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Production, Partial Purification and Antitumor Properties of Bioactive Compounds from Locally Isolated Actinomycetes (KH14)

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Abstract: The present study was conducted to evaluate the antimicrobial and antitumor activities of bioactive compounds produced from locally isolated actinomycetes (KH14), and analyzing these compounds by TLC, bioautograhgy and HPLC-MS. The isolate KH14 showed significant inhibition of human microbial pathogens, both Gram positive, Gram negative bacteria and yeast with an inhibition zone of 30, 13 and 12 mm, respectively. The MICs potential against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* were 64, 500 and 500 µl/ml, respectively. The antimicrobial compound was analyzed by TLC, which indicated the presence of four spots while only one of them exhibited antimicrobial activities and their position was determined by bioautography, via different solvents exhibiting different R_f values. HPLS-MS analysis of scraped active spot from the TLC indicated the presence of a large number of compounds when determining their mass chromatogram, while UV absorbance indicated the presences of only two peaks with UV absorbances 221.85and 264.85nm. The antitumor activity by 3-(4, 5-dimethylthiazol- 2-yl) -2, 5-diphenyl-tetrazolium bromide (MTT) assay indicated that KH14possesses a powerful effect against using cancerous cell lines, especially against Breast cancer cells (MCF-7) by inhibiting 75.84% with (400 µg), while against the rest of other cell lines, Human Prostate cancer cells (PC3)and Human malignant melanoma, skin cancer (A375), were shown moderate activities.

Key words: Antimicrobials, MICs, TLC, HPLC-MS, Antitumor activity

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Introduction

Actinomycetes are categorized by a multipart life cycle, filamentous Gram-positive bacteria fitting to the phylum *Actinobateria* that denotes one of the major taxonomic elements among the 18 main ancestries currently documented within the domain bacteria (1). Actinomycetes are broadly spread in most environment state, especially in terrestrial and aquatic ecosystems, particularly in soil, which play a vital role in the recycling of intractable biomaterials by decaying complex mixes of polymers in animal and fungal materials and dead plant. They are important in soil degradation and humus

formation by the reprocessing of nutrients related to intractable polymers such as lignocelluloses, keratin and chitin and yields numerous volatile materials like geosminliable of the characteristic "wet earth odor". They also display varied metabolic and physiological properties, such as the production of extracellular and intracellular enzymes (2, 3, 4). Actinomycetes produces bioactive compounds with angiogenic or wound healing properties, and new bioactive complexes of anthraquinone nature with powerful antimicrobial, antitumor, anti-inflammatory and antiviral activities have also been reported from soil actinomycetes and its antitumor activity has been reported (5, 6). Currently, there are little published and predictable evidence on the actinomycetes and their bioactivity for potential production of antimicrobial compounds and antitumor in Iraq, in addition the main objectives of this study are to characterize produced bioactive compounds, by TLC and HPLC-MS, and to investigate antimicrobial and antitumor properties of these compounds from isolated actinomycetes (KH14).

Materials and Methods

Actinomycetes Strain

The locally isolated actinomycetes strain (KH14) was obtained in a previous study (unpuplished paper), which characterized with optimum pH 8, NaCl salt tolerance 5%, and production of diffusible pigment (orange in color) and heavy mycelia growth with white and yellow color of aerial and substrate (reverse) mycelium color when grown on yeast extract malt extract agar (ISP2) (2).

Microbial Pathogens Used for Antimicrobial Activities

The human pathogenic microorganisms were used for testing antimicrobial activities such *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10231.

Fermentation Condition (Secondary Screening)

Secondary screening (fermentation) for production of antimicrobial metabolites was carried out by inoculating 150ml of production media (yeast extract, malt extract agar ISP2) in an Erlenmeyer flask with 1.5 ml of prepared stock suspension cultures then incubated at 29±1°C, 150rpm for 7days in shaking incubator. The broth was filtered with sterile Whatman No. 1 filter paper, and treated as extracellular crude extract (7).

In vitro Antimicrobial Activities of Isolated Actinomycetes (KH14)

Agar well diffusion method was done to screen antimicrobial activities against tested microbial pathogens. Using sterile swabs, Mueller Hinton agar plates inoculated with microbial pathogens, and dug wells of 6mm diameter using Pasteur pipette, 60 all of the extracts were loaded into the wells and the plates were incubated at 37^{o}C for 24 hours. The plates were observed for the inhibition zone, which recorded by a metric ruler (8).

