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Molecular Characterization and optimization of Bioactive Compounds Production of three Actinomycetes spp Isolated from Waste Dump Soil from Western Uganda

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Abstract

The study was designed to investigate the effect of growth culture conditions, namely: Media composition, incubation period, temperature and pH on the production of antimicrobial compounds from three selected actinomycetes isolated from waste dump soil in Western Uganda. Molecular characterizations of these selected isolates were also carried out. Optimization processes were assessed using shake-flask cultures on eight different media, Portion of filtered fermentation broths were assayed using agar well diffusion method. The remaining portions were extracted using water, ethanol, ethyl acetate and methanol solvents. The extracts were dried and re-dissolved in 2.5% dimethyl sulphoxide to concentration of 2.5mg/ml and tested for antimicrobial activity using agar well diffusion method. Three selected isolates were characterized using conventional PCR and sequenced using Sanger methods. The results showed that, Modified Nutrient broth supplemented with carbon sources (soluble starch and Glycerol at half each) at 12-18g/l, 08-16g/l and 10-18g/l for BRWDc (SP), KBMWDSb6 (M6) and KBRWDSa3 (RF) respectively produced optimum concentration of bioactive compounds. NaCl at concentration of 17-19g/l was found to be suitable for optimum bioactive compound production. The best optimized results were found when cultures were grown under the following conditions: temperature (30-35^{o}C), pH (7.0-7.5) and incubation period (168h). Aqueous and ethanol extracts gave optimum bioactive activity for all the three organisms. The identification of 16SrDNA gene showed that, the three isolates belong to phylum actinobacteria into the genus *Streptomycetes*. This study showed that media compositions, cultural conditions and solvents for extraction play role in bioactive compound production in these *actinomycetes* isolates.

Keywords: Optimization, Actinomycetes spp, Bioactive Compound, Molecular characterization, Waste dump soil, Western Uganda.

Introduction

Over the years, there has been increasing reports of infections with resistant micro-organisms and opportunistic pathogenic infections. This increase has been stated to be among immunocom promised patients with diseases such as HIV, organ transplant, cancer and other conditions (1, 2). In African countries for example in Uganda, there is growing concern on the increasing burden of infectious diseases

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among immunocompromised patients and drug resistant pathogens. Literature have shown that, among 35 million populations of Ugandans, 1.14% had HIV and an estimated 9.2% (101,000) have a CD4 count <200 cells/iL (3). Infections caused by bacteria were reported to be 20% and over 1 million cases of fungal infections per year (2, 3). These necessitated the need for search for new antimicrobial compounds to face these current global challenges.

Actinomycete has been shown to be a source of bioactive compounds with pharmaceutical, Agricultural and food industrial importance (4). The ability of this bacteria to produce these important compounds was linked to Non Ribosomal Polyketide Synthase (NRPK), Polyketide Synthase Pathways (PKS) and presence of Larger Genome (>55mol %) with adequate transcription factors which regulates the gene expression which acts in tune of specific environmental requirements (5). Literatures have shown that productions of these compounds depends on genus, species, strains of actinobacteria and could be decreased or increased by using different nutritional, growth conditions and time (6). Extraction solvents could also affect the amount of bioactive compounds (7). Taxonomy of antibiotics producing actinomycetes plays an important role in discovering new antimicrobial compounds (8). Isolation and identifications of actinomycetes using phenotypic methods remain the gold standard but in recent years the process seems to be more accurate using molecular methods (8). The later methods contributed greatly in characterizing novel actinomycetes species and strains from both terrestrial and aquatic environments (9).

This research studied actinomycetes isolated from waste dump soil from Western Uganda and found three isolates ((KBMWDSb6 (M6), BRWDSc (SP) and KBRWDSa3 (RF)) with ability to produce bioactive compounds. The three isolates were tested on bacteria and fungi including drug resistant clinical bacterial isolates (10, 11). This study optimized nutritional requirements, fermentation conditions; extracted fermented broths using different solvents and characterised the selected *actinomycetes* isolates using molecular methods.

MATERIALS AND METHODS

Isolation of Actinomycetes : The three actinomycetes spp were isolated using two different media: Starch casein nitrate agar (12) and yeast extract starch casein agar (YSCA) (13). The pure cultures were maintained on starch casein nitrate agar slants at 4^{o}C for short storage and 30% glycerol at -80^{o}C for long storage (14, 15).

Phenotypic identification of Actinomycetes : The active actinomycetes spp were screened using the API 20A kit (Biomerieux, France) (16) as described in manufacturer's guide and incubated for 48 hours at 37°C in an anaerobic jar after which the results were recorded. The identification was done using the apiweb™ software (V4.0). Other methods used for identification were macroscopic morphology of the colonies, microscopic and conventional biochemical methods (17, 18).

Molecular characterization of actinomycetes

DNA extraction : DNA was extracted from seven (7) day old actinomycetes cultures by suspending the isolates into 3ml of 1XPBS. The suspension was boiled at 90^{o}C for 40 mins and then centrifuged for 5mins at 13000rpm. Five hundred microliter (500µl) of the centrifuged suspension was transferred in to a sterile eppendorf after which 5 µl of RNAase enzyme was added and incubated at room temperature for 10 mins. One millilitre (1mL) of absolute ice cold ethanol was added to the solution and incubated at -80^{o}C for 30 mins. The solution was then centrifuged at 13000 rpm for 10 mins and washed by adding 1ml of 70% ethanol and centrifuged again at 13000 rpm for 10 mins. The solution was then inverted on tissue to dry and after drying, it was eluted in 50 µl of elution buffer.

Amplification of extracted DNA and sequencing : The 16S rDNA gene of three selected actinomycetes was amplified according to the method (19). The following primers 27F

(5'AGAGTTTGATCCTGGCTCAG3') and 1492R (5'ACGGCTACC TTGTTACGACTT 3') were used to amplify the extracted DNA. All PCR reactions were performed in a Perkin Elmer DNA thermal cycler (Perkin Elmer 480).

PCR products were washed by precipitation before sequencing and quantified using NanoDrop ND-1000 (Wilmington, DE, USA).Sequencing reactions were carried out using final volume of 7 µl solution containing 5.5µl of nuclease-free water, 0.5µl of (40 ng/µl) primer and 1µl of (100 ng/µl) of DNA template, BD terminator (ABI). The reaction products were analysed using ABI 3500XL Genetic Analyser, POP7™, BigDye® 3.1. Amplification and sequencing reactions were done at Inqab Biotechnological (Africa's Genomic Company) South Africa. The 16S rDNA sequence gene data of the potent actinomycetes were compared to the nucleotide sequences in Gene bank data base in National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nih.gov) using Basic Local Alignment Search Tool (BLAST).

