood erythrocytes

The antibiotic extracts from PAN 9, PAN 18, PAN 117, and PAN 138 were tested for hemolysis of sheep blood erythrocytes. PAN 19 and PAN 117 showed partial hemolysis while PAN 9 and PAN 138 exhibited complete hemolysis (Table 9).

Table 9: Hemolytic activity of ethyl acetate extracts of actinomycetes against sheep blood erythrocytes

Isolate	Type of hemolysis		
	A	B	Y
PAN 9	-	+	-
PAN 18	+	-	-
PAN 117	+	-	-
PAN 138	-	+	-

α; Partial hemolysis, β; Complete hemolysis, Y; No hemolysis, +; the presence of hemolysis and -; absence of hemolysis.

Discussion

Primary screening is a disposable technique for preliminary identification of actinomycetes with antagonistic properties to pathogens before more resources are applied in drug discovery (Davies-Bolorunduro *et al.,* 2019). There is a vast number of actinomycetes that do not have antagonism against pathogens (Janardhan *et al.*, 2015). In this study, a total of 20 actinomycetes were isolated from the geothermal vents of Menengai crater. Among them, 4 produced broad

spectrum antibiotics and presented the biggest zones of inhibition. The findings disagreed with a previous study by (Ahmad *et al*., 2015). The differences in the findings could be attributed to variations in the antibiotics produced by the actinomycetes isolates (Waithaka *et al.,* 2019).

Davies *et al*. (2015) indicated that not all actinomycetes that show antagonism against pathogenic microorganisms in primary screening do so in secondary screening. This is due to the fragmentation of the hyphal materials when actinomycetes are grown in liquid media during fermentation of the antibiotic metabolites for secondary screening (Waithaka *et al*., 2017). Contrary to the observations, the 4 actinomycetes screened for antagonistic properties against bacterial and fungal test pathogens in the present study demonstrated antagonism to the test microorganisms in secondary screening. This further contradicted with findings of another study by Yang *et al.* (2018). The differences could be attributed to variations in the genetic codes that dictate the antibiotics to be synthesized among actinomycetes. Four actinomycetes coded PAN 9, PAN 18, PAN 117, and PAN 138 were selected for further analysis based on the size of the zone of inhibition and the broad spectrum of activity.

According to previous studies, ethyl acetate is the solvent of choice used in the extraction of antibiotics from actinomycetes, and therefore it was used in the current study (Davies *et al*., 2015). The extracts produced zones of inhibition that varied from 22.67 ± 0.2 mm in isolate PAN 9 to 39.83 ± 0.2 mm in isolate PAN 117. The zones were bigger than those reported elsewhere (Sharma and Manhas, 2021). Waithaka *et al.* (2020) maintained that different actinomycetes yield different types of antibiotics.

Crude antibiotic metabolites were used to determine minimum inhibitory concentration against the selected test microorganisms. The minimum inhibitory concentration varied from 0.06 ± 0.1 mg/mL to 0.25 ± 0.2 mg/mL. These results contrasted with those obtained by Adam *et al.* (2018) against Gram-positive, Gram-negative, and fungal pathogens. Lavoie *et al*. (2017) reported activity against *S. aureus* (0.70 mg/mL) and *C. albicans* (0.85 mg/mL) but there was no activity against *E. coli*. Maciejewska *et al.* (2018) studied the minimum inhibition of antibiotic metabolites from *Streptomyces* of Cave Moonmilk of Mulu National park Borneo in Malaysia and obtained minimum inhibition of *S. aureus* (31.25 mg/mL) and *C. albicans* (16.62 mg/mL). A previous study by Ekundayo and Faniomi, (2017) obtained MICs that varied from *P. aeruginosa* (17.21 mg/mL) to *S. aureus* (21.31 mg/mL), which were higher than the ones obtained in this study. The minimum inhibitory concentrations observed in this study were lower than in the former studies. This may be attributed to higher amounts and concentrations of the active compounds in the extracted antibiotic metabolites (Bull *et al*., 2018).

Minimum inhibitory concentration was equal to minimum bactericidal and minimum fungicidal concentration (Maciejewska *et al*., 2015). As a result, the antibiotic metabolites were bactericidal and fungicidal rather than bacteriostatic or fungistatic respectively. This agreed with a previous study by Khattab *et al*. (2016) probably because the antibiotic metabolites used the same mechanisms in inhibiting the growth of the test pathogens.

This study indicated that the mortality of brine shrimp nauplii increased with an increase in the concentration of the antibiotic extracts. The LC_{50} of the antibiotic extracts varied from 2.438 ± 0.47 µg in PAN 9 to 9.3455 ± 0.41 µg in PAN 138. The antibiotic extracts had very high toxicity since the values were lower than 500 µg/mL (Lewin *et al*., 2016). The LC_{50} values suggested that the antibiotics had anticancer properties (Wang *et al*., 2017). Previous studies carried out by other researchers produced higher LC_{50} (Martins *et al*., 2016; Masad *et al*., 2018; Sharon, 2016). The variations could be attributed to differences in the active compounds present in the antibiotic extracts (Nofiani *et al*., 2022).

This study aimed at determining whether the antibiotic extracts caused oxidative damage to the erythrocyte membrane or not (Thirumurugan *et al.,* 2018). The results indicated that PAN 18 and PAN 117 caused partial hemolysis while PAN 9 and PAN 138 led to complete hemolysis of sheep blood erythrocytes. This concurred with previous studies carried out elsewhere (Janardhan *et al.*, 2014; El-Naggar and El-Shweihy, 2020). Blood erythrocytes bioassay is extremely sensitive to a wide range of antibiotic extracts from actinomycetes (Soyer and Tanul, 2020). This may be attributed to the wide array of compounds present in antibiotic crude extracts (Valipour *et al*., 2018).

Conclusions

Soils from Menengai crater geothermal vents have actinomycetes that produce antibiotics. The antibiotics are cytotoxic to brine shrimps. In addition, the antibiotics cause hemolysis of sheep blood erythrocytes.

Recommendations

There is a need to purify the antibiotics and structure elucidate them. The active antibiotic extract needs to be evaluated to determine whether the hemolysis is due to the compound itself or some other chemical constituent.

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