Minimum Inhibitory Concentrations (MICs)

MIC was determined by taking 1ml of nutrient broth into 13 test tubes, with a negative control and positive control

tube, then antimicrobial compounds (extracellular crude extract) were added into the first test tube and made a serial dilution to obtain a final concentration (1, 2, 4, 8, 16, 32, 64, 125, 250, 500, 600, 700, 800 ul /ml), 50µl of testing microbial pathogens were added into each test tube (except negative control) and they were incubated at 37^0 C for 24 hours. MICs were determined by naked, clear tubes were considered as MIC when compared with both positive and negative controls, adapted from (9) with little modification.

Bacteriostatic and Bactericidal Properties of Extracellular Crude Extract

To determine whether the bioactive compound is bactericidal or bacteristatic, a sample from the area of the inhibitory zone around the well was taken by the means of sterilized swab then inoculated into nutrient agar. If bacteria grow in the nutrient agar, the action of the extracellular crude extract was probably being bacteriostatic, while it will be bactericidal if there was no any growth, after 24 hours of incubation at 37^0 C (10).

Comparison between Synthetic Antibiotic Discs with Crude Extracellular Extract (KH14)

Ten synthetic antibiotic discs(E Erythromycin, CTX Cefotaxime, TE Tetracycline, CN Gentamicin, ST Streptomycin, AZM Azithromycin , AMC Amoxicillin/clavulanic acid, ME Methicillin, AX Amoxicillin, VA Vancomycin) were used for comparison of antimicrobial activity against tested microbial pathogens with extracellular crude extract metabolites especially against studied pathogen (*Staphylococcus aureaus* ATCC 25923, and *Escherichia coli* ATCC 25922), samples were spread on plates containing Mueller Hinton agar then the antimicrobial discs were placed on the media by the means of sterilized forceps, three discs per plates were used and incubated at 37°C for 24 hours. After that, plates were observed for the zone of inhibition using metric ruler to measure the zone of inhibition from (11) with slight modification.

Partial Purification by Thin Layer Chromatography (TLC)

The extracellular crude extract was dissolved in ethyl acetate and spotted by means of capillary tubes on TLC plates (TLC 20x20 cm, silica gel $60F_{254}$, Merk co, USA) and using different mobile phase solvents such Ethyl acetate 100%, methanol 100%, chloroform100%, water 100%, n-hexan 100%, toluene 100%, acetone 100%, ethanol 100%, n-butanol 100%, acetic acid 100%, ethyl acetate :methanol: water (10:1.5:1), toluene: ethyl acetate (93:7), methanol : water (6:4), Ethyl acetate : Chloroform : Water (95:5:5), Methanol : Chloroform (6:4), Ethyl acetate: Toluene: Methanol: Chloroform: Water (1:1:1:1:1) in order to determine the best solvent system that separates bioactive compounds , then determining the retention factor (R_f value) of active compounds After running the TLC plates, the plates were dried andTLC plates were observed visually under UV light, to detect the spot position according to (12). The retention factor (R_f), which is defined as the distance traveled by the compound divided by the distance traveled by the solvent, as follows

R_f = distance traveled by the compound / distance traveled by solvent

Detecting the Antimicrobial Metabolites Position (Bioautography)

Bioautography assay follows the thin layer chromatography to determine the active antimicrobial metabolites position, after the TLC plates running. Plates were seeded with microbial pathogens and the TLC strip containing the separated active spots were placed on the surface of agar media, then, incubated at 37°C for 24 hrs. After completing incubation period, inhibition zone around the active spot of antimicrobial metabolites were observed by eyes. The clear zone on the media indicated the presence of active antimicrobial compounds which inhibit the growth of the tested pathogens as described by (13). The active spot was visualized by UV, then scraped from the TLC plates by sterilized Scrapple and collected in a clean disposable test tube for High Performance Liquid Chromatography (HPLC) analysis.

High-Performance Liquid Chromatography (HPLC) Aanalysis and Partial Purification of Scraped Spot

The solution for HPLC were prepared by using 100 mg of spot scraped were dissolved in 1ml of methanol and shake well, then filtered by passing through a Millipore micro filter (0.2 µm pores) via disposable syringe. Separation was carried out with a HPLC-MS (Waters 2545-USA) quaternary gradient module equipped with system fluidics organizer (waters-SFO) coupled with SQ detector and operated in positive ionization mode at range from m/z = 200-900 with 2 scan/min, combined with photodiodes array detector (Waters 2998) with sampling rate 2 points/Sec (Lambda range 190-800 NM). A C18/4.5 x 155 mm RP column (X Bridge) was used for separating with a solvent system consisting of A: deionized water, B: methanol, each contain 0.1% formic acid. The following gradient was applied: 0-2 minutes 30% A, and 70% B, 2-10 minutes A linear from 30 to 95%, B linear from 70% to 5%. Ten microliter of the extract was injected using an auto - sampler (Waters 2767), and run with flow rate 1 ml/min using an auto - sampler (Waters 2767) from (14) with slight modification.