Phylogenetic analysis : The Phylogenetic and molecular evolutionary analyses were conducted using MEGA software (version 6) (20).

Optimization for antimicrobial production by three selected actinomycetes

Effect of different media and fermentation period on antimicrobial production : Fermentation was carried out using eight different media in shake-flask culture method (6) in Erlenmeyer flask (500ml). The media used included: **1.** Modified Nutrient broth (MNB) Thermo scientific oxoid (g/l: Lab-lemoco powder 1, Yeast extract 2, peptone 5, Soluble starch 8, Glycerol 3, K_2HPO_2 0.5, NaCl 5, $CaCO_3$ 0.75, $MgSo_4.7H_2O$ 0.5 and pH 7.0±4), **2.** Yeast extract starch broth (YESB) (Composition in media g/l: yeast extract 3, peptone 3, casein 3, starch 8, Glycerol 3, K_2HPO_4 0.5, $MgSO_4.7H_2O$ 0.5, NaCl 12 $CaCO_3$0.75, and pH 7.0±4), **3.** Modified Potato Dextrose broth (MNB) HiMedia-MO96-500G (g/l Potatoes infusion 200, Dextrose 20, Glycerol 3, $CaCO_3$ 0.75, NaCl 4.8, $MgSO_4.7H_2O$ 0.5, K_2HPO_4 0.5 and pH adjusted 7.0± 2**), 4.** Starch casein nitrate broth (SCNB) (Composition in media g/l: Starch 10, Casein 0.3, KNO_3 2, NaCl 2, K_2HPO_4 2, $MgSO_4.7H_2O$ 0.5, $CaCO_3$ 0.02, $FeSO_4.7H_2O$ 0.01 and pH adjusted 7.0 ± 2), **5.** Starch Casein broth (SCB) (the composition in g/l: Soluble starch 10.0; casein 0.3; $NaNO_3$ 2.5; K_2HP0_4 1.0; KH_2PO_4 1.0; $MgSo_4.7H_2O$, 0.5; KCl, 0.5; trace salt solution 1.0 ml : $CuSO_45H_2O$ 0.64; $FeSO_4.7H_2O$, 0.11; $MnCl_2.4H_2O$ 0.79, $ZnSO_4.7H_2O$ 0.15 and pH adjusted 7.0±2), **6.** Starch nitrate broth (SNB) (Composition in media g/l: Starch 20, KNO_3 1, NaCl 0.5, K_2HPO_4 0.5, $MgSO_4.7H_2O$ 0.5, $FeSO_4.7H_2O$ 0.1 and pH adjusted 7.0± 2), **7.** Glycerol Casein broth (GCB) (the composition in g/l: Glycerol 10.0; casein 0.3; $NaNO_3$ 2.5; K_2HP0_4 1.0; KH_2PO4 1.0; $MgSo_4.7H_2O$, 0.5; KCl, 0.5; trace salt solution 1.0 ml : $CuSO_45H_2O$ 0.64; $FeSO_4.7H_2O$, 0.11; $MnCl_2.4H_2O$ 0.79, $ZnSO_4.7H_2O$ 0.15 and pH adjusted 7.0 ±4) and **8.** Glycerol arginine broth (GAB) (the composition in g/l: Glycerol 12; arginine 1; NaCl 1 K_2HP0_4 1.0, $MgSo_4.7H_2O$, 0.5; $CuSO_45H_2O$ 0.001; $FeSO_4.7H_2O$, 0.001; $MnCl_2.4H_2O$ 0.001, $ZnSO_4.7H_2O$ 0.001 and pH adjusted 7.0 ±2). Seven days old culture of actinomycete sp was inoculated and incubated in a digital gas bath thermostats oscillator (THZ-82B) at 28°C and 200 ± 5 rpm. Aliquots of broth were obtained and checked for the antimicrobial activity at intervals: 24, 48, 72, 96, 120, 144 and 168h of incubation to study the effects of incubation period on antimicrobial production.

Effect of pH and temperature on antimicrobial production : At the end of the optimization studies, one media was selected to study the effect of pH and temperature on the production of bioactive compound(s). Modified Nutrient broth medium was selected for this study. The varying pH and temperature used were as shown pH (5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9) and incubation temperature (15°C, 20°C, 25°C, 30°C, 35°C, 40°C, 45°C and 50°C). Seven days old culture of actinomycete sp was inoculated and incubated in a digital gas bath thermostats oscillator (THZ-

82B) at 200 ± 5 rpm for 7 days after which the broth was centrifuged at 3000rpm for 20 minutes and filtered using filter paper (Whatman No. 1) and tested for antimicrobial activity using agar well diffusion method (21).

Effect of different carbon source on antimicrobial production : Effects of different carbon source were studied on Modified Nutrient broth according to the method (21). The different carbon sources used were: (10g/l): Soluble Starch, Glucose, Glycerol, Lactose, and Sucrose. A second trial was carried out by combining carbon sources as: (5g/l each): Starch and Glucose, Starch and Glycerol, Lactose and Sucrose were added to Nutrient broth medium while all other parameters were kept constant. The tests medium were inoculated with seven days old culture of actinomycete sp and incubated at 28°C in a digital gas bath thermostats oscillator (THZ-82B) at 200 ± 5 rpm for 7 days. After incubation the broth was centrifuged at 3000rpm for 20 minutes and filtered using filter paper (Whatman No. 1). The filtrate was tested for antimicrobial activity using agar well diffusion method.

Effect of different concentration of starch and glycerol combination on antimicrobial production : The effect of glycerol on media containing combine carbon were tested at different concentrations: soluble starch and glycerol at half concentration of each (g/l: 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20) were added to Modified Nutrient broth, other parameters remained constant. The broth was inoculated with seven days old culture of actinomycete sp and incubated at 28°C, 200 ± 5 rpm for 7days. After incubation the broth culture was centrifuged at 3000rpm for 20 minutes and filtered. The filtrate was tested for antimicrobial activity using agar well diffusion method (21).

Effect of sodium chloride and nitrogen source concentration on antimicrobial production : Effects of different concentrations of NaCl on production of bioactive compound were studied according to the method (22). The different concentration of the NaCl and Nitrogen source (Peptone) used were: (g/l: 5, 7, 9, 11, 13, 15, 17, 19, 21 and 23) on Modified Nutrient broth. All other parameters were kept constant. The broth media was inoculated with seven days old culture of actinomycete sp and incubated at 28°C, 200 ± 5 rpm for seven day. The broth cultures were centrifuged at 3000rpm for 20 minutes and filtered. The filtrate was tested for antimicrobial activity using agar well diffusion method (21).

