Egypt. J. Chem. **Vol. 66** No. 11 pp. 281 -303(2023)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Phenazine-Producing *Pseudomonas aeruginosa* OQ158909: A Promising Candidate for Biological Activity and Therapeutic Applications

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Abstract

Natural phenazines, as secondary metabolites of microorganisms, have recently gained much attention for their potential application as biologically active antimicrobial compounds. The present study was performed in order to investigate the production of a biologically active phenazine compound by a locally isolated strain of *Pseudomonas aeruginosa*. Thirty different strains were isolated from soil and water on a selective medium. From them, isolate 14-WA^{NRC}, was selected due to its high productivity of colored secondary metabolites after 5 days of incubation in King-A broth medium. The isolate was further identified using some morphological, biochemical and physiological tests as well as using 16S-rDNA which revealed a 99% similarity with *Pseudomonas aeruginosa* and was therefore registered in the Gene bank under the accession number of OQ158909. The production ability of the studied strain was then improved by studying the effect of different physiological and growth conditions. The results showed that the maximum output of phenazine compounds was obtained when the isolated strain was cultivated in mineral salt medium (MS), under shaking conditions of 100 rpm, for five days at 35°C. The secondary metabolites produced were finally extracted using ethyl acetate and subjected to TLC for compounds separation. The selected band exhibited potent antimicrobial, antioxidant and anticancer activities.

Keywords: *Pseudomonas aeruginosa*; phenazines; biologically active compounds; secondary metabolites; microbial natural products; isolation and bacterial identification

1. Introduction

Natural products are regarded as particularly significant sources for the development of new medicines. Nowadays, much interest for natural products had arose due to the quick development of technologies for separation, purification, and detection. Currently, 60% of medications on the market were derived from natural ingredients, including those of microbial origin. Diverse classes of molecules such as pigments, antibiotics and toxins, with a wide range of applications, are produced by bacteria and actinobacteria. From them, a broad category of secondary metabolites, known as phenazines, are mostly produced by *Pseudomonas* species specially *Pseudomonas aeruginosa*. The use of microbial phenazines in biotechnological and antibacterial applications is recently gaining much popularity.

The genus Pseudomonas bacteria, concerned with phenazine production, comprises an ubiquitous group of free-living bacteria generally found in most environments. They are characterized of being gram-negative, rod-shaped (1–5 µm long and 0.5–1 µm wide), non-spore former, mono-flagellate bacteria that respire aerobically at optimal conditions. However, they can also respire anaerobically in the presence of nitrate or other alternative electron acceptors which therefore, makes the *Pseudomonas* spp. a highly invasive group of bacteria that can be found in an array of environments such as soil, water, humans, animals, plants, sewage, and hospitals (**Abdul-Hussein and Atia, 2016 & Gahlout *et al.* 2017**). The importance of *Pseudomonads* bacteria relies, first of all, on their roles in the development of systemic résistance, against invading pathogens in addition to their ability to produce a variety of growth-promoting compounds as well as to solubilize inorganic phosphorus. These properties led to the enhancement of plant growth along with thier antagonistic effects against numerous

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Receive Date: 29 May 2023, Revise Date: 11 August 2023, Accept Date: 11 August 2023

DOI: 10.21608/EJCHEM.2023.214109.8045

phytopathogens. These bacteria therefor display excellent biological control and are consider very efficient for plant pathogen management (**Jain and Pandey, 2016**). The latter was proved to be refered to their ability to synthesize a range of coloured compounds called phenazines (**Gahlout *et al.*, 2017**).

Phenazines are a diverse class of heterocyclic nitrogen-containing compounds with various chemical and physical properties, depending on the type and location of the functional groups. More than 6,000 distinct phenazine-containing compounds have been synthesized, and more than 100 different phenazine structural variations have been discovered in nature. Phenazines were found to have broad-spectrum antibacterial activities along with different physicochemical characteristics, such as their oxidation-reduction (redox) capabilities, brilliant pigmentation, and ability to alter color within different pH and redox status. Other applications of phenazines include their use as electron acceptors and donors, building blocks for fuel cell technology, environmental and biological sensors as well as essential parts of anticancer drugs. Different studies reported that phenazines play a very important role in leukemia treatment (**Krishnaiah *et al.*, 2018**), and could represent a promising scaffold for the development of a new class of antibiotics (**Gahlout *et al.*, 2017**). All these characteristics are largely responsible for their ongoing biotechnological interest. For such vital properties, this study was carried out to isolate an Egyptian local Pseudomonas strain that have the ability to produce highly active pigmented secondary metabolites, and investigate the factors needed to optimize their production. The most active metabolites were extracted using a suitable solvent and their antimicrobial, antioxidant, and cytotoxicitic activities were also studied.

2. Materials and methods

2.1. Sample collection and isolation technique

Samples were collected from an Egyptian village called Zur Abu-aliall, Awlad Saqr center, Al-Sharkia governorate (Latitude N30.93276, Longitude E 31.76496). Some of the samples were retrieved from a freshwater channel and the others were obtained from the rhizosphere of a maize field (*Zea mays*). The samples were collected in sterile containers and immediately delivered to the lab in an ice box. The soil samples were air dried at room temperature prior to isolation while the water samples were kept in the refrigerator until use.

The Phenazine-producing microorganisms were isolated on king medium (Merck^{TM}), (**Jain and Pandey, 2016**) using the ten-fold serial dilution technique. One ml was used to inoculate three plates for each diluted sample. All inoculated plats were incubated at 37 °C for 24-48 hrs. The growing colonies, characterized by their distinctive diffusible pigments, were picked up and further cultured on Pseudomonas agar base medium enriched with CN X_{107} supplement (Neogen^{TM}) as a *Pseudomonas aeruginosa* selective medium.

2.2. Morphological and biochemical characterization of isolated bacteria

Pseudomonas isolates were selected according to their morphological appearance, Gram stain as well as catalase and oxidase assays.

2.3. Primary Screening of isolates with the highest antimicrobial activities

The most antimicrobially effective *Pseudomonas* isolates were chosen. Their antimicrobial activity was tested against 7 microbial pathogens including 2 Gram-negative bacteria: *Escherichia coli* (ATCC 25922) and *Salmonella enterica* (ATCC 25566), 3 Gram-positive bacteria: *Bacillus cereus* (ATCC 6629), *Bacillus subtilis* (ATCC 6633) and *staphylococcus aureus* (ATCC 6538) and 2 pathogenic fungal strains: *Candida albicans* (ATCC 10231) and *Aspergillus niger* (ATCC 18666). These tested pathogens were American Type Culture Collection (ATCC) strains obtained as courtesy of the Biological Assessment of Natural and Microbial Products Unit, Lab. 174, Chemistry of natural & microbial products department, National Research Centre. The antimicrobial activities of the produced microbial secondary metabolites were assessed using the agar disc diffusion method using a sterile cork borer of internal diameter of 6 mm **(Devnath *et al.*, 2017, Elboraey *et al.*, 2021 & Sultan *et al.*, 2022).**

2.4. Secondary screening of the most potent isolates for secondary metabolite production

The three promising bacterial isolates (4-SA^{NRC}, 14-WA^{NRC} & 15-WA^{NRC}), selected based on their highest antimicrobial activities against all the tested pathogens, were screened for their secondary metabolites production ability on a modified King-A broth medium (Glycerol 1 ml, $MgCl_2$ 0.14g, K_2SO_4 1g, Asparagine 0.1%, Peptone 2 g, Distilled water upto100 ml) (**Devnath *et al.*, 2017 & Karthik and Kalyani, 2023**). The above mentioned isolates were first of all individually cultivated in nutrient broth medium for 24 hours at 37°C and 150 rpm in order to obtain homogenous cultures. These cultures were then adjusted at 0.5 McFarland (1.5 x 10^8 CFU) and

used to inoculate modified King-A broth medium (50 mL / flask) as 10% (v/v) inoculum size. The inoculated flasks were investigated for the presence of pigmented secondary metabolites. The investigation was performed after different incubation periods of 1, 3, 5, and 7 days, using spectrophotometric analysis at of the clear supernatant of the fermentation medium, obtained by centrifugation of the fermentation broth at 12500 rpm for 10 minutes, at the adjusted wave length of 362 nm **(Jain and Pandey 2016 & Devnath *et al.*, 2017)**.

2.5. Identification of the selected isolate

2.5.1. Morphological, biochemical and physiological characterization

Characterization of the most potent isolate was primarily carried out based on Bergey's Mannual of Determinative Bacteriology (**Boone *et al.*, 2001 & Devnath *et al.*, 2017**). The colony morphology and Gram staining , as well as a number of biochemical tests , were performed on a 24 hours old bacterial culture of the 14-WA^{NRC} isolate. These biochemical tests included oxidase, catalase, methyl red, Voges-Proskaure and deep glucose agar tests as well as citrate utilization, indole and H_2S production, nitrate reduction, in addition to casein, gelatin and starch hydrolysis tests. Moreover, the ability of the selected strain to tolerate wide ranges of pHs and temperatures over 24 to 48 hours incubation period, were also studied.