Cytotoxicity Activity of Extracellular Crude Extract

Cancer Cell Lines

Three cell lines **MCF-7** (Breast cancer cells), **PC3** (Human Prostate cancer cells) and **A375** (Human malignant melanoma, skin cancer) obtained from Pharmacology Department/Medicine College/Malayia University, were used in this study to determine the cytotoxic activity of extracellular crude extract (KH14).

In vitro **Evaluating the Cytotoxic Activity of Extracellular Crude Extract of KH14**

Cytotoxic properties were determined by MTT assay for KH14 according to (15,16) with little modification against different cell lines. Cell lines were seeded in 96-well tissue culture plates (5000 cells/well) using Eagles Minimum Essential Medium (EMEM). A serial dilution (400, 200, 100, 50, 25, 12.5ug/m) was prepared from lypholized stock solutions of extracellular crude extract. All samples were done in triplicates.

The cells were cultivated at 37°C with 5% CO_2 and 95% air in 100% relative

humidity in (Thermo Forma Series II Water Jacketed CO_2 incubator). After 72 hours of incubation the solution in the medium was removed. An aliquot amount of 100 µl of medium containing 1 mg/ml of 3-(4, 5-dimethylthiazol- 2-yl) -2, 5-diphenyl-tetrazolium bromide (MTT) was loaded to the plate. The cells were cultured for 4 hours in a dark chamber for the conversion of MTT to formazan, and then the solution in the medium was removed, each well were loaded an aliquot amount of 100 µl of DMSO were added to the plate, then measuring the absorbance of the converted dye at 570 nm in a (Hidex *Candida albicans* ATCC 10231 (13 and 12 mm inhibition zone respectively), this was in agreement with (17) that most of the bioactive compounds extracted from actinomycetes were performed against Gram-positive bacteria with strong activity, against Gram-negative bacteria with moderate activity and against yeasts with weak activity.

The data presented in Table (1) summarizes minimal inhibitory concentration (MIC) against microbial pathogens, the MIC for tested microbial pathogens (*Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC25922 and *Candida albicans* ATCC 10231, were exhibited 64 ul /ml, 500 ul /ml and 500 ul /ml respectively. It reveals that the extracellular crude extract was very strong against *Staphylococcus aureus*, as well as having a moderate activity against both *Escherichia coli* and *Candida albicans*. Our results were in agreement with that described by (18, 19, 20, 21, 22 and 23) Chameleon plate reader). Cytotoxicity of each sample was expressed as % cell variability, as follows

Cell viability % = (mean OD control x mean OD treated) / (mean OD control) *100%

Results and Discussion

As shown in Figure (1), *in vitro* antimicrobial activities of isolated actinomycetes (KH14) has been studied, the highest results achieved against *Staphylococcus aureaus* ATCC 25923 (30mm) inhibition followed by *Escherichia coli* ATCC25922 and for their activities of the active substance in case of antimicrobial activities against both gram positive and negative bacteria, while in the study of (24, 25) they reported the activity of their isolated actinomycetes against only *Staphylococcus aureus* and *Candida albicans* without obtaining any activity against *Escherichia coli*.

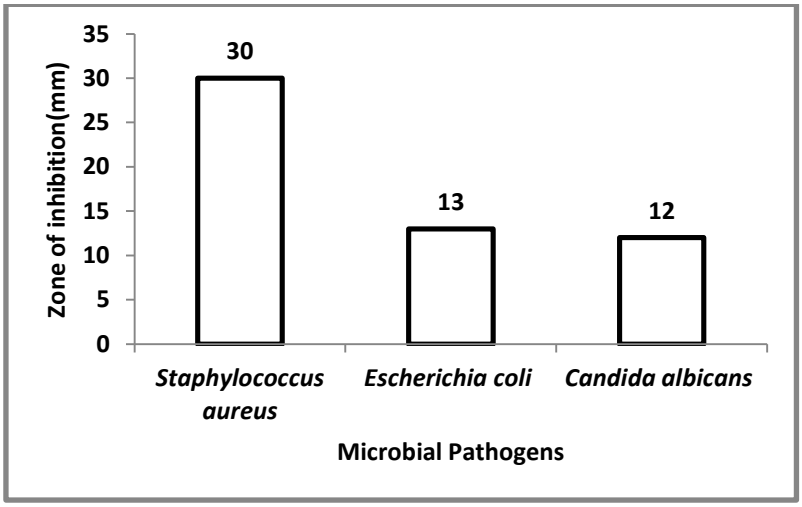

Figure 1: *in vitro* antimicrobial activities of isolate (KH14)

