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Regular Article

Sensitivity testing, yield and stability of antimicrobial metabolites obtained from soils of Menengai crater in Kenya

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Abstract
This study sought to determine the effectiveness of antimicrobial metabolites extracted using ethyl acetate. The antimicrobial metabolites were obtained from previously isolated actinomycetes coded as PAN 30, PAN 37, PAN 41 and PAN 154. The study also determined the yield of the extracts from the actinomycetes in addition to testing the stability of the antimicrobial metabolites to degradation by enzymes, temperature and pH. To obtain the antimicrobial metabolites from the actinomycetes, fermentation was carried out for a month using an orbital shaker. Extraction of the metabolites was carried out using ethyl acetate. The metabolites were concentrated using a vacuum evaporator. The metabolites were separately dried in pre-weighed containers using a hot air oven. To determine the weight of the antimicrobial metabolites, the concentrated antimicrobials were placed in pre-weighed containers followed by evaporation of the solvent to dryness in a hot air oven. The containers having the antimicrobial metabolites were reweighed and the weight of the antimicrobials determined by subtracting the weight of the empty container from that of the container plus the metabolites. The stability of the antimicrobial metabolites was determined by exposing the metabolites to enzymes, varying levels of temperatures and pH. The antimicrobial metabolites yield of PAN 30 was 253.6mg, PAN 37 (231.4mg), PAN 41 (258.0mg) and PAN 154 (259.0mg). There was no significant difference in the weights of the antimicrobial metabolites among the four actinomycetes. The zones of inhibition presented by *Staphylococcus aureus, Bacillus subtilis, Escherichia faecalis, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi, Xanthomonas campestris, Erwinia carotovora, Candida albicans, Aspergillus niger, Fusarium oxysporum and Ustilago maydis* were significantly different ($F=6.6046$ $P=0.001338$). The zones of inhibition produced by the pathogens after incubating the antimicrobial metabolites to enzymes trypsin, lysozyme, pepsin and lipase in addition to temperatures of 60ºC, 70ºC and 80ºC were not statistically different. However, there were significant differences when the antimicrobial metabolites were tested for stability against pH in bacterial ($F = 319.0101$ $P=<0.05$) and fungal ($F=55.58353$, $P<0.05$) pathogens. The antimicrobial metabolites obtained from PAN 41 and PAN 154 were not stable in trypsin, lysozyme, pepsin and lipase. All the antimicrobial metabolites remained stable at 60ºC, 70ºC and 80ºC. In addition, the antimicrobial metabolites remained effective in pH 2, 4, 5 and 7 but were not effective in pH of 9 and 11. There is need to further purify the metabolites and determine their structure.

Key words: actinomycetes, antimicrobials, enzyme, pH, stability, testing.

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1. Introduction

World health organization's working document on stability testing of active substances and pharmaceutical products maintains that the purpose is to provide evidence of how the quality of an active substance varies under the influence of environmental factors [17]. Product related factors such as chemical and physical factors, pharmaceutical excipients, dosage, form and composition and manufacturing process are also considered [13]. In addition, other factors that can lead to reactive degradation of the active substance should also be tested [4].

[4] asserts that stability of the active substance should go through a series of re-testing. The shelf life of the compound is then indicated which helps in recommending the storage conditions of the substance [9]. Stress testing of the active compound is an integral part of stability testing. It helps in determining the cause of degradation of the compound if any [14]. Through stress testing, the degradation pathways and intrinsic stability are indicated. Stress testing depends on the individual substance and type of the product involved [25]. Stress testing involves testing of the effect of temperature in 10^{o}C increments, humidity, oxidation and photolysis of the active ingredient [1]. Susceptibility of the active substance to hydrolysis across varying levels of pH is also done [6].

Data for formal stability studies of the active compound is provided to at least three primary batches of the active substance [18]. The batches of the compound should be of the same quality as the final product made on large scale [16].

Another important element of stability testing is the test of the active substance when packed in a closed container [22]. The container should emulate the final container that will be used in packaging and distribution of the final product [29].