2.5.2. Phylogenetic identification

2.5.2.1. DNA isolation and polymerase chain reaction (PCR) amplification

A culture of the 14-WA^{NRC} strain, grown overnight at 28 °C, was used for the extraction of the genomic DNA using PCR (9700 (ABI), SolGent company), following the kit's manufacturer instructions (Solgent EF-Taq protocol). The PCR amplification of the 16S-rDNA region was carried out via Big Dye® Terminator v3.1 Cycle Sequencing Kits and Veriti™ 96-Well Thermal Cycler (Applied Biosystems).

The 16S-rDNA was amplified by PCR using primers designed to amplify a 1500- bp fragment of the 16S-rDNA region. The domain bacteria-specific primer 27F (Universal forward primer) was 5'-AGA GTT TGA TCC TGG CTC AG-3' and the universal bacterial primer 1492R (reverse primer) was 5'-GGT TAC CTT GTT ACG ACT T-3'. The reaction mixture, of 50-µl total volume, was composed of 1 µl of 16S-rRNA forward primer, 1 µl of 16S rRNA reverse primer, 18 µl nuclease free water, 25 µl of 2X Red master Mix (2×) as well as 5 µl of genomic DNA as a template. The PCR reaction was performed as follows: first of all, the activation of the 2 Taq polymerase enzyme as well as the pre denaturation of the DNA template were achieved at 95 °C for 3 minutes. This was followed by a group of 3 major steps that were repeated for 35 cycles. These steps included: the second denaturation step at 95 °C for 30 seconds, the annealing cycle at 50 °C for 30 seconds and the extension step at 72 °C for 90 seconds. After that, the final extension step was performed at 72 °C for 5 minutes (**Atwa *et al.*, 2021 & Mohamed and Awad, 2021**).

To confirm the amplification of the targeted PCR, 5 µl of the PCR product was electrophoresed, along with DNA of molecular weight 100bp, in 1% agarose gel, in 1X TAE buffer, containing ethidium bromide, at the rate of 0.5µg/ml and constant 80V power for 30 minutes. The amplified product was visualized as a single compact band, of an expected size, under UV light and imaged using Samsung Note 4 smart phone.

2.5.2.2. DNA sequencing, phylogenic analysis, and tree construction

The amplified PCR product was submitted to Solgent Co Ltd (South korea) for gel purification and sequencing. The resulting sequences were trimmed and assembled in Geneious software (Biomatters) then identified through the National Center of Biotechnology Information (NCBI) GenBank website, using the BLAST basic local alignment search tool (www.ncbi.nlm.nih.gov/BLAST).Nucleotides sequence was downloaded from the GenBank website and compared to the previously identified sequences, using MAFFT alignment (**Katoh and Standley, 2013**). The phylogenetic tree was then constructed using the Neighbor joining method (**Saitou and Nei, 1987**), employing the Tamura-Nei Model (**Tamura and Nei, 1993**). The tree was assessed using 1000 bootstrap replicate.

2.6. Evaluation of the optimum culture medium for secondary metabolite production

Different broth media, having a broad range of nutrient sources were tested for maximum phenazine compounds production. The tested media included, *Pseudomonas* agar base enriched with CN X_{107} supplement (Neogen^{TM}), King B (Merck ^{TM}), King A (Merck ^{TM}), tryptone yeast extract (TY) (Neogen^{TM}), mineral salt (MS) and nutrient media . The most efficient broth was chosen based on the color intensity of the produced pigment measured after five days of incubation at 37°C in an orbital shaker incubator adjusted at 120 rpm.

2.7. Factors affecting biological activities of produced Phenazine compounds

2.7.1. Different incubation periods

In order to study the effect of different incubation periods on the production of Phenazine compounds by *P. aeruginosa* OQ158909, 50 ml of MS medium were inoculated with 10 % (v/v) of 0.5 McFarland ($1.5x10^8$ CFU) concentration of the tested strain.
The inoculated flasks were incubated at 37°C on a shaking incubator, at 120 rpm agitation speed, for different incubation periods of 1, 2, 3, 4 & 5 days.

2.7.2. Different inoculum sizes

Six different inoculum sizes of a 24 hours *P. aeruginosa* OQ158909 culture adjusted at 0.5 McFarland, (1.5×10^8 CFU) (1, 2.5, 5, 10, 15 and 20 % (v/v)) were tested. Each of these inoculum sizes was used to inoculate 50 ml of MS medium which were then incubated at 37 °C and 120 rpm for 5 days. The inoculum size resulting in the highest antibacterial activity was chosen.

2.7.3. Different agitation speeds

The effect of different agitation speeds (80, 100 and 120 rpm) on the antimicrobial activities of secondary metabolites produced by *P. aeruginosa* OQ158909 was detected. This factor was studied using the inoculum size concentration of 10 % and applying the same above mentioned conditions.

2.7.4. Different initial pHs

The initial pH of the selected fermentation medium was adjusted at six different pH values (1.5, 3, 5.2, 7.2, 9.2 and 11.2) including both acidic and alkaline ranges. This experiment was carried out using mineral salt medium (MS), inoculated with 10 % (v/v) ($3.0 x10^8$ CFU) of the selected bacterial culture. The inoculated flasks were incubated at 37°C under shaking cultivation conditions of 100 rpm agitation speeds. The most effective initial pH value that enhanced the secondary metabolites production, with the maximum antimicrobial activities was selected.

2.7.5. Different incubation temperatures

Four different incubation temperatures of *P. aeruginosa* OQ158909 (30, 35, 40, and 45 °C) were tested under the previously set conditions in order to investigate the optimal temperature for the production of high levels of secondary metabolites having strong antimicrobial activities.

2.8. Extraction and evaluation of crude secondary metabolites produced by *P. aeruginosa* OQ158909

Mineral salt medium (MS) was cultivated with the selected bacterial strain, *P. aeruginosa* OQ158909, using the most appropriate cultural conditions, in order to obtain twelve liters of fermentation broth containing crude bacterial secondary metabolites. The both was subjected to centrifugation at 12,500 rpm for 10 minutes and the resulting cell-free supernatant was acidified to pH 2 with concentrated HCL. Afterward, successive extraction with equal volumes of n-hexane, di-chloromethane, chloroform, ethyl Acetate, and methanol, were performed twice for each solvent. The resulting fractions were tested for their antimicrobial activities. The ethyl acetate fraction was selected and its active components were separated using thin layer chromatography (TLC) eluted with 2:3 n-hexane/ethyl solvent system. Spot virtualization was done under UV light.

2.9. Biological activities of the ethyl acetate fraction

2.9.1. Antimicrobial activity

In order to determine the antimicrobial activity of the ethyl acetate extract, a series of tests were conducted over various human pathogens. These microorganisms included both Gram-positive bacteria such as *Bacillus cereus* (ATCC 6629), *Micrococcus luteus* (ATCC 10240), *Enterococcus faecalis*(ATCC 19433), *Staphylococcus aureus*(ATCC 6538) and *Staphylococcus epidermidis*(ATCC 12228), as well as Gram-negative bacteria such as *Escherichia coli*(ATCC 25922), *Klebsiella pneumoniae* (ATCC 10031), *Salmonella enterica* (ATCC 25566) and *Helicobacter pylori* (ATCC 43504). Additionally, pathogenic fungi including *Candida albicans* (ATCC 10231) *and Aspergillus niger* (ATCC 18666) were also tested. All microorganisms were prepared from fresh overnight broth cultures in nutrient broth medium incubated at 37°C, as described by **El-Anssary *et al.* (2021) & Sultan *et al.* (2022)**. The methodology used in this study involved the inoculation of sterile petri dishes containing 20 ml of nutrient agar medium (NA), each with 25 µL of 0.5 McFarland standard culture ($1.5x10^8$ CFU/ml) of each pathogenic strains **(McFarland, 1907)**, previously cultivated for 24 hours in nutrient broth medium at convenient cultivation conditions. Wells, of 0.9 mm internal diameter, were then made in the nutrient agar medium layer. The antimicrobial activities of the tested fermentation medium were evaluated by the addition of aliquots of 100 µL, from a solution of 300 mg of concentrated ethyl acetate extract in 6 ml DMSO, in each well. This step was

performed three times, (**El-Anssary *et al.*, 2021 & Elboraey *et al.*, 2021)**. After 24 hours of incubation, the resulting inhibition zones in the growth of each tested pathogen were measured.

2.9.2. Antioxidant activity

The free radical scavenging activity of the ethyl acetate fraction was measured using 1, 1-diphenyl-2-picryl-hydrazil ($DPPH^{\cdot}$) according to the method of **Shimada *et al.* (1992)**. The ethyl acetate fraction was tested in different concentrations ranging from 100µg/mL, as a starting concentration, to 25µg/mL in order to detect the IC_{50}, which is the most potent active concentration able to scavenge 50 % of the DPPH free radicals.