Table 1 minimum inhibition concentrations (MICs), bacteriostatic and bactericidal assay of isolated strain (KH14), + :growth, - :No growth

Extracellular Crude concentration ul /ml	*Staphylococcus aureus* ATCC 25923	*Escherichia coli* ATCC25922	*Candida albicans* ATCC 10231
1	+	+	+
2	+	+	+
4	+	+	+
8	+	+	+
16	+	+	+
32	+	+	+
64	(MIC)	+	+
125	-	+	+
250	-	+	+
500	-	(MIC)	(MIC)
Bacteriostatic assay	**+**	**+**	**+**
Bactericidal assay	-	-	-

Figure 2 summarized comparison between synthetic antibiotic discs with *in vitro* antimicrobial activities of extracellular extract (KH14) against Escherichia coli ATCC25922 among all used antibiotics disc the highest result was exhibited by Cefotaxime 30 ug (30mm inhibition zone), while Erythromycin, Amoxicillin/clavulanic acid, Methicillin, Amoxicillin and Vancomycin don't showed any activities, in contrast our strain exhibited the zone of inhibition (13mm), which meaning better than 50% of all used antibiotic discs.

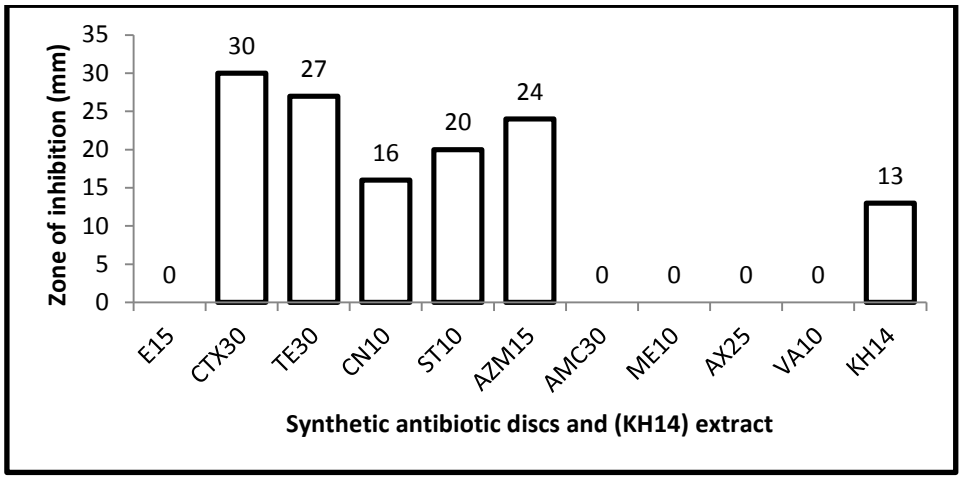

Figure 2: Antibiotic synthetic discs compared with (KH14) antimicrobial activity
(E Erythromycin, CTX Cefotaxime, TE Tetracycline, CN Gentamicin, ST Streptomycin, AZM Azithromycin , AMC Amoxicillin/clavulanic acid, ME Methicillin, AX Amoxicillin, VA Vancomycin)

synthetic antibiotic discs activities compared with *in vitro* antimicrobial activities of (KH14). The extracellular extract (KH14) against *Staphylococcus aureus* ATCC 25923, among all used antibiotic discs result only Tetracycline 30 ug gave better results (35mm inhibition zone) than our isolate activity as shown in Figure (3) , that KH14 exerted was 30mm inhibitory zone, which is similar as Azithromycin and Amoxicillin whereas other tested antibiotic showed lower activities.