Antimicrobial activity of fermented broths

Test organisms : The following test organisms were used to determine the antimicrobial activity of all fermented broths and different solvents extracts: drug (s) resistant clinical bacterial isolates (*Escherichia coli* 2966, *Pseudomonas aeruginosa* 2929, *and Staphylococcus aureus* 2876) and Standard drug sensitive fungus *Candida albicans* ATCC1023), (obtained from Department of Medical Microbiology Makerere University, Kampala).

Antibacterial and antifungal assay : Cell concentration of test organisms was adjusted to 0.5 McFarland turbidity standards. Bacterial cultures were inoculated on Mueller Hinton agar (HIMEDIA M173-500G), fungal cultures on potato dextrose agar plate. Wells were bored using sterile 1000 µl micro pipette tip (17). The wells were filled with 100 µl of supernatant and the plates were incubated at 37°C for 24 h and 72h at 28°C for bacteria and fungus respectively. Freshly prepared broth media was used as negative control. All experiments were performed in triplicates.

Extraction of bioactive compounds using different solvents : Extraction of bioactive compounds was carried out according to the method earlier described by (8, 17). The remaining centrifuged fermented filtered broth of each isolate was divided into four. Three parts were extracted using three solvents by adding equal volume (1:1) of ethanol (95%), ethyl acetate (95%) and Methanol (95%). The solution was shaken vigorously on a rotatory shaker. The solvent phase was collected and evaporated in hot air oven at 40°C. The remaining one part of the filtered broth was concentrated to get aqueous

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extract. The filtrates were dried and stored at 4°C for further studies.

Antimicrobial activity of different solvent crude extracts : The dried crude extracts (0.43g/200ml, 0.38g/200ml and 0.40g/200ml) for BRWDc (SP), KBMWDSb6 (M6) and KBRWDSa3 (RF) respectively, were re-dissolved in 2.5% dimethyl sulphoxide (DMSO) (THOMAS BAKER) (23, 24) to a concentration of 2.5mg/ml of each extract which was used for antibacterial and antifungal activity (17). Cell concentration of test bacteria and *C. albicans* was adjusted to 0.5 McFarland turbidity standards and inoculated on Mueller Hinton agar (HIMEDIA M173-500G) for bacteria and potato dextrose agar plate for fungus. The test organisms were then spread on to respective media plates. Wells were bored using sterile 1000 µl micro pipette tip and filled with 100µl of 2.5mg/ml ethanol crude extract, ethyl acetate crude extract, Methanol crude extract and aqueous crude extract and 2.5% DMSO was used as negative control. Plates were incubated at 37°C for 24 h and 72 h at 28°C for bacteria and fungus respectively. At the end of incubation, the zones of inhibition were measured. All experiments were performed in triplicates.

Statistical analysis : The data was analysed using GraphPad software (Version 5.04). Results of mean zone of inhibition on the effects of different media used, carbon source, growth temperature, incubation time, pH, nitrogen source, NaCl concentration and different solvents crude extracts were analysed using one way ANAVO using multiple comparison and $p \leq 0.05$ was considered to be significant.

Results and discussion

The results of Analytical profile index using API 20A showed that only actinomycete isolate KBRWDSa3 (RF) can ferment the sugar anaerobically (Table 1). The analysis of this isolates using APIwab™ showed that it's belonging to actinomycetes genus. This also showed that isolate KBRWDSa3 (RF) is a facultative organism. The inability of the isolates BRWDSc (SP) and KBMWDSb6 (M6) to ferment the sugars anaerobically showed that they are either obligate aerobic organisms or other genus of actinobacteria. However, this could also be as result of kit used for the identification which is selective to only actinomycetes genus.

The molecular characterization of the three broad spectrum active actinomycetes isolates were determined and PCR amplification of genomic extracted DNA using actinomycetes forward and reverse primers produced 1600bp for all the three isolates (Fig. 1). The amplified PCR products were also sequenced and produced 885bp, 881bp and 860 bp, of sequences for KBMWDSb6 (M6), BRWDSc (SP) and KBRWDSa3 (RF) respectively. The resultant sequences were BLAST using MEGA software with nucleotide BLAST search in NCBI. The sequences that produced significant alignment

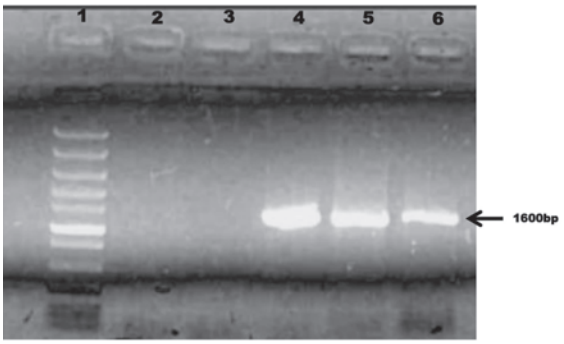


Fig. 1 : Agarose gel showing PCR amplification of 16S rDNA of the actinomycetes isolates: 1: DNA molecular weight ladder, 2: Negative control, 3: MBJ, 4: FR: KBRWDSa3 (RF), 5:KBMWDSb6 M6), 6: BRWDSc (SP).

results showed that actinomycetes isolates KBMWDSb6 (M6) and BRWDSc (SP) were 98% and 97% respectively similar to *Streptomyces* sp AQB.SKKU 20 with accession number JN836957.1. However, these were also found be to be 97% similar to *Streptomyces* sp. MSU 2261 with accession number AY232829.1. Isolate KBRWDSa3 (RF) was found to be 99% similar to *Actinomycete* TGsR-01-04 with accession number AB775551.1 and 33 *Streptomycetes* spp including

Uncultured *Streptomyces* sp. clone 2516 FJ429559.1. The sequences were deposited to Genbank with accession numbers: MG594793, MG594794 and MG594795 for KBMWDSb6 (M6), KBRWDSa3 (RF) and BRWDSc (SP) respectively. The phylogenetic tree analysis based on 16S rDNA was constructed using the Maximum Likelihood method (Fig. 2). The tree result showed that the two isolates (KBMWDSb6 (M6), and BRWDSc (SP)) formed cluster with *Streptomyces* sp. MSU 2261 while KBRWDSa3 (RF) isolates occupied new position in the tree but belong to genus *Streptomycetes*. Base on the phenotypic and molecular data, the isolate KBRWDSa3 (RF) was suggested to be new *Streptomycetes* MG594794 sp