Briefly, 0.1 mM solution of $DPPH^{\cdot}$ in methanol was prepared. Then, 1.0 mL of this solution was transferred to different test tubes to which different concentrations of the ethyl acetate fraction (25-100 µg/mL) were added. The final volumes of these solutions were adjusted at 3 mL using methanol. The mixtures were then vigorously shaken and allowed to stand, in the dark, at room temperature for 30 min. The absorbance was then measured at 517 nm using a microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA). Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. DPPH scavenging activity percentage (A %) was calculated by the following equation:

$$A\ (\%) = [(A_0-A_1)/A_0]\ X100.0$$

Where: A_0 was the absorbance of the control DPPH sample and A_1 was the absorbance of the tested sample.

2.9.3. Cytotoxic activity

Cell viability was evaluated via the mitochondrial dependent reduction of yellow MTT (3-4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide) to purple formazan (**Mosmann, 1983 & Wood *et al.*, 2015**). All the following steps were performed under aseptic conditions throughout a sterile area using a laminar flow cabinet biosafety class II level (Baker, SG403INT, Sanford, ME, USA).

The cells were suspended and mixed in DMEM-F12 medium which was used for the growth of the tested cancer cell lines ($HePG_2$, MCF_7, $PACA_2$ and HCT_{116}) in comparison to a normal cell line (BJ1). The medium was supplemented with 1 % antibiotic-antimycotic mixture (10,000U/ml Potassium Penicillin, 10,000 µg/ml Streptomycin Sulfate and 25.0 µg/ml Amphotericin B) and 1 % L-glutamine. The cultures were incubated at 37 ºC under 5 % CO_2 for anaerobic conditions.

The cells were then cultivated in a batch culture and incubated for 10 days followed by their inoculation, at the concentration of 10 x 10^3 cells/well, in a fresh complete growth culture medium in 96-well microtiter Eliza plates then incubate in a water jacketed Carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA), at 37 ºC for 24 hours under 5 % CO_2 for anaerobic conditions.

The medium was aspirated by the addition of fresh medium (without serum). The cells were incubated either alone, without any additives and labeled as (negative control), or mixed with different concentrations of ethyl acetate extracte sample (ES) to give a final concentration of 100 -50 -25 -12.5 -6.25 -3.125- 1.56 and 0.78 µg/mL.

After 48 hours of incubation period, the inoculated medium was aspirated again by the addition of 40 µl MTT salt (2.5 µg/mL), to each well, then the inoculated Eliza plate was incubated for four more hours at 37 ºC under 5 % CO_2 for anaerobic conditions. This reaction was ended, and the formed crystals were dissolved, by the addition of 200 µL of 10 % Sodium dodecyl sulphate (SDS) dissolved in deionized water, to each well, mixed well then incubated overnight at 37 ºC.

The positive control was composed of 100 µg/mL of a known cytotoxic natural agent (doxorubicin) which resulted in 100 % lethality under the same above conditions **(Thabrew, *et al.*, 1997, El-Menshawi *et al.*, 2010, Goldufsky *et al.*, 2015, Abo-Alkasem *et al.*, 2019 & Amer *et al.*, 2021).** The results were measured by detecting the absorbance using a microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at 595 nm and a reference wavelength of 620 nm. A statistical significance was evaluated between the treated and negative control samples (cells with vehicle) using independent t-test in SPSS 11 program.

DMSO was the vehicle used for dissolving ES and its final concentration on the cells was less than 0.2 %. The percentage of change in the cell viability (V %) was calculated according to the formula:

V % = [(absorption of ES / absorption of negative control) -1] x 100

The Probit analysis was carried out in order to determine the IC_{50} and IC_{90} values using SPSS 11 program. The degree of selectivity of the tested sample (SI) was calculated as follows:

SI=IC_{50} of sample tested against normal cell line/IC_{50} of the same sample tested against cancer cell line. Where IC_{50} is the concentration required to kill 50 % of the cell population.

2.10. Statistical analysis

All the data recorded were subjected for ANOVA test to assess the statistical significance of the measured parameters. T-test and Duncan's multiple range tests were also performed to compare between the measured data. The software program utilized for this analysis was SPSS version 16. The data was presented in the form of mean values ± standard error (SE).

3. Results and discussion

3.1. Isolation, selection and characterization of promising Phenazine producer bacterial strain

Thirty bacterial isolates were recovered from soil and fresh water samples, of which fourteen were found to belong to the genus *Pseudomonas* (Table 1). Morphological characterization showed that only fourteen of these isolates were bacilloid in form and lack the ability to Gram stain but had significant capabilities for the formation of diffusible pigments. In meantime these isolates were catalase and oxidase positive. These microscopic characters, physiological traits, and biochemical properties were found to be typical of those described in "Bergey's Manual of Determinative Bacteriology" (**Boone *et al.*, 2001 and Devnath *et al.*, 2017**) for the genus *Pseudomonas*. Therefore, the majority of the bioactive secondary metabolites produced were expected to belong to the phenazine group of compounds.

3.2. Primary Screening of isolates with the highest antimicrobial activities

The results, illustrated in table 2, showed that only six of the fourteen previously chosen *Pseudomonas* isolates displayed antibiotic activities, of them, three isolates (4-SA^{NRC}, 14-WA^{NRC}, and 15-WA^{NRC}) recorded significant inhibition zones ranging from 10-18 mm against some of the tested pathogenic microorganisms. According to the results showed in figure 1, the the production of secondary metabolites by the three selected isolates was greatly affected by the period of incubation as it was found to have a substantial impact on the intensity of the produced color, which was a function of the presence of active compounds. These results proved that the production of the secondary metabolites was improved with the extension of the incubation period. This improvement began on the third day and more or less increased steadily through the subsequent four days of incubation. These data are in agreement with those obtained by **Naik *et al.*, 2015, Jain and Pandey, 2016 & Devnath *et al.* (2017).** However, the maximum production of pigmented phenazine compounds (5.8) was obtained when isolate 14-WA^{NRC} was incubated for 5 days in the king A medium and consequently this isolate was selected for further study.

3.3. Identification of the selected isolate

3.3.1. Morphological, biochemical and physiological characterizations

The microbiological examination showed that 14-WA^{NRC} was aerobic, Gram-negative, rod-shaped and motile, (Table 3). The isolate also showed positive results for oxidase and catalase tests, ability for citrate utilization, gelatin and casein hydrolysis as well as a positive deep glucose agar test. However, it showed negative results for the rest of the tested biochemical analysis. The results of table 3 also revealed that strain 14-WA^{NRC} tolerated various pH ranges and that its specific growth rate was directly proportional to the medium's pH, with an optimum pH rang of pH 7-9. The ability to grow on wide ranges of temperatures was also studied. The results revealed that the temperature of 37°C recorded the highest growth rate. However, weak growth was observed at lower temperatures of 15-25^{o}C, and negative growth was detected at extreme temperatures of 5 and 45^{o}C. (Table 3).

Table 1: Biochemical and phenotypic characterizations of all isolates recovered from soil rhizosphere and water channel located at Awlad Sakr center, Al-Sharkia governorate, Egypt.

Isolate No.	Isolate Code	Biochemical test		Phenotypic Characterization			
				Pigmentation		Gram stain	Morphology
		Oxidase test	Catalase test	Pigment color	Diffusible pigment		
1	1SA^{NRC}	-	-	-	-	-	Bacilloid
2	2SA^{NRC}	+*	+	Golden yellow	-	-	Bacilloid-short rods
3	3SA^{NRC}	+	+	Golden yellow	-	-	Bacilloid-short rods
4	4SA^{NRC}	+	+	Green	+	-	Bacilloid-short rods
5	5SA^{NRC}	-	-	-	-	+	Bacilloid
6	6SA^{NRC}	+	+	Yellow	-	-	Bacilloid-short rods
7	7SA^{NRC}	-	+	Yellow	-	-	Bacilloid-short rods
8	8SA^{NRC}	-	-	-	-	-	Bacilloid
9	9SA^{NRC}	-	+	Pale yellow	-	-	Bacilloid-short rods
10	10SA^{NRC}	-	+	-	-	+	Cocci
11	11SA^{NRC}	-	-	-	-	-	Bacilloid-short rods
12	12SA^{NRC}	-	+	-	-	+	Cocci
13	13SA^{NRC}	-	+	-	-	+	Cocci
14	14SA^{NRC}	-	-	-	-	+	Bacilli
15	15SA^{NRC}	-	-	-	-	+	Bacilli
16	16SA^{NRC}	-	-	-	-	+	Cocci
17	1WA^{NRC}	-	-	-	-	+	Cocci
18	2WA^{NRC}	-	-	-	-	+	Bacilli
19	3 WA^{NRC}	-	-	-	-	+	Cocci
20	4 WA^{NRC}	-	+++	Yellow	-	+	Cocci
21	5 WA^{NRC}	-	-	-	-	+	Bacilli
22	6 WA^{NRC}	-	-	-	-	+	Bacilli
23	7 WA^{NRC}	-	++	Pale yellow	-	+	Cocci
24	9 WA^{NRC}	-	-	-	-	+	Strepto coccoid
25	10WA^{NRC}	-	-	Green	+	-	Bacilloid-short rods
26	11WA^{NRC}	+++	-	Bluish green	+	-	Bacilloid-short rods
27	13WA^{NRC}	+	+	Yellow	-	-	Bacilloid-short rods
28	14WA^{NRC}	+++	+++	Dark bluish green	+	-	Bacilloid-short rods
29	15WA^{NRC}	+	+	Bluish green	+	-	Bacilloid-short rods
30	16WA^{NRC}	+++	+	Pale yellow	-	-	Bacilloid-short rods

SA, soil is the isolation source; WA, water is the isolation source; -, negative result; +, positive result; ++, moderate positive result; +++, strong positive results.