Specificative testing involves testing the attributes of the active ingredients that may change during storage [26]. Factors that may change quality, safety and or efficacy of the active substance are also determined. The testing should cover the physical, chemical and biological attributes of the active compound [20].

Retesting of the active substance is important to determine whether it decomposes with time [8]. Re-testing should be done after every three months in the first year, 6 months in the second year and annually thereafter. A minimum of one year is recommended for re-testing [7].

In storage conditions of the active compound, thermal stability and sensitivity to moisture is carried out [10]. This determines the right temperature and humidity for storage of the compound. For safety reasons, a minimum of 12 Months thermal stability and humid requirements are recommended [21].

The current study sought to determine the weight of metabolites produced by four actinomycetes isolated from Menengai crater in Kenya, carry out sensitivity test of the test pathogens to the metabolites and test the stability of the metabolites different enzymes, temperature and pH levels.

2. Materials and methods

2.1 Actinomycetes isolates

Four actinomycetes isolates (PAN 30, PAN 37, PAN 41 and PAN 154) previously isolated from the soils of Menengai crater in Kenya (Figure 1) were used in this study. Isolation of actinomycetes was carried out using starch casein agar, Luria Bertani agar and starch nitrate agar and characterized using biochemical means.

2.2 Extraction of crude metabolites

The selected antagonistic actinomycetes strain were separately inoculated into 3L Luria Bertani (M1) broth, and incubated at 28°C in an orbital shaker (Gallenkamp, Model 10X 400) at 200 rpm for seven days. After incubation, the broths were filtered through Millipore filter (Millipore Millex-HV Hydrophilic PVDF 0.45 Zm). The filtrates were separately transferred aseptically into a conical flask and stored at 4°C for further assays. To the culture filtrates, equal volume of ethyl acetate were separately added and centrifuged at 5000 rpm for 10 min to extract the antimicrobial metabolites [27]. The compounds obtained from each actinomycetes strain were tested for their activity against the test pathogens by agar well diffusion method [31].

2.3 Separation of antimicrobial metabolites

Ethyl acetate was used in separately dissolving the selected antimicrobial metabolites. The solvent was evaporated using a vacuum evaporator (Heidolph Laborota, 4001, Büchi Vacuum Controller V-805) at 40°C (50 rpm). The concentrated antimicrobials were placed in pre-weighed containers before

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evaporating the solvent in a hot air oven. The dark gummy substances obtained were dissolved in ethanol, following which the crude antimicrobial metabolite powders were obtained. The crude antimicrobials were collected and dried in vacuum oven (Schwabach, DIN 40050-IP20, V=240, Hz 50/60) at 40°C overnight. The weights of the antimicrobial metabolites were obtained by subtracting the weight of the pre-weighed containers from the weight of the containers having the antimicrobials.

2.4 Determination of stability of the antimicrobial

2.4.1 Thermal stability of the antimicrobials

To determine the effect of temperature on stability of the antimicrobials, screw capped ampoules, each with 100 µg/ml of the antimicrobial in water were kept at temperatures 60, 70 and 80°C for 1 h in a water bath. The antimicrobial solutions were cooled to room temperature (20ºC ± 2) and the residual antimicrobial activity was determined against *Staphylococcus aureus* (ATCC 25923), *Streptococcus pneumoniae* (ATCC 49617), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus vulgaris* (ATCC 49990), *Candida albicans* (ATCC 10231), *Aspergillus niger* (ATCC 1015), *Fusarium oxysporum* (ATCC 16608) and *Ustilago maydis* (ATCC 14826) using agar well diffusion method [11].

2.4.2 Effect of pH on activity and stability of the antimicrobial s

To determine the effect of pH on stability of the antimicrobials, 100 µg/ml of the antimicrobials were separately mixed with 1 ml of 0.1 M phosphate buffer of varied pH (2, 4, 5, 7, 9 and 11) in various tubes incubated for 1 h at 30°C and the residual antimicrobial activity in each tube determined against *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Streptococcus pneumoniae* (ATCC 49617),*Proteus vulgaris* (ATCC 49990), *Aspergillus niger*(ATCC 1015),*Fusarium oxysporum* (ATCC 16608) and *Ustilago maydis* (ATCC 14826). Positive control involved use of antimicrobial without the buffer while in the negative control the buffer mixed with distilled water was used.