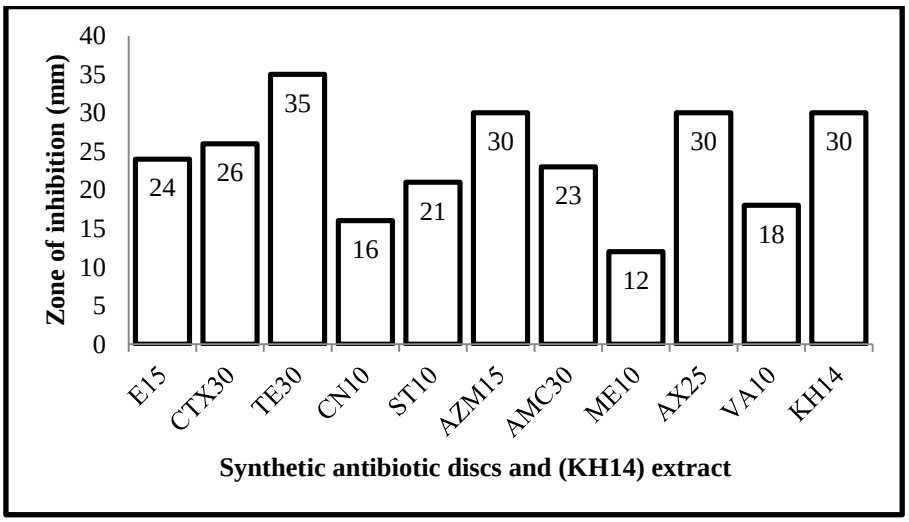

Figure 3: Antibiotic synthetic discs compared with (KH14) antimicrobial activity

The result in Figure (4 A) shows the separation of bioactive materials on TLC plates. It has been shown that four separate spots with different R_f values were chosen, after applying bioautography, only one spot exhibited

antimicrobial activity against tested microbial pathogens Figure (4B).The data in Table (2) summarized the ability of different solvents for separating the active spots, all used mobile phase was able to separate the active spots with different R_f values except n-Hexan 100%, Toluene 100% and Toluene: Ethyl acetate (93:7) which they fail for separating the bioactive compounds. Our results were in agreement with that described by (26, 14 and 27), they reported a single active spot in their isolated actinomycetes with different R_f values using different organic solvents in the mobile phase, after applying direct bioautography assay for determining their position, (28) obtained three spots on TLC plate also only one of them showed antimicrobial activity against *Staphylococcus aureus*.

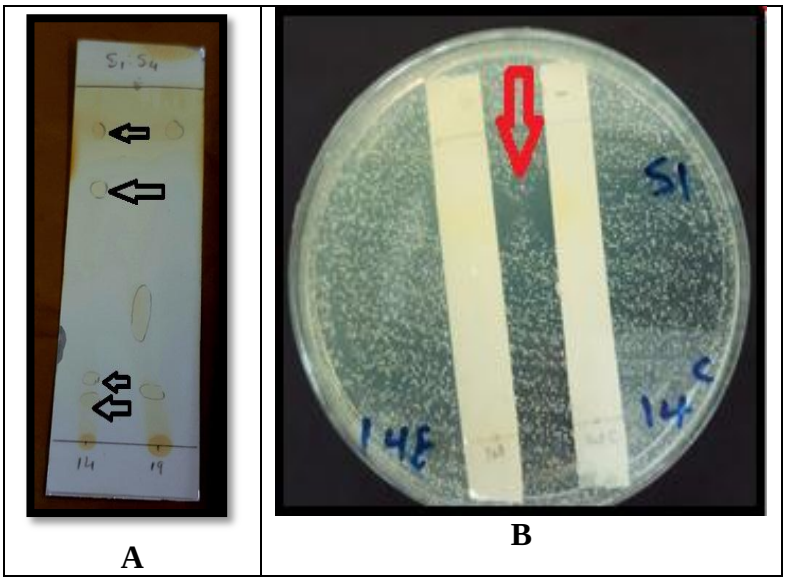

Figure 4: A separations of bioactive compounds, B bioautography

Table 2 R_f values of bioactive spot, exhibited antimicrobial activities by bioautography

Solvents (mobile phase)	R_f value (cm)
Ethyl acetate 100%	0.81
Methanol 100%	0.84
Chloroform100%	0.14
Water 100%	0.3
n-Hexan 100%	0.0
Toluene 100%	0.0
Acetone 100%	0.9
Ethanol 100%	0.92
n-Butanol 100%	0.6
Acetic acid 100%	0.87
Ethyl acetate: Methanol: Water (10:1.5:1)	0.69
Toluene: Ethyl acetate (93:7)	0.0
Methanol: Walter (6:4)	0.65
Ethyl acetate : Chloroform : Water (95:5:5)	0.40
Methanol: Chloroform (6:4)	0.38
Ethyl acetate: Toluene: Methanol: Chloroform: Water (1:1:1:1:1)	0.59

The results presented in Figure (5.A) represents the mass chromatogram of scraped spot extract exhibited different peaks at a different retention time (where the overall time for separation were only 10 minutes) most of the peaks appear to be interacting together, however, the highest signal intensity was appeared at retention time 5.46 minutes.