3.3.2. Phylogenetic identification

The PCR product of the strain indicated a band at 1500 kb (data not shown). The Blast search tool demonstrated an elevated level of sequence similarity (99%) with numerous *Pseudomonas aeruginosa* species. The phylogenetic tree, illustrated in figure 2, was performed using the fifteen most similar species, having the closer distance nucleotides sequences matrices, adapting the neighbor-joining technique. The missing data and gaps in all positions have been wiped out.

3.4 Detection of the optimum fermentation medium

Screening of various media for maximum production of secondary metabolites by the selected *Pesudomona* isolate, identified as *P. aeruginosa* OQ158909, showed significant ($p \leq 0.05$) variation in color intensities measured at 362 nm, which is a function for the presence of biologically active metabolites (**Vandermolen *et al.*, 2013**). The highest light absorbance of 8.08, recorded after 5 days of incubation in MS medium, revealed that the highest color intensity, and consequently the greatest amount of phenazine compounds produced, was obtained upon using this medium and which could be referred to its low nutrients content.

Table 2: Antimicrobial activities of the fourteen tested isolates, selected based on their Gram-stain ability and biochemical tests results, against different human pathogens. All data are represented in means ±SE. Means followed by the same letter per column are not significantly different at level $p \leq 0.05$ using Duncan's multiple range tests.

Isolate No.	Designation Code	Source	Antimicrobial activity					
			Diamter of inhibition zone (mm)					
			Tested microbial pathogenes					
			E.coli	*Salmonella entirca*	*Bacillus Cereus*	*Bacillus subitilus*	*Staphyllococcus aureus*	*Candida albicans*
1	2SA-NRC	Soil	-	-	6.00±0.58^{e}	8±0.58^{e}	-	-
2	3SA-NRC	Soil	-	-	-	-	-	-
3	4SA-NRC	Soil	-	-	13.00±1.16^{c}	12.00±0.58^{b}	10.00±0.0^{b}	-
5	6SA-NRC	Soil	-	-	6.00±0.0^{e}	10.00±0.0^{c}	9.00±1.16^{c}	-
6	7SA-NRC	Soil	-	-	-	-	-	-
8	9SA-NRC	Soil	-	-	-	-	-	-
20	4WA-NRC	Water	-	-	-	-	-	-
23	7WA-NRC	Water	-	-	-	-	-	-
25	10WA-NRC	Water	-	-	-	-	-	-
26	11WA-NRC	Water	-	-	9.00±0.0^{d}	10.00±1.16^{c}	6.00±0.0	-
27	13WA-NRC	Water	-	-	-	-	-	-
28	14WA-NRC	Water	-	-	18.00±0.0^{a}	14.00±0.0^{a}	12.00±1.16^{a}	-
29	15WA-NRC	Water	-	-	15.00±0.0^{b}	12.00±0.0^{b}	13.00±0.58^{a}	-
30	16WA-NRC	Water	-	-	-	-	-	-

-, no activity.

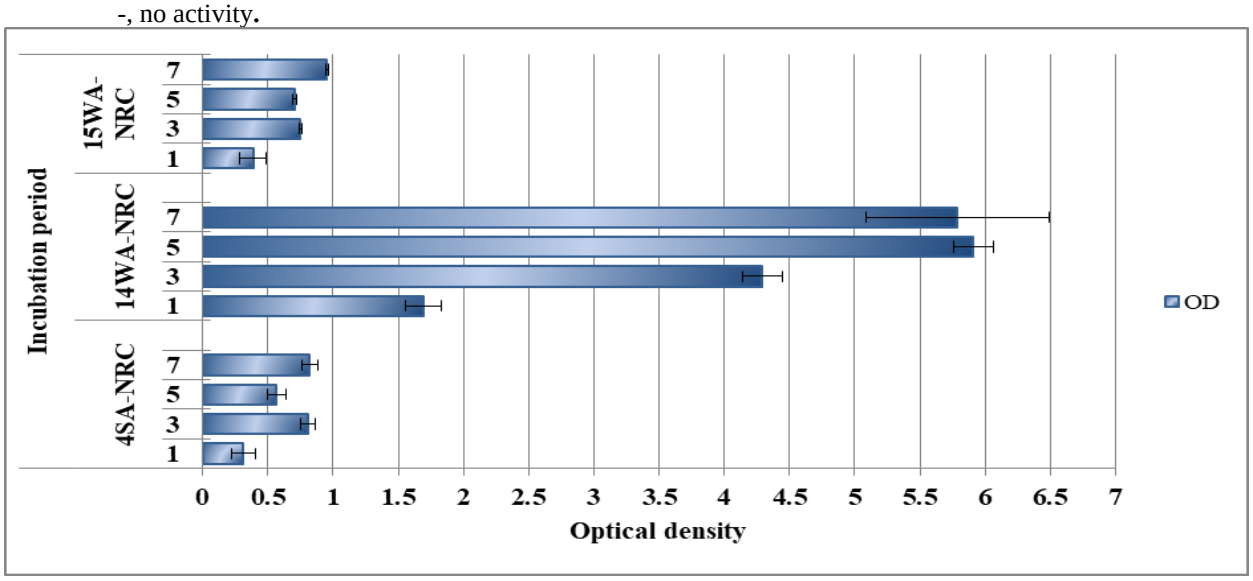

Figure (1): Optical densities of colored secondary metabolites produced by three selected *P. aeruginosa* strains at different incubation periods, Data is represented in means ±SE.

The cultivated strain therefore exhibited a shorter growth curve and consequently the incubation time required for maximum production of secondary metabolites decreased (Figure 3). In other words, media containing more nutrients could result in a delayed production of the same amount of secondary metabolites. This result is in according to that of **Ramakrishna and Ravishankar (2011**) who stated that low salts broth, which contains minimal nutrients, may create a stress factor that could promote the building-up of secondary metabolites.

The antimicrobial activities of the secondary metabolites produced by *P. aeruginosa* OQ158909, cultivated in each of the 5 tested fermentation media were evaluated against a wide range of human pathogens. The data revealed that, although all the tested fermentation broths had significant antimicrobial effects on all the tested pathogenic strains, the secondary metabolites produced in the MS medium mainly exerted higher antimicrobial activities against most gram positive tested bacteria resulting in the largest inhibition zones of more than

26 and 27 mm (as compared to those obtained when other fermentation broths were tested) for the growth of *Bacillus cereus* and *staphylococcus aureus* respectively ($p \leq 0.05$). However, the biologically active phenazine compounds produced in nutrient broth were found to be more or less specific for gram negative bacteria as they resulted in larger inhibition zones of 12 and 17 mm for the growth of *E. coli* and *S. enterica* respectively ($p \leq 0.05$), compared to those obtained when other fermentation broths were used. On the other hand significantly large inhibition zones of 15 and 23 mm were recorded for the growth of *C. albicans* and *A. niger* respectively ($p \leq 0.05$) showing that the secondary metabolites produced upon using the king A medium were more active against the fungal tested strains (Figure 4).

These results confirmed that the tested strain have the ability to produce a consortium of phenazine compounds which synergistically act against a variety of microorganisms resulting in a broad spectrum of activity and that the ratio of these compounds differs according to the medium composition. MS medium was consequently selected for further studies as it clearly supported the production of the largest amounts of phenazines significantly active against all the tested pathogens. It was worth noting that the OD of the produced colored compounds, recorded in this experiment using MS medium, represented almost double that reported by **Jain and Pandey (2016)** using the same fermentation medium and comparable cultivation conditions for *Pseudomonas chlororaphis* GBPI 507 (MCC2693).

3.5. Factors affecting bioactivity of secondary metabolites: Production Optimization

3.5.1. Different incubation periods

The effect of different incubation periods on the biological activities of secondary metabolites produced by *P. aeruginosa* OQ158909 was investigated against a wide range of human pathogens (Figure 5). Antimicrobial activities obtained when the bacterial culture was incubated at 37°C for various durations, ranging from 1 to 5 days, revealed that the growth inhibition of all the tested strains was found to gradually increase with the increase of the incubation period up to 4 days ($p \leq 0.05$). At this incubation period the recorded inhibition zone of the growth of Gram positive bacteria ranged between 22–39 mm while those of Gram negative bacteria were approximately 14 mm and those of fungi recorded 11 and 24 mm against C. *albicans* and *A. niger* respectively. However further incubation of the selected strain resulted in a slight decrease in the measured inhibition zones of all the tested pathogens.These findings suggested that the optimal incubation period for the production of secondary metabolites with strong biological activities was critical and should be carefully considered in future studies.