2.4.3 Effect of enzymes on activity of the antimicrobials

The sensitivity of the antimicrobials to denaturation by enzymes trypsin, lysozyme, pepsin and lipase (Himedia) were tested. All the enzymes were dissolved in distilled water at a concentration of 1 mg/ml. One hundred microliters (100µg/ml) of the antimicrobial solution were separately mixed with 100 µl enzyme and incubated at 30°C for 1 h. In each case, antimicrobial solution without any enzymes and use of distilled water served as controls. The residual antimicrobial activity of the mixtures was tested against *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Streptococcus pneumoniae* (ATCC 49617), *Proteus vulgaris* (ATCC 49990), *Aspergillus niger*(ATCC 1015), *Fusarium oxysporum* (ATCC 16608) and *Ustilago maydis* (ATCC 14826) using agar well diffusion method [11].

2.5 Data analysis

The data obtained was analyzed using statistical package for social sciences (SSPS) version 17.0 software. Single factor ANOVA was used in determining the level of significance at a P value of 0.05.

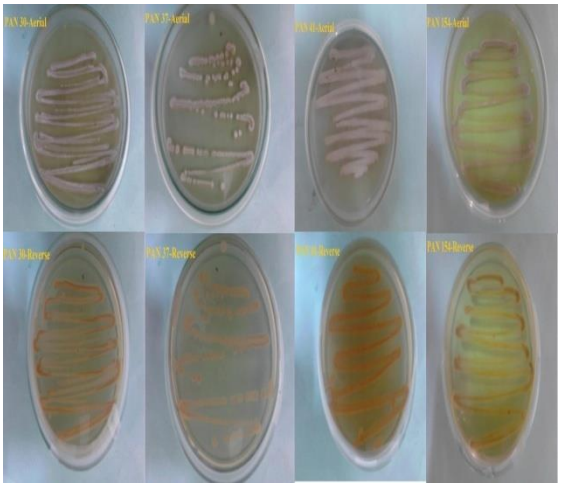

Figure 1: Morphological characteristics of the selected actinomycetes.

3. Results

3.1 Zones of inhibition (mm) after exposure of selected pathogens to antimicrobials from four actinomycetes

The zones of inhibition among the four extracts varied significantly (F = 6.6046 P = 0.001338). Among bacteria pathogens, the zones of inhibition of PAN 30 were biggest in *Streptococcus pneumoniae* (42±1), *Escherichia faecalis* (41±3),

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Escherichia coli (39±3), *Proteus vulgaris* (37±3), *Staphylococcus aureus* (35±2) and *Pseudomonas aeruginosa* (28±2) (Table 1). The biggest zones of inhibition in PAN 37 were *Streptococcus pneumoniae* (41±2), *Escherichiafaecalis* (38±2), *Proteus vulgaris* (36±3), *Escherichia coli* (34±1), *Staphylococcus aureus* (32±1) and *Pseudomonas aeruginosa* (25±2). In PAN 41 the zones of inhibition were biggest in *Escherichia faecalis* (43±3), *Proteus vulgaris* (41±3), *Streptococcus pneumoniae* (40±1), *Escherichia coli* (39±3), *Staphylococcus aureus* (39±1) and *Pseudomonas aeruginosa* (35±2). However, in PAN 154 the biggest zone of inhibition was observed in *Proteus vulgaris* (28±2), *Staphylococcus aureus* (26±2), *Pseudomonas aeruginosa* (25±3), *Escherichia faecalis* (25±2), *E. coli* (24±2) and *Streptococcus pneumoniae* (20±3). In addition, among the fungi, PAN 30 exhibited the biggest zone of inhibition in *Fusarium oxysporum* (32±20, *Candida albicans* (30±3), *Aspergillus niger* (29±1) and *Ustilago maydis* (28±3). PAN 37 had the biggest zone of inhibition in *Candida albicans* (30±1), *Aspergillus niger* (27±2), *Fusarium oxysporum* (26±3) and *Ustilagomydis* (26±1). Besides, PAN 41 indicated the biggest zone of inhibition in *Fusarium oxysporum* (33±1), *Aspergillus niger* (30±3), *Candida albicans* (25±2) and *Ustilago mydis* (24±1). In PAN 154, the biggest zone of inhibition was observed in *Aspergillus niger* (31±2), *Ustilago mydis* (31±2), *Fusarium oxysporum* (30±1) and *Candida albicans* (28±2).