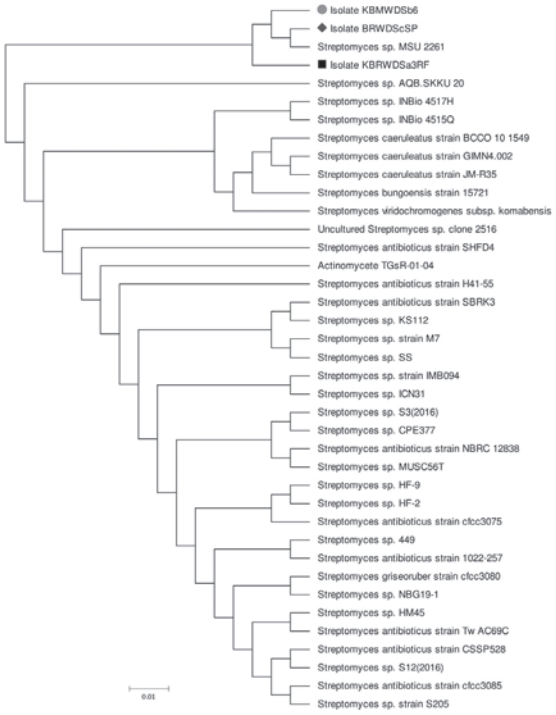


Fig. 2: A phylogenetic tree was constructed using Maximum Likelihood method bootstrap test (100 replicates). The tree showed the position of isolates KBMWDSb6 (M6), BRWDSc (SP) and KBRWDSa3 (RF) with closely related *Streptomycetes* species.

The ability of actinomycetes spp to grow and produce antimicrobial compounds depends on the media compositions and incubation conditions (6, 21). This research has also shown that, selection of media and incubation conditions for production of antimicrobial compounds is necessary.

Out of the eight different fermentation media : Modified Nutrient broth(MNB), Yeast extract starch broth (YESB), Modified Potato Dextrose broth (MPDB), Starch casein nitrate broth (SCNB), Starch casein broth (SCB), Starch casein arginine broth (SCAB), Glycerol casein nitrate broth (GCNB), Glycerol arginine broth (GAB), three actinomycetes spp (BRWDc (SP), KBMWDSb6 (M6) and KBRWDSa3 (RF) isolates grown on two media MNB and YESB were found to support the production of antimicrobial compound (Fig.3). Actinomycete sp KBMWDSb6 (M6) fermented in all the media tested but did not show activity to resistant clinical isolates *S. aureus* 2876 while actinomycete spp (BRWDc (SP) and KBRWDSa3 (RF) fermented in all media showed no activity against two clinical resistant isolate *P. aeruginosa* 2929 and *E. coli* 2966. It was also observed that, all the three actinomycetes fermented broths showed activity to standard test fungus *Candida*

Table 1: Analytical profile index of active actinomycetes isolates (Using API20A kit)

Active Isolates	IND	URE	GLU	MAN	LAC	SAC	MAL	SAL	XYL	ARA	GEL	ESC	GLY	CEL	MNE	MLZ	RAF	SOR	RHA	TRE	CAT
BRWDSc (SP)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
KBMWDSb6 (M6)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
KBRWDSa3 (RF)	-	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	-	+	+	+

KEY: IND: Indole, URE: Urease, GLU: Glucose, MAN: Mannitol, LAC: Lactose, SAC: Saccharose, MAL: Maltose, SAL: Salicin, XYL: Xylose, ARA: Arabinose, GEL: Gelatin, ESC: Esculin, GLY: Glycerol, CEL: Cellobiose, MNE: Mannose, MLZ: Melezitose, RAF: Raffinose, SOR: Sorbitol, RHA: Rhamnose, TRE: Trehalose, CAT: Catalase.

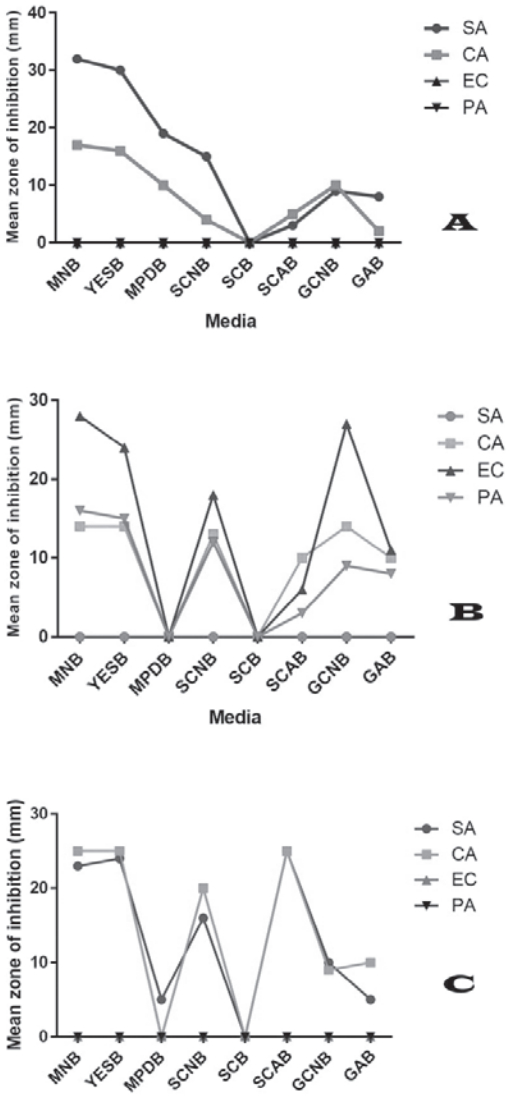
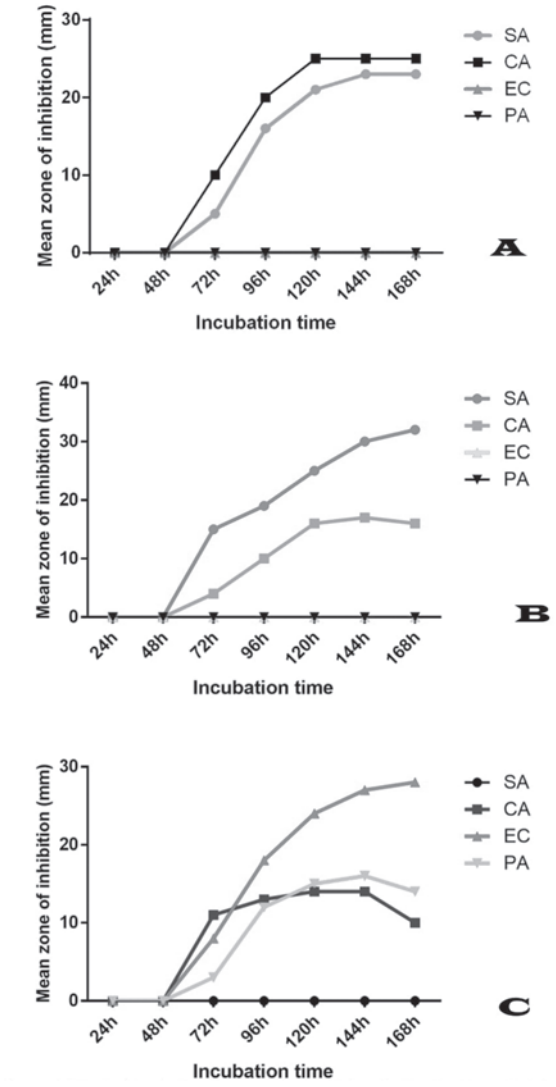