3.5.2. Different initial pHs of the selected fermentation medium

The biological activity of secondary metabolites of the fermentation medium produced by *P. aeruginosa* OQ158909 was found to be greatly influenced by the initial pH of the fermentation medium. The study showed that no antimicrobial activity was detected when the producing strain was cultivated under highly acidic conditions (pH of 1.5 and 3.2).

Table 3: Morphological, biochemical and physiological characterizations of the selected 14-WA^{NRC} isolate.

Test	Result
Morphological study	
Micromorphology	Bacilloid form, short rods
Gram staining	-
Motility test	Motile
Biochemical study	
Oxidase test	+++
Catalase test	+++
Indole test	-
Methyl red test	-
H_2S production test	-
Voges-Proskauer test	-
Nitrate reduction test	-
Citrate utilization	+
Gelatin hydrolysis	+
Deep glucose agar test	+
Casein hydrolysis	+
Physiological study	
Growth response at different pHs	
pH-1	-
pH-5	+
pH-7	+++
pH-9	+++
pH-11	+
Growth response at different temperatures (°C)	
5°C	-
15°C	±
25°C	++
37°C	+++
45°C	-

-, negative; ±, very weak; +, weak; ++, moderate; +++, strong.

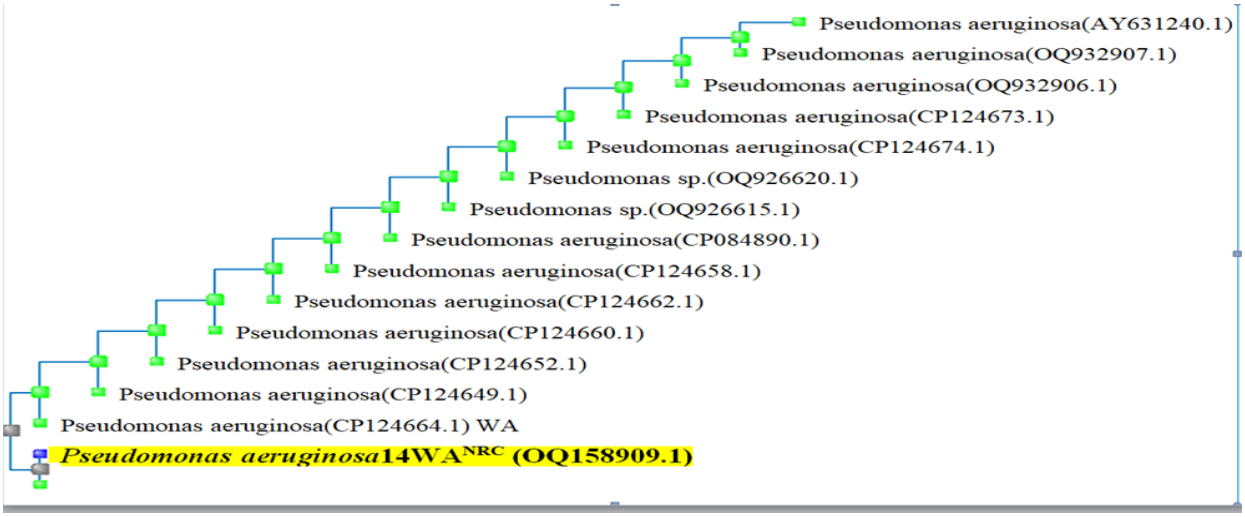

Figure (2): Phylogenetic tree for *P. aeruginosa* OQ158909.

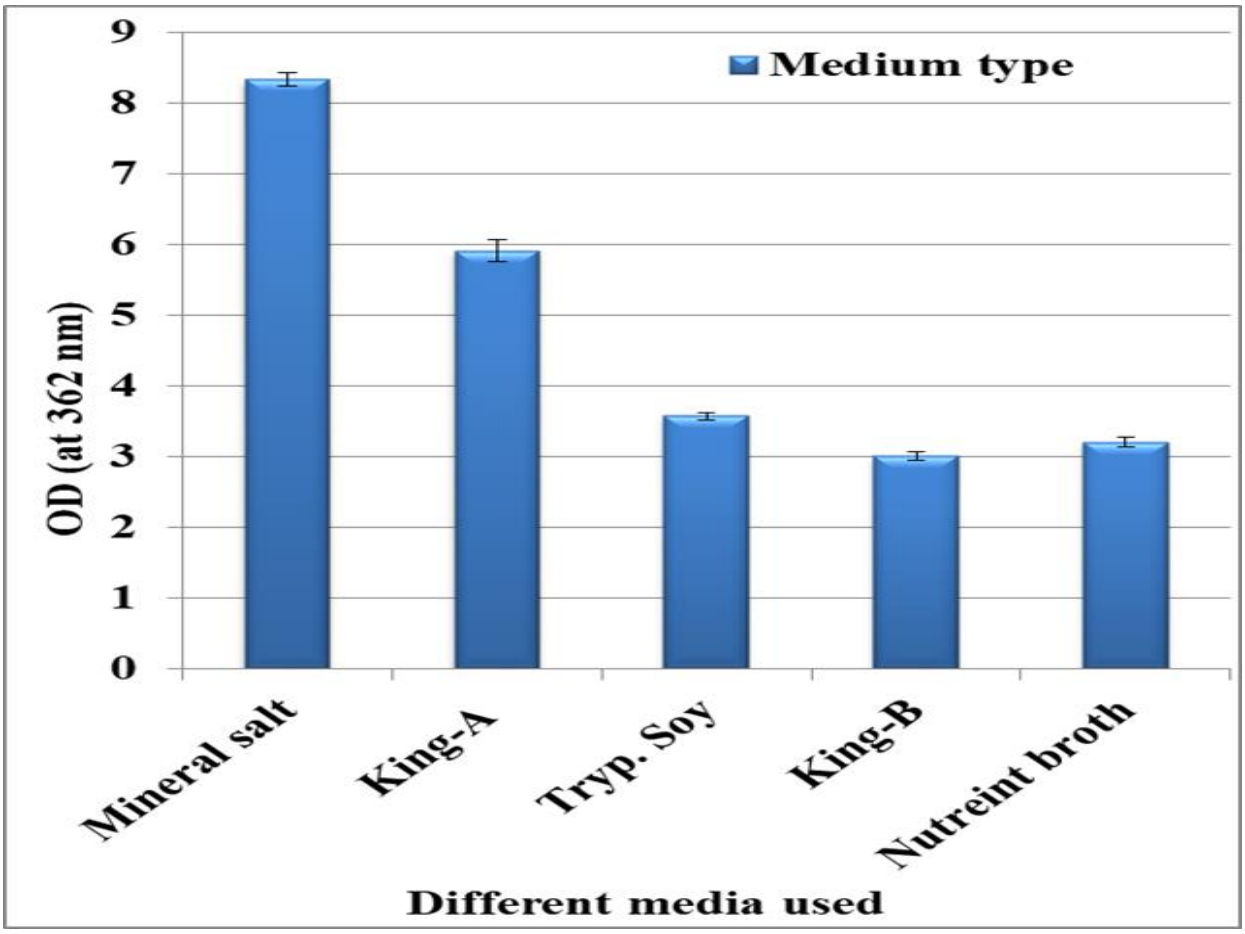

Figure (3): Effect of different fermentation media on the production of secondary metabolites by the selected 14-WA^{NRC} strain, Data are in mean ±SE.