3.2 Yield of the metabolites

There was no significant difference in the yields of antimicrobial metabolites among the four actinomycetes isolates (F = 0.000294, P = 0.999992). The antimicrobial metabolites were extracted from the actinomycetes using fermentation method. The extraction was carried out using ethyl acetate and concentrated using a vacuum evaporator. After drying of the metabolites, PAN 154 gave the highest yield (0.2590±0.000807g) while PAN 37 gave the lowest (0.2314±0.00033g) yield (Figure 2).

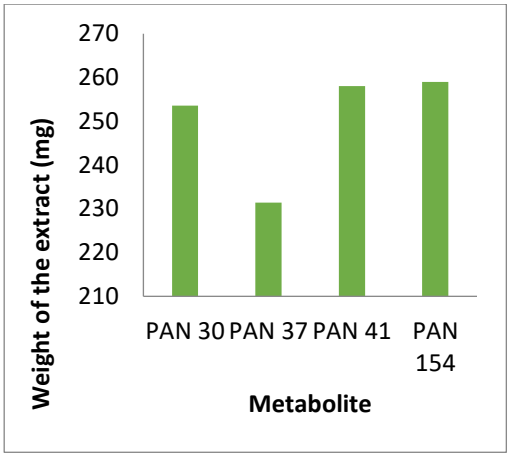

Figure 2: Weights (g) of purified metabolites after evaporation of ethyl acetate

3.3 Stability of the antimicrobial metabolites

3.3.1 Effect of enzymes on the stability of the metabolites against bacterial pathogens

The zones of inhibition shown by the antimicrobial metabolites against the pathogens did not vary significantly (F=0.0132 P=0.99792). The zones of inhibition in *Staphylococcus aureus* ranged from 0 in PAN 41 and PAN 154 when tested against trypsin, lysozyme, pepsin and lipase to 48±2 in PAN 30 when tested against trypsin (Table 2). In *Streptococcus pneumoniae* the range was 0 in PAN 41 and PAN 154 when tested against trypsin, lysozyme, pepsin and lipase to 43 ± 3 in PAN 37 when tested against lipase.

The zones of inhibition in *Escherichia faecalis* varied from 0 in PAN 41 and 154 in all the enzymes tested to 45±1 when the antimicrobial metabolites were tested against lipase. However, in *Escherichia coli* the zones of inhibition ranged from 0 in PAN 41 and 154 when tested against trypsin, lysozyme, pepsin and lipase to 37 ± 2 in PAN 30 when tested against trypsin and PAN 37 against lipase. The zones of inhibition in *Pseudomonas aeruginosa* varied from 0 in all the tested enzymes to 36 ± 2 in lysozyme.

3.3.2 Thermal stability of the metabolites against bacterial pathogens

The zones of inhibition among the bacterial pathogens did not vary significantly (F = 0.03194, P = 0.968579). The zones of inhibition in *Staphylococcus aureus* varied from 25 ± 2 at 60ºC in PAN 154 to 47 ± 2 at 60ºC in PAN 30 (Table 2). The zones of inhibition in *Streptococcus pneumoniae* varied from 25 ± 1 in PAN 154 at 70ºC

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to 42 ± 2 at 80ºC in PAN30. In addition, *Escherichia faecalis* produced zones of inhibition that ranged from 22 ± 1 at 60º C in PAN 154 to 40 ± 3 at 60ºC in PAN 41. *Escherichia coli* produced zones of inhibition varying from 20 ± 2 at 60ºC in PAN 154 to 28 ± 2 at 70ºC in PAN 37. The zones of inhibition observed when the antimicrobial metabolites were tested against *Pseudomonas aeruginosa* varied from 24 ± 2 at 60ºC in PAN 154 to 34 ± 3 at 60ºC in PAN 37 and PAN 30 at 80ºC. However, the zones of inhibition in *Proteus vulgaris* varied from 23 ± 3 at 60ºC in PAN 154 to 34 ± 2 at 70ºC in PAN 30.