Fig. 3: Media selection for antimicrobial productions. MNB: Modified Nutrient Broth, YESB: Yeast extract starch broth, MPDB: Modified Potato Dextrose broth, SCNB: Starch casein nitrate broth, SCB: Starch casein broth, SCAB: Starch casein arginine, GCNB: Glycerol casein nitrate broth and GAB: Glycerol arginine broth. A: BRWDSc(SP), B:KBMWDSb6 (M6), C: KBRWDSa (RF), EC: *E. coli* 2966, PA: *P. aerugenosa* 2929, CA: *C. albicans* ATCC1023, SA: *S. aureus* 2876. The results of multiple comparisons showed statistical significance at p value ≤ 0.05 using one way ANOVA.

*albicans*ATCC1023. The zone of inhibition of the three actinomycetes spp from all test media ranged from 02 to 32mm. The results showed that the three selected isolates produced low amount of antimicrobial compound in 71.42% of the media used. The p values (0.0051, 0.0002, and 0.0003 for KBMWDSb6 (M6), KBRWDSa3 (RF) and BRWDc (SP) respectively) of multiple comparison between the different media used for extraction of antimicrobial compound showed statistical significance at $p \leq 0.05$. This confirmed the reports of (6, 21) who also found out that, different actinomycetes isolates from different environments do not produce bioactive compounds in all test media.

The results on different incubation periods showed that antimicrobial production of the three isolates started after 72h of incubation but maximum production was obtained after 168h (Fig. 4). This could be as a result of the depletion the nutrients from medium after 168h of incubation which enhance the production of antimicrobial compounds by actinomycetes (25). This was contrary to the findings of (6) who reported that, maximum productions of antimicrobial compounds by *Streptomyces* strain ERI-1, ERI-2 and ERI-3 were obtained after 96h of incubation. The p values (0.0133, 0.0012, and 0.0006 for KBMWDSb6 (M6), KBRWDSa3 (RF) and BRWDc (SP) respectively) of multiple comparisons on the influence of incubation time on antimicrobial production showed statistical significance at $p \leq 0.05$. This showed that different actinomycetes isolates require different growth conditions for optimum production of bioactive compounds.

Modified Nutrient broth medium was selected for study on the effect of different carbon source on the production of antimicrobial compounds. The combined carbon source (Starch and Glycerol) gave a higher production of antimicrobial compounds when compared to the other carbon sources used (Fig.5). The p values (0.1026, for KBMWDSb6 (M6) and 0.0001 for KBRWDSa3 (RF) and BRWDc (SP)) of multiple comparisons on the effects of different carbon source on antimicrobial production showed statistical



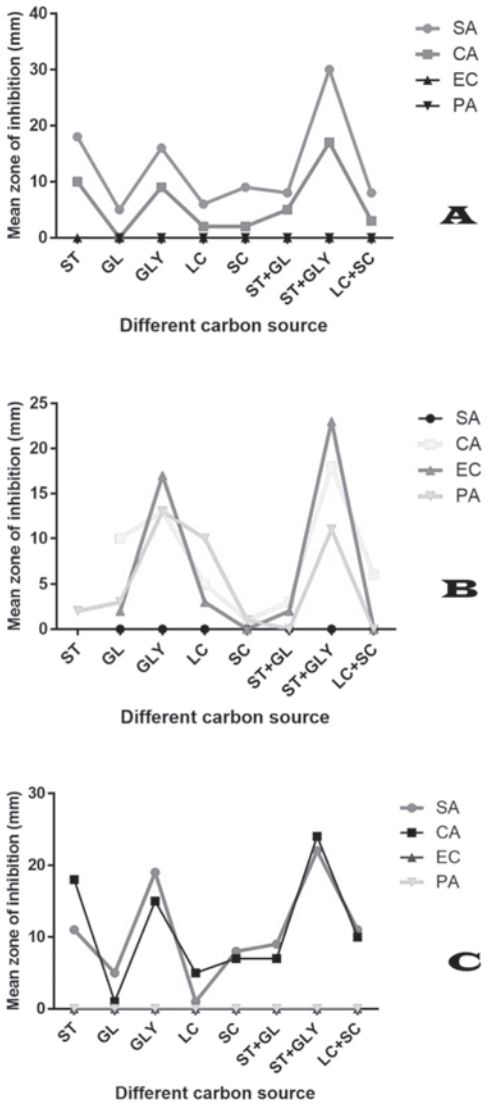


Fig. 4: Effect of incubation time on antimicrobial production by three selected actinomycetes spp. on modified nutrient broth medium, A: BRWDSc (SP), B: KBMWDSb6 (M6); C: KBRWDSa (RF). EC: *E. coli* 2966, PA: *P. aerugenosa* 2929, CA: *C. albicans* ATCC1023, SA: *S. aureus* 2876.The results of multiple comparisons showed statistical significance at p value ≤ 0.05 using one way ANOVA.

Fig. 5: Effect of different carbon source on production of antimicrobial compound in modified nutrient broth medium. A: BRWDSc (SP), B : KBMWDSb6(M6); C : KBRWDSa (RF). EC: *E. coli* 2966, PA: *P. aerugenosa* 2929, CA: *C. albicans* ATCC1023, SA: *S. aureus* 2876, ST: starch, GL: Glucose, GLY: Glycerol, LC: Lactose, SC: Sucrose, ST+GL: Starch and Glucose, ST+GLY: Starch and Glycerol, LC+SC: Lactose and Sucrose. The results of multiple comparisons showed statistical significance at p value ≤ 0.05 using one way ANOVA.

significance at $p \leq 0.05$ for KBRWDSa3 (RF) and BRWDc (SP) while statistical insignificance was observed on the isolate KBMWDSb6 (M6). This result was similar to those of (26, 27, 28) though they used the two carbons separately. Arasu*et al.* (6) reported the maximum antimicrobial production by *Streptomyces* spp from medium with glucose and fructose combinations. However, studies by (29, 30) reported that media containing single carbon such as Glucose, Sucrose, and Lactose supported higher production of bioactive compounds.