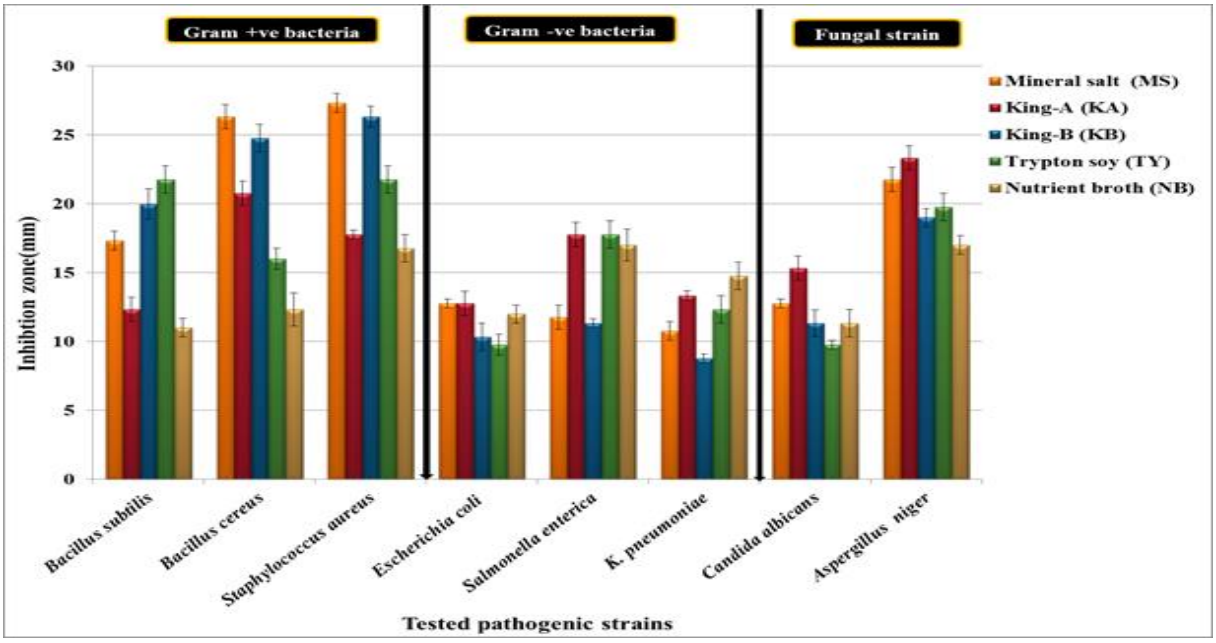

Figure (4): Effect of different fermentation media on the antimicrobial activities of the cell free fermentation broths as an essential function for secondary metabolites production by *P. aeruginosa*. Data are in mean ±SE.

However, when the initial pH of the fermentation medium was shifted towards the neutral zone, the obtained inhibition zones gradually increased reaching their maximum when the latter was adjusted at 7.2($p \leq 0.05$). These obtained maximum inhibition zones measured 20-29 mm against Gram positive bacteria, 13-14 against Gram negative bacteria, 13 mm against *C. albicans* and about 25 mm against *A. niger*. Lower inhibitions zones results were obtained when the pH of the medium was adjusted at more alkaline values of 9.2 and 11.2 (Figure 6). This could be attributed to the fact that the changes in the pH of the fermentation medium had a significant ($p \leq 0.05$) impact on the chemical composition and structure of the produced secondary metabolites, which in turn influenced their biological activities (**Leinberger *et al.*, 2021**). These findings confirmed that the pH of the fermentation medium used for the production of secondary metabolites is crucial in order to optimize their biological activities and potential use as antimicrobial agents.

3.5.3. Different agitation speeds

Agitation speed, known to impact cell development and metabolism, is one of the many parameters that can influence the production of secondary metabolites (**Mustafa *et al.*, 2019**). The study findings revealed that, when the agitation was raised from 80 to 100 rpm, the obtained inhibition zones diameters increased significantly ($p \leq 0.05$) to 19-26 mm for Gram positive bacteria, 12-15 mm for Gram negative bacteria and 17-26 mm for fungi, followed by a more or less slight decrease in the inhibition zones recorded as the agitation speed was further increased to 120 rpm (Figure 7). This proved the crucial effect of the change in the agitation speed which can be referred to the increase in the interaction between microorganisms and nutrients present in the media, ultimately resulting in higher cell density and production of metabolites **(Tadijan *et al.*, 2017 & Al-ghazali and Omran, 2017)**. These findings confirmed that optimizing the agitation speed during the production of secondary metabolites by *P. aeruginosa* can enhance their biological activity and potential use as antimicrobial agents.

3.5.4. Different inoculum sizes

The size of the inoculum had a significant impact on the production of secondary metabolites that consequently enhanced

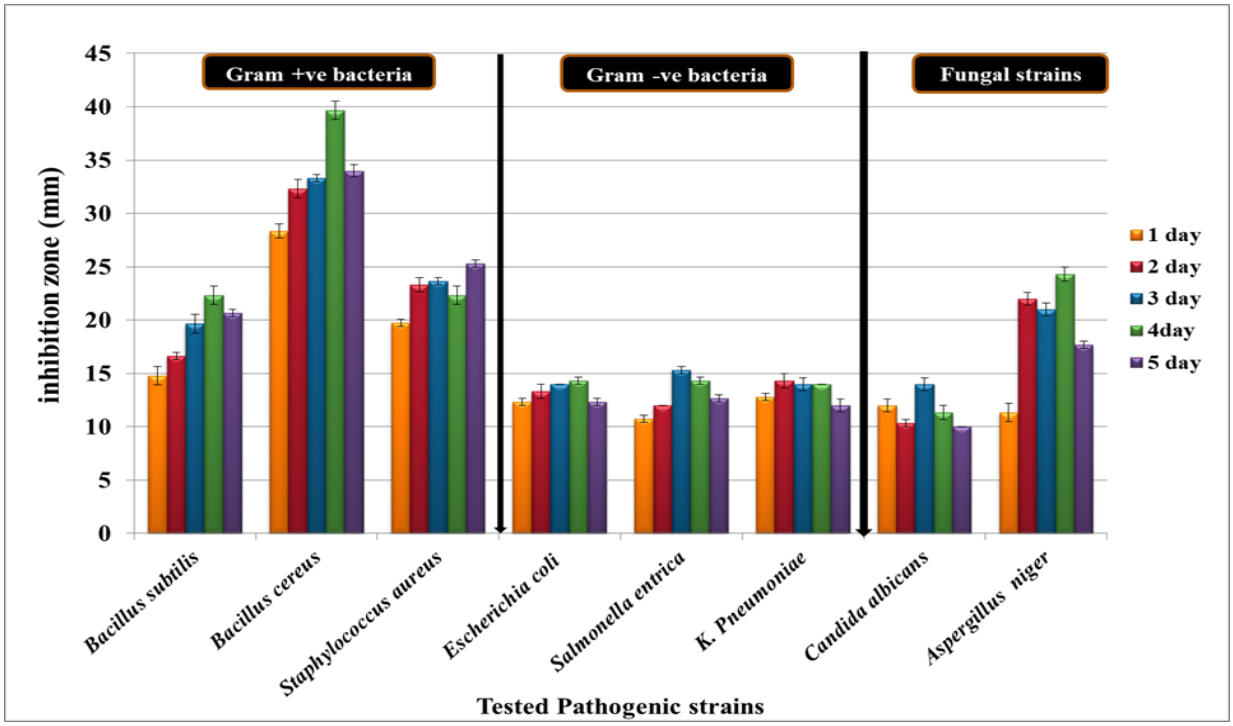

Figure (5): Effect of different incubation periods (day) on the antimicrobial activities of cell free MS fermentation broth as an essential function for secondary metabolites production by *P. aeruginosa*. Data are in mean ±SE.

their antimicrobial activities. The present study showed that the inoculum size of 10% v/v (3.0 x10^8cfu) led to the highest yields of secondary metabolites. This resulted in the highest inhibition zones of 25-31 mm for Gram positive bacteria, 15-23 mm for Gram negative bacteria and 22–26 mm for fungi, as shown in Figures 8 (A & B). However, when the inoculum size was increased beyond the optimal size, there was a decrease in the metabolite activities. This can be explained as a result of increased competition for nutrients and space **Lichtenberg *et al.* (2022)**.

3.5.5. Different incubation temperatures

The production of active metabolites by *P. aeruginosa* was found to be influenced by various environmental factors, with temperature being a key determinant. In order to determine the best temperature for producing secondary antimicrobial active metabolites, the *P. aeruginosa* OQ158909 was exposed to a range of temperatures between 30°C and 45°C while maintaining optimal values for all previously studied parameters.

The study findings revealed that the optimal temperature for the production of active antimicrobial compounds by *P. aeruginosa* was 35°C. At this temperature, *P. aeruginosa* has been observed to result in maximum antimicrobial activities of 22-29 mm against Gram positive bacteria, 13-23 mm for Gram negative bacteria and 11-14 mm for fungi (Figure 9). However, when the incubation temperature was raised to 40°C, the antimicrobial activity was more or less reduced, while at 45°C, the *P. aeruginosa* strain was unable to grow and consequently no pigments were produced. This result can be explained as the temperature can affect the growth and metabolism of the bacteria. Generally, to a certain level, high temperatures can accelerate bacterial growth and metabolism, which led to increased production of the antimicrobial compounds. However, higher temperatures can also denaturate or degrade these compounds, resulting in reduced antimicrobial activities results. On the other hand, lower temperatures can slow down bacterial growth and metabolism, which can also results in reduced production of antimicrobial compounds.