3.3.3 Effect of pH on stability of the antimicrobials using bacterial pathogens

The zones of inhibition exhibited by the tested bacterial pathogens against the metabolites varied significantly (F = 319.0101, P = <0.05). The zones of inhibition after testing the antimicrobials for stability against pH in *Staphylococcus aureus* varied from 0 at pH of 9 and 11 in PAN 30, 37, 41 and 154 to 48 ± 2 at pH of 2 in PAN 30 (Table 2). *Streptococcus pneumoniae* produced zones of inhibition ranging from 0 at a pH of 9 and 11 in PAN 30, PAN 37, PAN 41, PAN 154 to 46 ± 2 at pH of 2 in PAN 30. The zones of inhibition in *Escherichia faecalis* varied from 0 at pH 9 and 11 in PAN 30, PAN 37, PAN 41 and PAN 154 to 38 ± 2 at pH 7 in PAN 41. In addition, the zones of inhibition in *Escherichia coli* ranged from 0 at pH of 9 and 11 to 40 ± 2 at pH of 2 in PAN 41. The zones of inhibition in *Pseudomonas aeruginosa* varied from 0 at pH 9 and 11 in PAN 30, PAN 37, PAN 41 and PAN 154 to 37 ± 3 at pH 5 in PAN 37. However, the zones of inhibition in *Proteus vulgaris* ranged from 0 at pH 9 an 11 in PAN 30, PAN 37, PAN 41 and PAN 154 to 38 ± 1 at pH 7 in PAN 41.

3.3.4 Effect of enzymes on the stability of the metabolites against fungal pathogens

The zones of inhibition indicated by the fungal pathogens when tested against the metabolites did not vary significantly (F = 0.004851, P = 0.999529). When the antimicrobial metabolites were tested for stability against fungi, they gave different zones of inhibition (Table 3). The zones of inhibition shown by *Candida albicans* ranged from 0 when tested against trypsin, lysozyme, pepsin and lipase to 45 ± 3 in lysozyme. In *Fusarium oxysporum* the zones of inhibition in ranged from 0 in PAN 41 and PAN 154 in all the tested enzymes to 46 ± 2 in trypsin. In addition, when tested against *Ustilago maydis* the zones of inhibition varied from 0 in PAN 41 and PAN 154 when tested against trypsin, lysozyme, pepsin and lipase to 43 ± 2 in PAN 30 when tested against lipase. The zones of inhibitions exhibited by *Aspergillus niger* against PAN 30, ranged from 0 when tested against trypsin, lysozyme, pepsin and lipase to 28 ± 2 when tested against lysozyme.

3.3.5 Thermal stability of the metabolites using fungi

The zones of inhibition indicated by the fungal pathogens against the metabolites were not statistically different (F = 0.05985, P = 0.94198). When tested for thermal stability against *Candida albicans*, the zones of inhibition varied from 25 ± 1 at 60ºC in PAN 154 to 45±3 at 80ºC in PAN 30 (Table 3). The zones of inhibition exhibited by *Fusarium oxysporum* ranged between 26 ± 2 at 60ºC in PAN 154 and 45 ± 2 at 60ºC in PAN 30. In addition, the zones of inhibition shown by *Ustilago maydis* varied from 26 ± 2 at 60ºC in PAN 154 to 40 ± 1 at 70ºC. However, in *Aspergillus niger* the

Table 1: Zones of inhibition (mm) after exposure of selected pathogens to antimicrobials from four actinomycetes