Different concentrations of selected carbon source (Soluble starch and Glycerol) were also studied for their influence on the production of antimicrobial compounds. The results are shown on (Fig. 6). The influence of combine Starch and Glycerol concentrations on the production of antimicrobial compounds ranged from 12-18g/l, 08-16g/l and 10-18g/l for BRWDc (SP), KBMWDSb6 (M6) and KBRWDSa3 (RF) respectively. These combinations gave better results than those of single carbon sources. The p values (0.0081, 0.0057, and 0.0011for KBMWDSb6 (M6), KBRWDSa3 (RF) and BRWDc (SP) respectively) of multiple comparisons on the effects of different concentration of combine carbon source (Starch and Glycerol) on antimicrobial production showed statistical significance at $p \leq 0.05$. Similar finding were reported by Sengupta *et al.*(27). He used, yeast extract medium combined with two carbon source (starch 8g/l and Glycerol 3g/l) though different concentration on antimicrobial production was not established.

The effects of temperatures on the production of antimicrobial compounds are shown on (Fig. 7). The minimum temperature for the antimicrobial production was found to be 25°C while the maximum temperature was 35°C. It was observed that, higher antimicrobial activity was when cultures were grown at 30-35°C. The ability to produce antimicrobial compounds below room temperature (20°C) could be due to their adaptations to the environments where they were isolated. The p values (0.0945, 0.0400, and

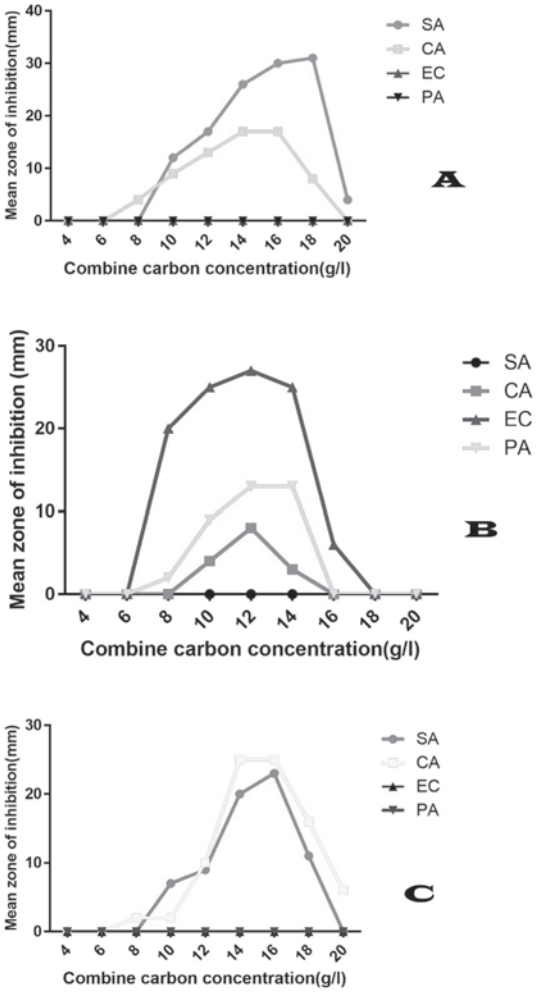


Fig. 6: Effects of different combine carbon source(Starch and Glycerol) on antimicrobial production from three selected actinomycetes spp. A: BRWDSc (SP), B : KBMWDSb6(M6); C : KBRWDSa (RF). EC: *E. coli* 2966, PA: *P. aerugenosa* 2929, CA: *C. albicans* ATCC1023, SA: *S. aureus* 2876.The results of multiple comparisons showed statistical significance at p value ≤ 0.05 using one way ANOVA.

0.0403for KBMWDSb6 (M6), KBRWDSa3 (RF) and BRWDc (SP) respectively) of multiple comparisons on the effects of growth temperature on antimicrobial production showed statistical significance at $p \leq 0.05$ for KBRWDSa3 (RF) and BRWDc (SP), but no statistical difference was observed from isolate KBMWDSb6 (M6). Other

studies have reported high production of antimicrobial compounds by most actinomycetes spp when grown at temperature 30 - 35°C (6, 22, 28, 30; 31).

Furthermore, this study showed that the production of antimicrobial compounds were high when the three isolates were grown under pH 6.7 to 8.5. Higher zone of inhibitions were observed from organisms grown at pH 7.0-pH 7.5 (Fig. 8).

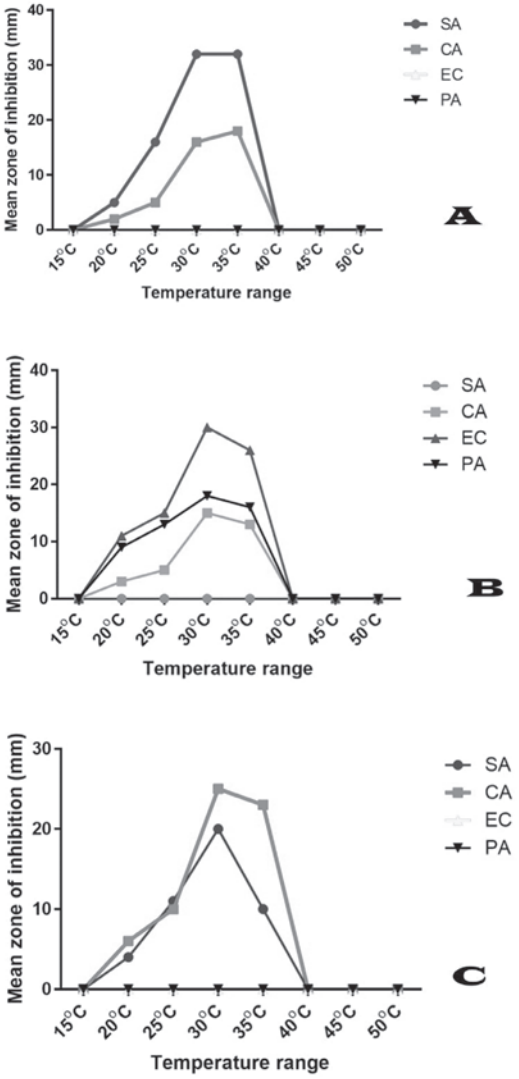


Fig. 7: Effect of temperature on the production of antimicrobial compound from three selected actinomycetes ssp. **A**: BRWDSc (SP), **B**: KBMWDSb6 (M6); **C**: KBRWDSa (RF). **EC**: *E. coli* 2966, **PA**: *P. aerugenosa* 2929, **CA**: *C. albicans* ATCC1023, **SA**: *S. aureus* 2876. The results of multiple comparisons showed statistical significance at p value ≤ 0.05 using one way ANOVA.