The results presented in Figure 5.B summarized all compounds that having UV absorbance potential at these peaks, Scraped spot extract possessing two sharp peaks appeared at 221.85nm and 264.85nmUV absorbance. Our results were in agreement with that obtained by (29) that the bioactive compound of them isolated strain with ethyl acetate extract exhibited a maximum UV absorption at 217–221 nm, exactly for the first peak of spot scraped, in addition (31 and 32), they reported that the maximum UV absorbance for most of peptide antibiotics exhibited at 210-230 nm and 270-280 nm, further more (22) also described that that maximum absorption UV as a spectroscopic characteristics for their bioactive compound shown at 269 nm., in addition, (33) obtained maximum absorbance at 210 -260 nm.

The results presented in Figure (6) summarize the effects of different extracellular crude extract concentration on PC3 (Human Prostate cancer cells), the concentration (400ug/mL) was showed the highest results that (30.84%) of the PC3 cells was inhibited, However, 69.18% of the cells remained viable during the entire incubation process, on the other hands, the lowest results were recorded by 12.5 ug/mL that means only 3.69%of the cancerous PC3 were inhibited during the incubation process.

The results shown in (Figure 7) represent the anticancer activity of extracellular crude extract (KH14) on the MCF7 (Breast cancer cells), the highest cell inhibition (75.84%) was recorded by 400ug/mL exactly of MCF-7 breast cancerous cells after 72hr incubation which reveals that only 24.16% of MCF-7 were able to form insoluble formazan products and the remaining cells were viable. On the other hands, the 12.5ug/mL concentration was shown the lowest results that only 13.71% was inhibited of the breast cancer cells. Our results were better than that of (34) since they obtained 56% inhibition of MCF-7 cell line by the activity of them isolated strain of actinomycetes EI-4 after 72 hours incubation via MTT assay, while our extracts inhibited75.84% of MCF-7 cell line after 3days of incubation, also our obtained results confirmed higher inhibitory effects than (35). They reported that the actinomycetes strain HP411 only inhibited 39.8% of MCF-7 cell line.