Isolate	Zone of inhibition (mm)										Mean
	S. aur	*S. pne*	*E. fae*	*E. col*	*P. aer*	*P. vul*	*C. albica*	*A. niger*	*F. oxy*	*U. maydis*	
PAN 30	35±2	42±1	41±3	39±3	28±2	37±3	30±3	29±1	32±2	28±3	34.08±2
PAN 37	32±1	41±2	38±2	34±1	25±2	36±3	30±1	27±2	26±3	26±1	32.33±2
PAN 41	39±1	40±1	43±3	39±3	35±2	41±3	25±2	30±3	33±1	24±1	35.33±2
PAN 154	26±2	20±3	25±2	24±2	25±3	28±2	28±2	31±2	30±1	31±2	26.67±2

Each value represents the means (±SD) of five independent experiments, *S. aur; Staphylococcus aureus, S. pne; Streptococcus pneumoniae, E. fae; Escherichia faecalis, E. col; Escherichia coli, P. aer; Pseudomonas aeruginosa, P. vul; Proteus vulgaris, C. albica; Candida albicans, A. niger; Aspergillus niger, F. oxy; Fusarium oxysporum and U. mydis; Ustilago mydis.*

zones of inhibition ranged from 19±1 at 60ºC in PAN 154 to 27±1 at 70ºC in PAN 37.

3.3.6 Effect of pH on activity and stability of the antimicrobials using bacteria

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The zones of inhibition indicated by the fungal pathogens varied significantly (F = 55.58353, P<0.05). Among the fungi, the zones of inhibition in *Candida albicans* varied from 0 in PAN 30, PAN 37, PAN 41 and PAN 154 to 45±3 at pH 7 in PAN 30 (Table 3). The zones of inhibition in *Fusarium oxysporum* ranged from 0 at pH in PAN 30, PAN 37, PAN 41 and PAN 154 to 48±3 at pH 4. However, in *Ustilago maydis*, the zones of inhibition varied from 0 at pH in PAN 30, PAN 37, PAN 41 and PAN 154 to 44 ± 2 at pH 2 in PAN 30. 37, PAN 41 and PAN 154 to 44 ± 2 at pH 2 in PAN 30. In addition, in *Aspergillus niger* the zones of inhibition ranged from 0 at pH 9 and 11 in PAN 30, PAN 37, PAN 41 and PAN 154 to 27 ± 2 at pH 7 in PAN 37.

Discussion

[32] carried out a study on sensitivity of antimicrobial metabolites extracted from actinomycetes. The test pathogens exhibited varying zones of inhibition such as *E. coli* (34.73 ± 0.22mm), *Staphylococcus aureus* (33.56 ± 0.35mm) and *Aspergillus niger* (28 ± 0.19mm). In a similar study done by Phan *et al.* (2016), *Staphylococcus aureus* yielded a zone of inhibition of 26 ± 20.42mm, *Pseudomonas aeruginosa* (0mm) and *Fusarium oxysporum* (17 ± 0.76mm). These values were lower than those of the current study. Differences in the strain of the isolated actinomycetes which produce varying antimicrobial metabolites could be a contributing factor [28].

In a similar study on extraction and purification of antibacterial metabolites from actinomycetes, [12] obtained 0.019g from 500µl of crude extract. This was higher than the level of antimicrobial metabolites obtained in the current study after drying of the crude extract. This may have emanated from differences in temperature, the speed of the orbital shaker, and broth composition during fermentation of the actinomycetes for production of the metabolite.

The results obtained in the current study on stability of the metabolites to enzymes differ with a previous study curried out in Tamilnadu [23]. In their findings, [23] obtained varying zones of inhibition such as amalyse (24.1mm), protease (21mm), lipase (14.5mm) and Esterase (11.8mm) when testing metabolites obtained from

Table 2: Zones of inhibition (mm) after exposure of the metabolites to enzymes, temperature and pH using bacteria