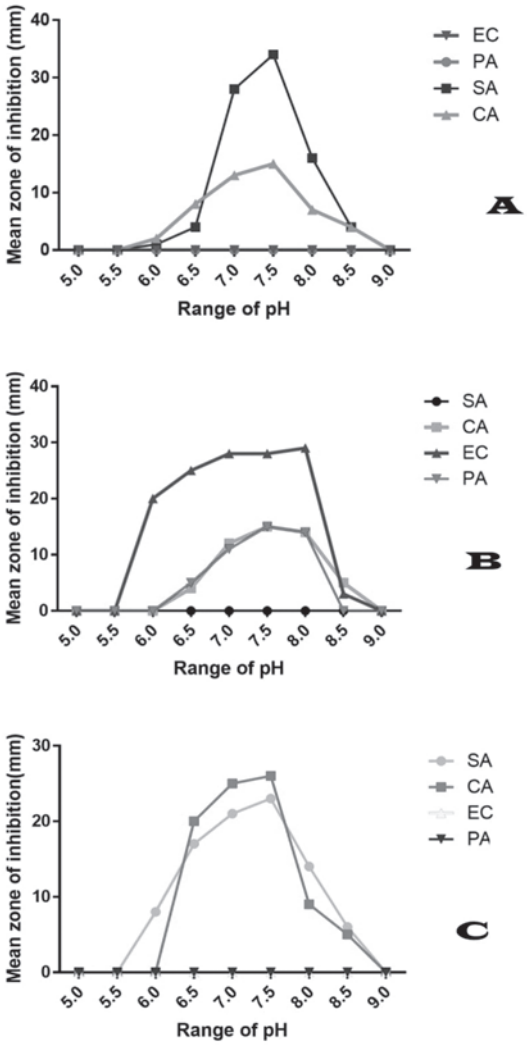


Fig. 8: Effect of pH on the production of antimicrobial compounds by three selected actinomycetes isolates. **A**: BRWDSc(SP), **B**: KBMWDSb6(M6); **C**: KBRWDSa(RF). **EC**: *E. coli* 2966, **PA**: *P. aerugenosa* 2929, **CA**: *C. albicans* ATCC1023, **SA**: *S. aureus* 2876. The results of multiple comparisons showed statistical significance at p value ≤ 0.05 using one way ANOVA.

The p values (0.0054, 0.0042, and 0.0186 for KBMWDSb6 (M6), KBRWDSa3 (RF) and BRWDc (SP) respectively) of multiple comparisons on the effects of pH on antimicrobial production showed statistical significance at $p \leq 0.05$. Our finding was in line with previous results reported in similar studies (21, 22, and 28). However, other studies reported high zone of inhibition at neutral to slightly acidic pH (30, 31).

Figure 9 below showed the results of the effect of different NaCl concentrations on the production of antimicrobial compounds. The suitable concentration of NaCl for the production of antimicrobial compounds ranges 15-21g/l for the selected actinomycetes spp but highest antimicrobial activity was observed at 17 and 19g/l. The p value (0.0001 each for KBMWDSb6 (M6), KBRWDSa3 (RF) and BRWDc (SP)) of multiple comparisons on the effects of different NaCl concentration on antimicrobial production showed statistical significance at $p \leq 0.05$. Different actinomycetes spp produced antimicrobial compounds at different concentration of NaCl (22). Other studies have shown optimum antimicrobial production by actinomycetes spp with NaCl concentration of: (20g/l) Singh *et al*. (32) and (10g/l): El-Refai*et al.* (33). Mangamuri*et al.* (22) findings showed that the optimum NaCl concentration for the production of bioactive compounds from *Streptomyces tritolerans* DAS 165T was equivalent to 50g/l.

Figure 10 showed the effect of different concentration of nitrogen source (peptone) on antimicrobial production by three actinomycetes spp. The suitable nitrogen source (peptone) for the production of antimicrobial compounds ranged from 9-21g/l and 11-21g/l for BRWDc (SP) and KBRWDSa3 (RF), andKBMWDSb6 (M6) respectively. The optimum activity was observed at 11-21g/l for all three isolates. The p value (0.0001 each for KBMWDSb6 (M6), KBRWDSa3 (RF) and BRWDc (SP)) of multiple comparisons on the effects of different nitrogen source (peptone) on antimicrobial production showed statistical significance at $p \leq 0.05$. The concentration obtained from this study was higher than that of Al-Ghazali and Omran (34) who reported 0.05g/100ml as the required concentration for maximum antimicrobial production by *Streptomyces* sp. LH9.

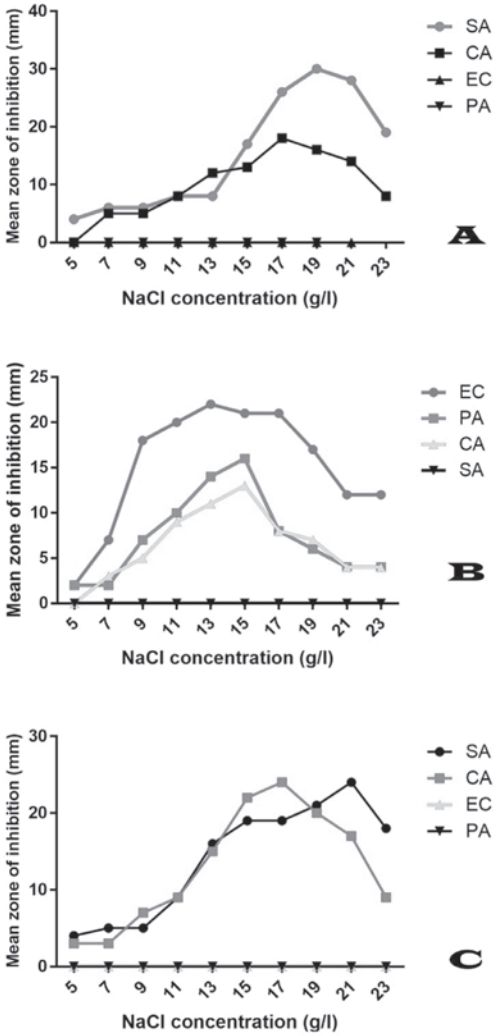


Fig. 9: Effect of NaCl concentration on the production of antimicrobial compounds by three selected actinomycetes isolates. **A**: BRWDSc (SP), **B**: KBMWDSb6 (M6); **C**: KBRWDSa (RF).). **EC**: *E. coli* 2966, **PA**: *P. aerugenosa* 2929, **CA**: *C. albicans* ATCC1023, **SA**: *S. aureus* 2876. The results of multiple comparisons showed statistical significance at p value ≤ 0.05 using one way ANOVA.