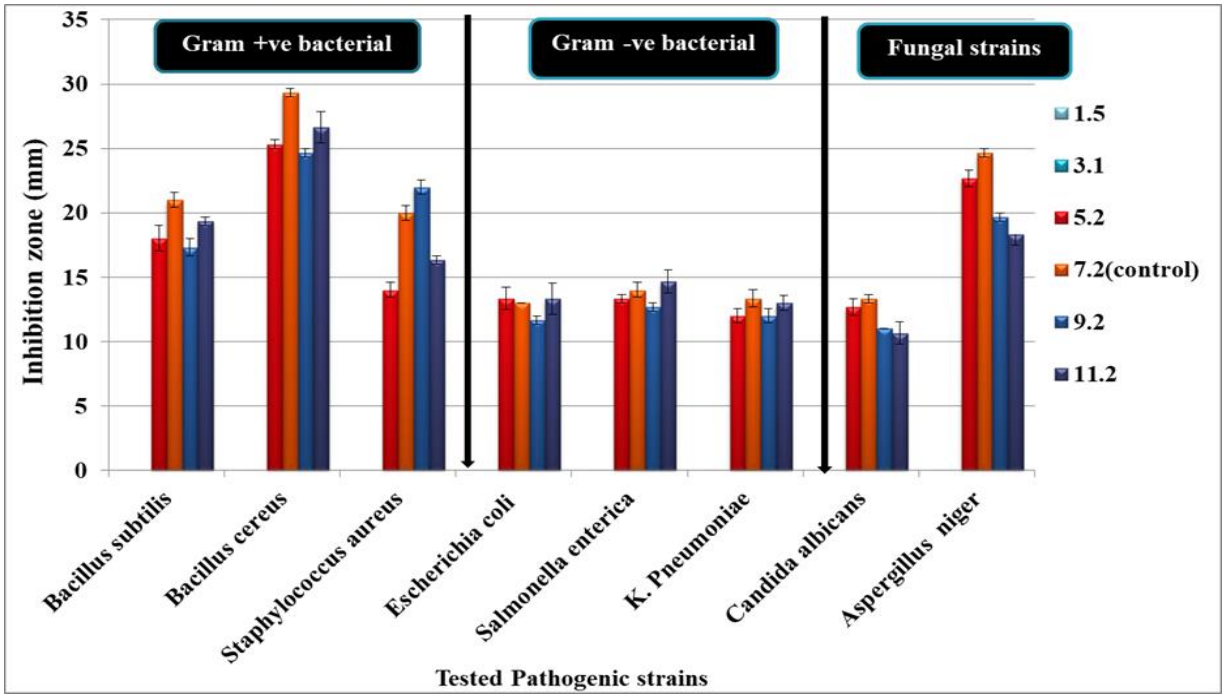

Figure (6): Effect of different initial pHs on the antimicrobial activities of cell free MS fermentation broth as an essential function for secondary metabolites production by *P. aeruginosa*. Data are in mean ±SE.

Therefore, it could be stated that incubation temperature plays an important role in the antimicrobial activity of *P. aeruginosa* strain under study by affecting the specific antimicrobial compound production.

3.6. Extraction of crude secondary metabolites produced by *P. aeruginosa* OQ158909

The result of the successive extraction of the cell free fermentation broth with different solvents of different polarities resulted in the isolation of different secondary metabolites, mainly phenazine compounds, each having a different spectrum of antimicrobial activities. This result confirmed the previously observed effect of fermentation medium's composition on the type of pathogen affected (Figures 10 & 11).

Ethyl acetate was selected as the most appropriate solvent for the extraction of the highest amounts of biologically active phenazine compounds with the broadest spectrum of activity. The use of ethyl acetate for the extraction of bioactive compounds was previously reported by many investigators such as **Arif *et al.* (2022) & Tumpa *et al.* (2023)**. The ethyl acetate fraction was obtained by extracting 12 liters of cell free MS fermentation medium with using equal volume of ethyl acetate in successive steps (**Jain and Pandey, 2016 & Devnath *et al.*, 2017)**. Prior to extraction, the pH of the cell free supernatant was acidified using conc. HCL. As a result of this procedure, the color of the latter changed from dark blue green to dark pink. The resulting dark brown ethyl acetate upper layers, obtained after several successive extraction processes were then collected, concentrated using a rotary evaporator and adjusted at 40°C. The retrieved ethyl acetate fraction, which was viscous and dark brown, represented a concentrated solution of phenazine compounds with a final volume of 20 ml.

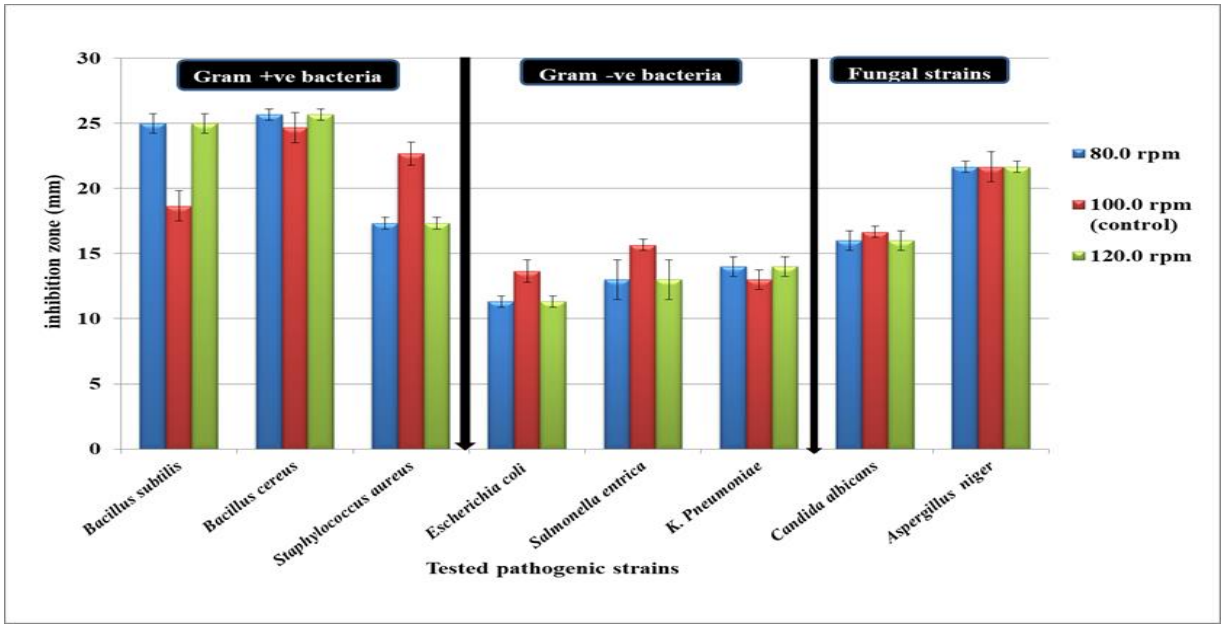

Figure (7): Effect of different agitation speeds (rpm) on the antimicrobial activities of cell free MS fermentation broth as an essential function for secondary metabolites production by *P. aeruginosa*. Data are in mean ±SE.

3.7. Evaluation of the ethyl acetate fraction's biological activities

3.7.1. Antimicrobial activity

The ethyl acetate fraction showed high antimicrobial efficacy against many human pathogens including Gram-positive and Gram-negative bacteria as well as fungi. The recorded inhibition zones against most tested Gram-positive bacteria were in the range of 15.0-25.0 mm, with an excellent inhibition zone of 41mm that was detected against *Bacillus cereus*. However, the inhibition zones recorded against Gram-negative bacteria ranged between 15-36 mm. The later inhibition zone was obtained against *Helicobacter pylori* which are highly contagious bacterial species that affect human stomach causing numerous stomach ulcers. Moreover the results also showed that the ethyl acetate fraction caused inhibition zones of 15-19 mm in diameter against both tested pathogenic fungi (Figures 12 & 13). These antimicrobial activities results of the ethyl acetate fraction, exceeded by far those obtained by **Jain and Pandey (2016)** who reported much lower inhibition zones of 7-9 mm against Gram positive bacteria and 0.3-1.7 mm against Gram negative bacteria.

3.7.2. Antioxidant activity

The ethyl acetate fraction showed a highly efficient antioxidant activity as well, since a small concentration, of only 100 µg/ml of the fraction, was found to successfully scavenge 100 % of the DPPH free radicals present in the solution. The recorded IC_{50} of only 10.4 µg/ml, was considered as an excellent result when compared to the considerably higher required IC_{50} values of 53.9±3.1 and 42.80±1.5 µg/ml obtained when butylated hydroxyanisole (BHA) and vitamin C were used as positive controls (Figure 14). These results revealed the much higher antioxidant affinity of the ethyl acetate fraction compared to that of the BHA and vitamin C which are the standard compounds used to evaluate the effectiveness of any tested compound. This IC_{50} result was also much lower than that obtained by **Yan *et al.*, 2021, Zhang *et al.*, 2021 & Liandi and Cahyana (2022)** who reported a higher required IC_{50} of 14.26 µg/ml for the scavenging of the same concentration of DPPH free radicals.