Pathogen	Metabolite	Enzyme				Temperature			pH					
		Try	Lyso	Pep	Lip	60°C	70°C	80°C	2	4	5	7	9	11
S. aureus	PAN30	48±2	41±3	47±1	43±2	47±2	46±2	42±3	48±2	35±2	42±3	40±3	0±0	0±0
	PAN37	46±1	44±2	41±2	45±2	43±3	43±2	42±2	45±1	48±2	44±3	46±2	0±0	0±0
	PAN41	0±0	0±0	0±0	0±0	40±1	38±1	39±1	40±2	44±3	43±2	45±2	0±0	0±0
	PAN154	0±0	0±0	0±0	0±0	25±2	27±1	26±2	27±1	25±2	30±2	27±2	0±0	0±0
S. pneu	PAN30	40±2	43±1	41±2	40±2	40±1	39±3	42±2	46±2	42±1	43±2	40±1	0±0	0±0
	PAN37	43±1	39±2	43±2	43±3	39±3	35±2	30±3	34±2	33±2	34±1	37±2	0±0	0±0
	PAN41	0±0	0±0	0±0	0±0	31±2	35±3	37±2	38±3	35±3	39±2	37±1	0±0	0±0
	PAN154	0±0	0±0	0±0	0±0	27±1	25±1	29±2	27±1	24±2	26±2	28±2	0±0	0±0
E. fae	PAN30	44±2	42±2	40±1	45±1	30±2	35±3	32±3	33±2	36±1	35±1	34±2	0±0	0±0
	PAN37	40±1	44±1	40±1	44±2	31±2	38±2	35±1	34±2	35±2	35±3	38±3	0±0	0±0
	PAN41	0±0	0±0	0±0	0±0	40±3	37±2	34±1	37±3	36±1	34±1	38±2	0±0	0±0
	PAN154	0±0	0±0	0±0	0±0	22±1	25±1	27±3	27±2	25±2	28±3	26±3	0±0	0±0
E. coli	PAN30	37±2	35±2	30±2	33±3	27±2	25±1	23±2	30±2	34±2	36±2	35±3	0±0	0±0
	PAN37	34±1	35±2	35±1	37±2	25±1	28±2	24±3	35±3	37±3	34±1	37±1	0±0	0±0
	PAN41	0±0	0±0	0±0	0±0	25±1	21±2	27±2	40±2	36±2	37±2	33±1	0±0	0±0
	PAN154	0±0	0±0	0±0	0±0	20±2	21±3	25±2	38±1	31±2	33±2	34±3	0±0	0±0
P. aeru	PAN30	37±2	35±1	30±2	33±1	33±3	27±2	34±3	32±1	35±1	33±3	30±2	0±0	0±0
	PAN37	36±3	33±2	33±1	32±2	34±3	32±1	35±2	31±2	32±1	37±3	33±2	0±0	0±0
	PAN41	0±0	0±0	0±0	0±0	38±2	32±2	38±1	35±2	30±2	37±1	35±2	0±0	0±0
	PAN154	0±0	0±0	0±0	0±0	24±2	27±1	26±1	22±3	25±2	27±2	24±1	0±0	0±0
P. vul	PAN30	32±2	36±2	30±2	34±2	31±1	34±2	33±2	31±2	34±3	30±3	36±1	0±0	0±0
	PAN37	30±1	35±1	35±1	32±1	31±2	30±2	24±3	32±2	37±2	36±2	37±2	0±0	0±0
	PAN41	0±0	0±0	0±0	0±0	30±2	32±1	29±2	31±1	34±3	30±2	38±1	0±0	0±0
	PAN154	0±0	0±0	0±0	0±0	23±3	25±3	28±2	26±2	24±3	27±1	26±2	0±0	0±0

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Each value represents the means (±SD) of five independent experiments, *S. aureus; Staphylococcus aureus, S. pneu; Streptococcus pneumoniae, E. fae; Escherichia faecalis, E. coli; Escherichia coli, P. aeru; Pseudomonas aeruginosa, P. vul; Proteus vulgaris.*

actinomycetes against *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and pathogenic fungi which included *Candida albicans*, *Fusarium oxysporum* and *Aspergillus niger*. The possible reason for the difference could be the type of antimicrobial metabolite extracted from the actinomycetes [19].

[24] observed that temperature greatly influences the potency of an antimicrobial metabolites. In addition, [30] indicated that temperature at which an antimicrobial remains potent affects it's storage conditions. A study carried out in Japan obtained the maximum storage temperature of antimicrobials screened from actinomycetes to be 80ºC [29] which agree with the findings of the present study. The possible reason could be similarity in the nature of the antimicrobials [2].