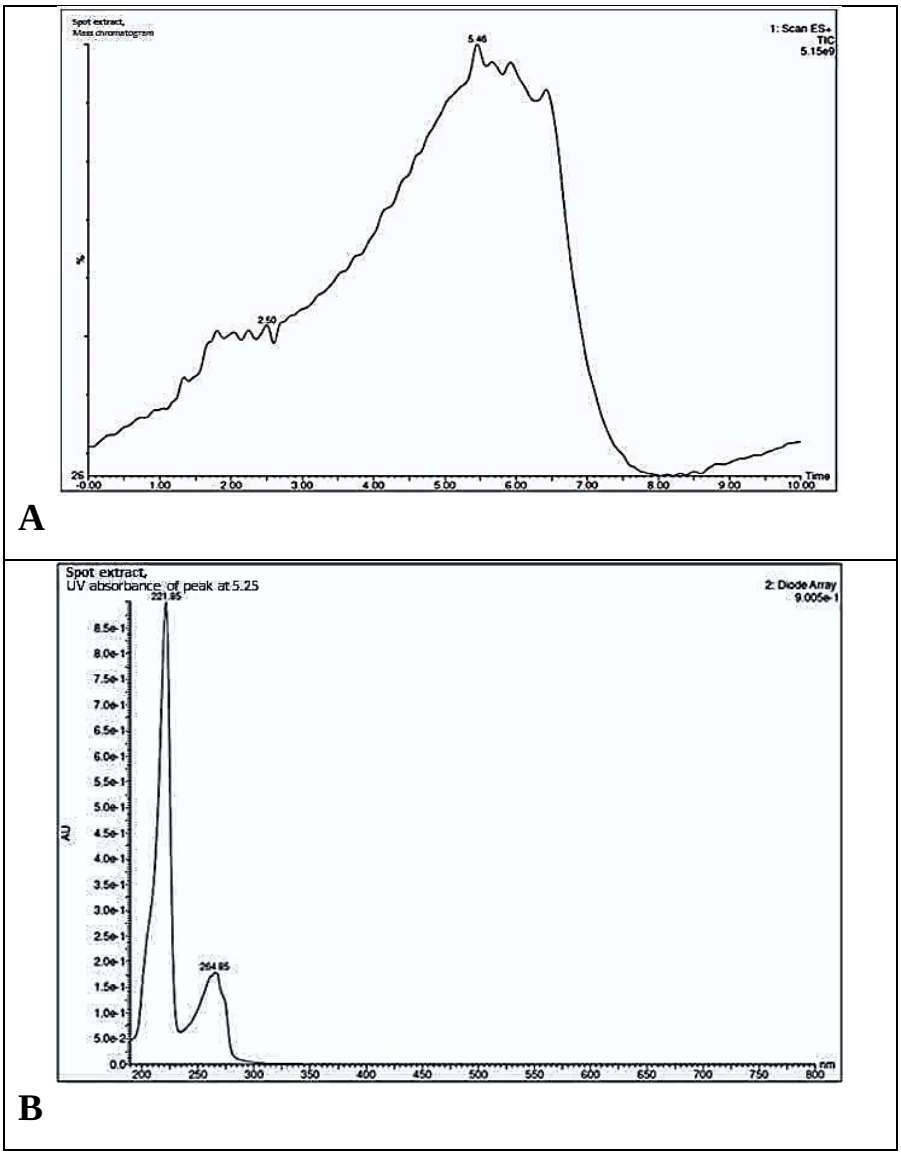

Figure 5. A: mass chromatogram of scraped spot, B UV absorbance of peak (5.25 min)

The results shown in Figure (8) represents the anticancer activities of extracellular crude extract (KH14) on the **A375** (Human malignant melanoma, skin cancer), the highest result (cell inhibition) was recorded by 400ug/mL exactly 53.1% of **A373** skin cancerous cells were inhibited after 72hr incubation which meaning only 46.9% of **A375** were able to form insoluble formazan products and the remaining as viable cells, as well as, the lowest result was only 2%inhibition of the skin cancerous cell which was produced by 12.5 ug/mL. Our results were in agreement with that obtained by (16) in case of the effect of extracellular crude extract was in a concentration - dependent manner (dose dependent and they obtained the highest activity with 500 µg/ml of ethyl acetate extract against A549 lung adenocarcinoma cancer cell line which inhibited 84.9% of the lung cancer cells.

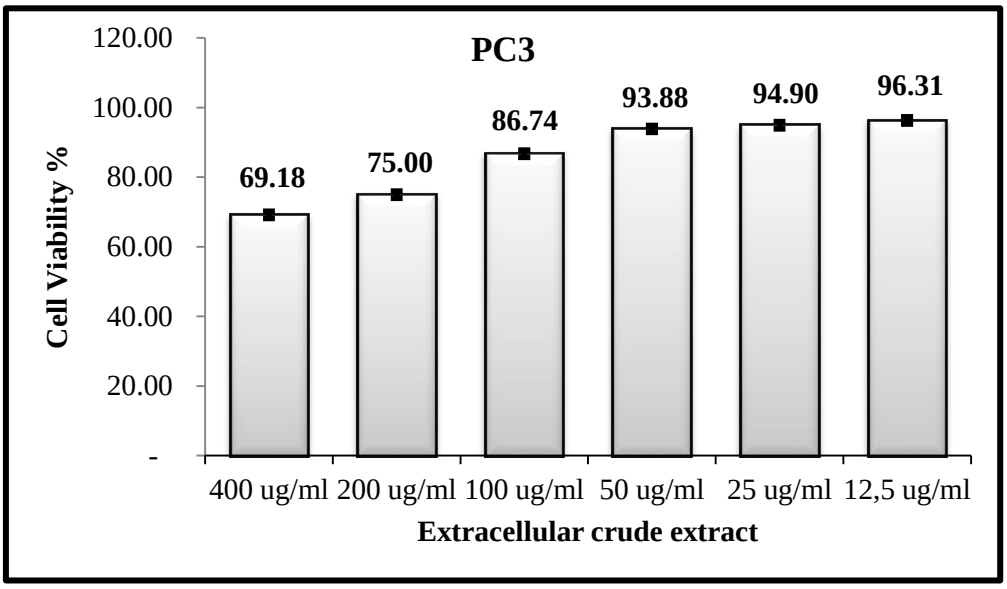

Figure 6: Anticancer activities of extracellular crude extract (KH14) against PC3 cell lines

The antitumor activities of extracellular crude extracts (KH14) among all cancer cell lines were used, the MCF-7 (breast cancer cells) was the most one that highly responded to extracellular crude extract (KH14) through inhibiting the metabolically active cancer cells, while, A375 and PC3 cancerous cell lines become as second and third responded respectively. Increasing the extracellular crude extract concentration from 12.5 to 400 µg/ml against PC3, MCF-7 and A375 cell lines the cytotoxicity value was increased as well as the cell viability decreased. Our results were in agreement with that obtained by (36) from their study for screening new bioactive compounds against MCF-7 and A549 cell lines from crude extract fraction in which the effect was dose dependent. (37 and 38) they described that the antitumor antibiotics (anticancer antibiotics /cytotoxic) are medications that prevent and fighting the growth of tumors, and the antitumor antibiotics which produced by Streptomyces species are precious in the medical field.