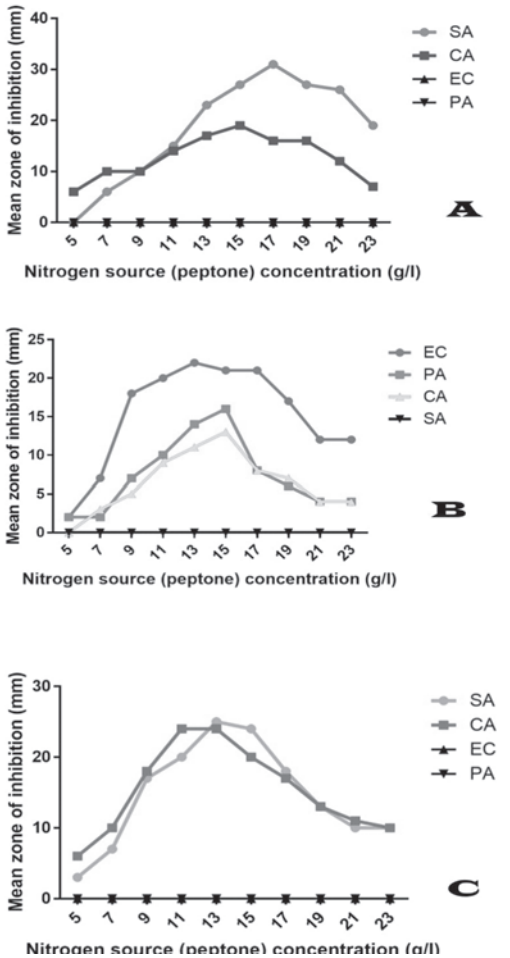


Fig. 10: Effect of nitrogen source (Peptone) on the production of antimicrobial compounds by three selected actinomycetes spp. **A**: BRWDSc (SP), **B**: KBMWDSb6 (M6); **C**: KBRWDSa (RF).). **EC**: *E. coli* 2966, **PA**: *P. aerugenosa* 2929, **CA**: *C. albicans* ATCC1023, **SA**: *S. aureus* 2876. The results of multiple comparisons showed statistical significance at p value ≤ 0.05 using one way ANOVA.

The results on antimicrobial activity from different solvents crude extracts showed that aqueous and ethanol crude extracts had the highest antimicrobial activity (Figure 11). The mean zones of inhibition from all crude extracts ranged from 8.33 - 31.67, 3.66 - 22.33 and 9.67-24.16±0.17 for BRWDc (SP), KBMWDSb6 (M6) and KBRWDSa3 (RF) respectively. Aqueous crude extract of actinomycetes isolate KBMWDb6 (M6) did not show activity against *S. aureus* 2876, but had activity when ethanol and methanol were used for extraction. The p values (0.0001 each for KBMWDSb6 (M6), BRWDc (SP) and 0.0019

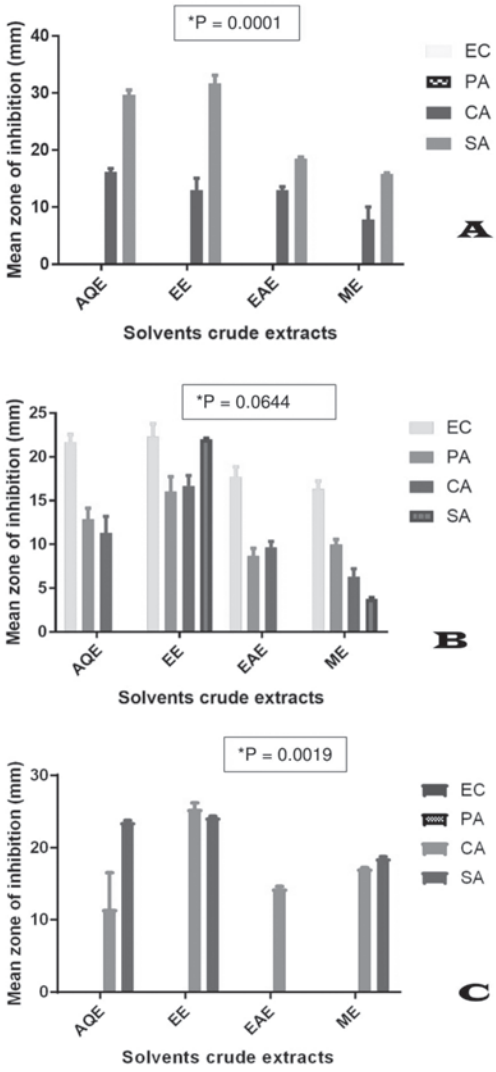


Fig. 11: Mean and standard error zone of inhibition of different solvents crude extracts from three actinomycetes spp. A: BRWDSc (SP), B: KBMWDSb6 (M6); C : KBRWDSa (RF). EC: *E. coli* 2966, PA: *P. aerugenosa* 2929, CA: *C. albicans* ATCC1023, SA: *S. aureus* 2876. AQE Aqueous extract, EE: Ethanol extract, EAE: Ethyl acetate extracts, ME: Methanol extract, * P value of multiple comparisons using one way ANOVA.

forKBRWDSa3 (RF)) of multiple comparisons between the crude extracts showed statistical significance at ($P \leq 0.05$). The ability of ethanol crude extract to produce higher activity could be as result of universal polarity of the solvent which make it to extract both polar and weak polar compounds (35).

CONCLUSION

Production of antimicrobial agents by actinomycetes species is dependants on media compositions, growth conditions and extraction methods. This study revealed that suitable medium, optimum concentration of combine carbon sources, growth conditions, and solvent are important for optimum production of antimicrobial compounds by actinomycetes spp. The molecular identification of 16S rDNA gene showed that all the three isolates belong to phylum Actinobacteria into the genus *Streptomycetes*.

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