[3] showed that the activity of metabolites from actinomycetes increases with increase in pH reaching optimum at a pH of 5-8. This disagrees with the findings of the present study since optimum zones of inhibition were observed at low pH. This suggests that the antimicrobials can be taken orally since they can resist the low pH that occurs in the stomach. The differences in stability of the antimicrobials observed in the two studies can be attributed to the nature of the antimicrobials. [15] observes that the bonding that exists between the active ingredients of a given compound dictates the way it will respond to variations in pH.

Table 3: Zones of inhibition (mm) after exposure of the metabolites to enzymes, temperature and pH using fungi

Path	Meta	Enzyme				Temperature			pH					
		Try	Lyso	Pep	Lip	60ºC	70ºC	80ºC	2	4	5	7	9	11
C. albi	PAN30	40±2	45±3	40±2	45±1	41±2	42±3	45±3	41±3	40±1	42±2	45±3	0±0	0±0
	PAN37	39±1	43±2	41±2	42±2	40±3	38±2	35±2	40±2	42±2	41±3	43±2	0±0	0±0
	PAN41	0±0	0±0	0±0	0±0	37±3	40±1	42±3	41±2	42±3	40±2	43±2	0±0	0±0
	PAN154	0±0	0±0	0±0	0±0	25±1	27±1	29±2	25±1	24±2	22±2	25±1	0±0	0±0
F. oxy	PAN30	46±2	43±2	45±3	42±2	45±2	43±2	41±3	45±1	40±3	41±3	42±1	0±0	0±0
	PAN37	40±2	41±1	43±2	45±3	40±1	37±3	42±2	41±1	45±2	40±2	43±3	0±0	0±0
	PAN41	0±0	0±0	0±0	0±0	37±1	40±2	41±2	45±2	48±3	44±1	41±2	0±0	0±0
	PAN154	0±0	0±0	0±0	0±0	26±2	27±1	28±1	23±3	25±2	21±2	23±1	0±0	0±0
U. may	PAN30	42±2	40±2	41±2	43±2	39±2	40±1	38±2	44±2	43±2	43±1	42±1	0±0	0±0
	PAN37	39±1	42±1	43±1	38±1	37±1	33±3	35±1	37±3	38±3	39±2	40±3	0±0	0±0
	PAN41	0±0	0±0	0±0	0±0	38±3	34±2	44±2	38±1	39±2	40±1	42±2	0±0	0±0
	PAN154	0±0	0±0	0±0	0±0	26±2	28±2	27±2	23±1	20±3	21±2	24±2	0±0	0±0
A. niger	PAN30	24±1	28±2	21±2	27±2	25±1	26±2	24±3	23±3	26±1	20±1	23±1	0±0	0±0
	PAN37	23±2	29±2	24±4	21±1	24±2	27±1	25±2	24±2	21±2	25±2	27±2	0±0	0±0
	PAN41	0±0	0±0	0±0	0±0	20±1	23±1	22±2	23±2	25±1	21±2	24±1	0±0	0±0
	PAN154	0±0	0±0	0±0	0±0	19±1	21±3	20±1	23±2	24±2	20±1	22±2	0±0	0±0

Each value represents the means (±SD) of five independent experiments. Path; pathogen, meta; metabolite, *C. albi; Candida albicans, F. oxy; Fusarium oxysporum,U. may; Ustilago maydis and A. niger; Aspergillus niger*.

Conclusion

Sensitivity of bacterial and fungal pathogens to antimicrobial metabolites extracted from actinomycetes coded PAN 30, PAN 37, PAN 41 and PAN 154 were carried out. The amounts of antimicrobial metabolites that the actinomycetes produce in 3 L of broth were determined. The antimicrobials obtained were stable against trypsin, lysozymes, pepsin and lipase. However, antimicrobials from PAN 41 and 154 could not tolerate the tested enzymes. In addition, the antimicrobials were stable at temperatures of 60ºC, 70ºC, and 80ºC. They were also stable at pH 2,4,5,7 but not pH 9 and 11.

Conflict of interest

The authors declare that there are no conflicting parties.

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