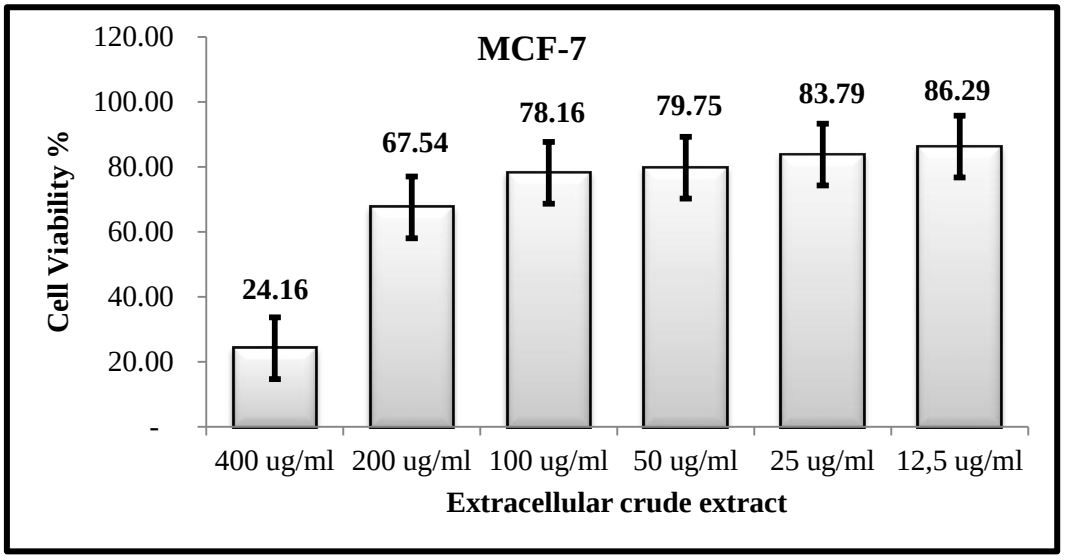

Figure 7: Anticancer activities of extracellular crude extract (KH14) against MCF-7 cell lines

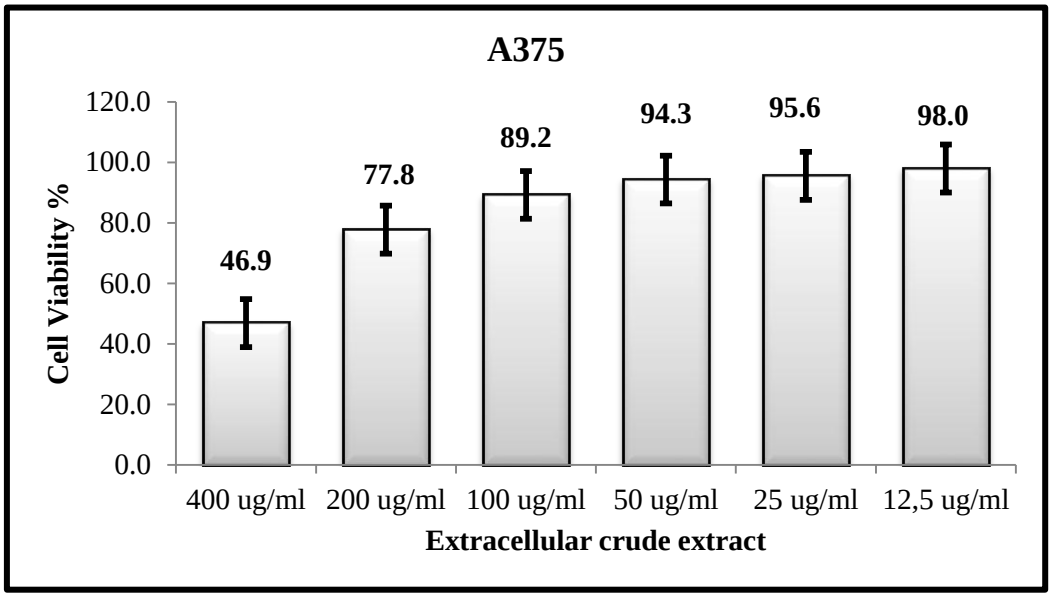

Figure 8: Anticancer activities of extracellular crude extract (KH14) against A375 cell lines

Conclusion

Our isolate, KH14, was exhibited numerous bioactive metabolites properties, which emphasized its importance as a potential antimicrobials activities against numerous human microbial pathogens, and can be compared with different synthetic antimicrobial discs, as well as which might offer a basis for additional improvement of novel compounds, furthermore, these are attractive biological features especially for their

achieving the powerful antitumor activity against various cancerous cell lines and for the development of potential anticancer drugs with precise cellular objectives, this requiring more research to identify its importance therapeutic properties to occur in the instant prospect

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