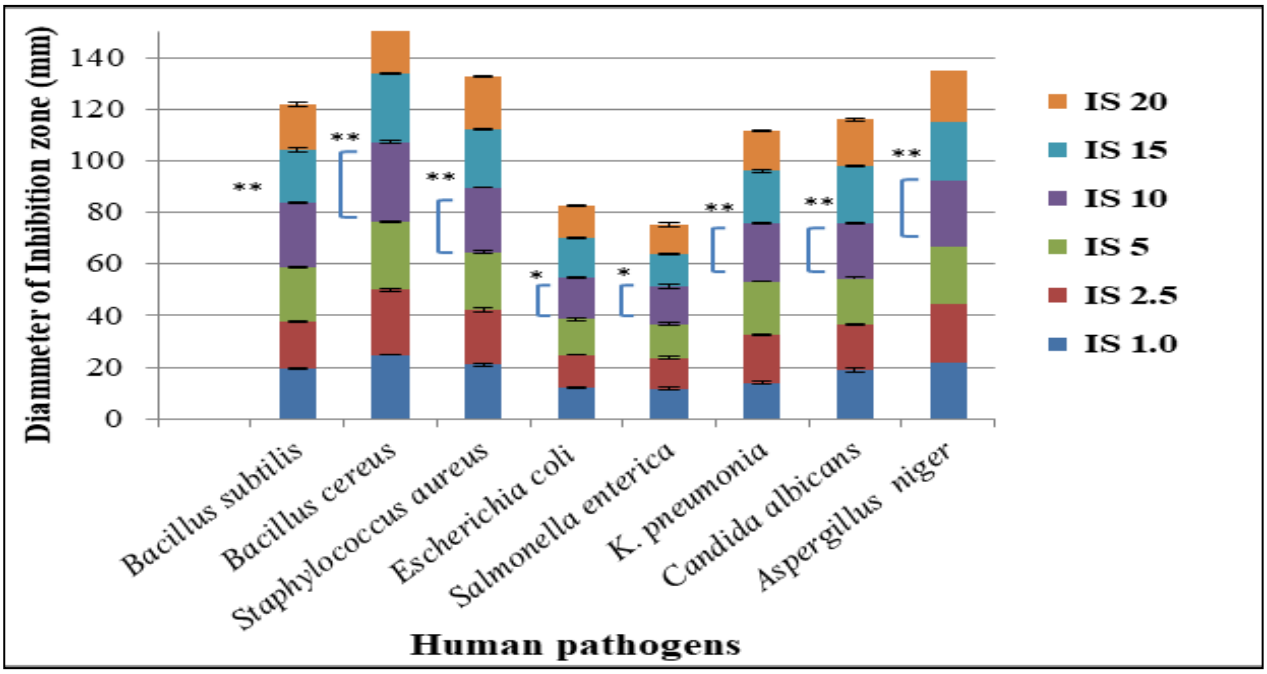

Figure (8A): Effect of different inoculum sizes (v/v) on the antimicrobial activities of cell free MS fermentation broth as an essential function for secondary metabolites production by *P. aeruginosa*. Data are in mean ±SE. Means with ** are highly significant at p≤0.01; means with * are significant at p≤0.05.

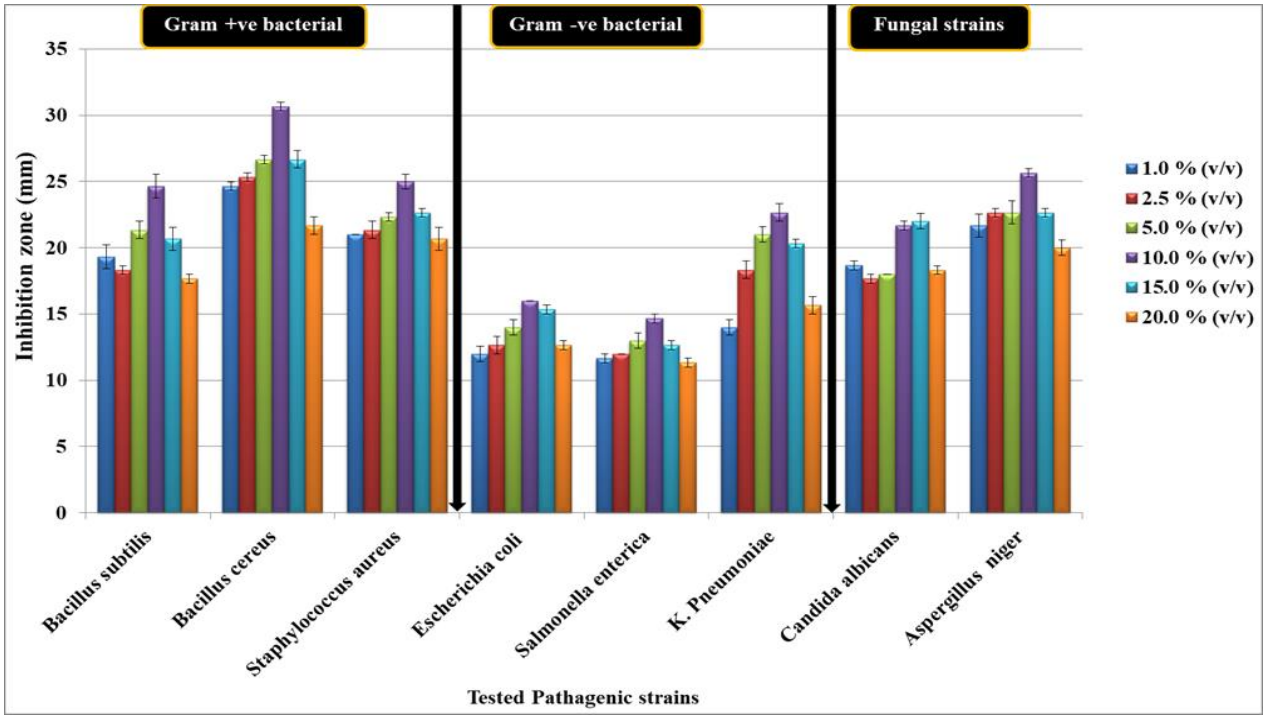

Figure (8B): Effect of different inoculum sizes (v/v) on the antimicrobial activities of cell free MS fermentation broth as essential function for secondary metabolites production by *P. aeruginosa*. Data are in mean ±SE.

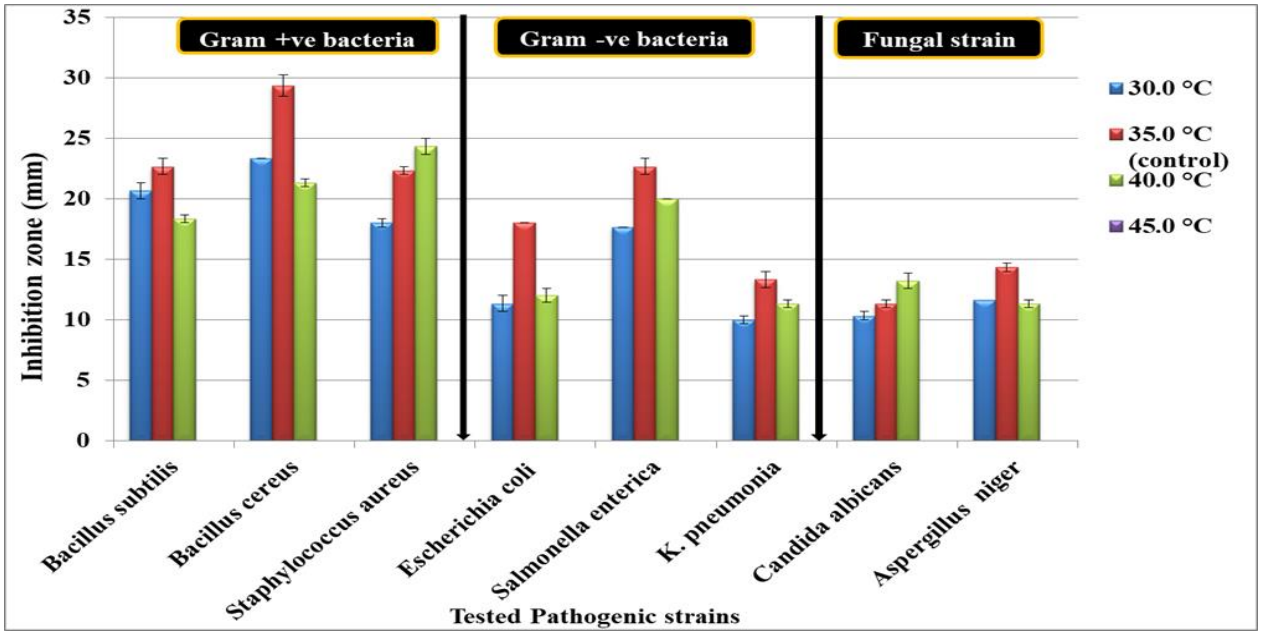

Figure (9): Effect of different incubation temperatures (°C) on the antimicrobial activities of cell free MS fermentation broth as an essential function for secondary metabolites production by *P. aeruginosa*. Data are in mean ±SE.

Figure (10): Successive extraction of the cell free MS fermentation broth with different solvents of different polarities.

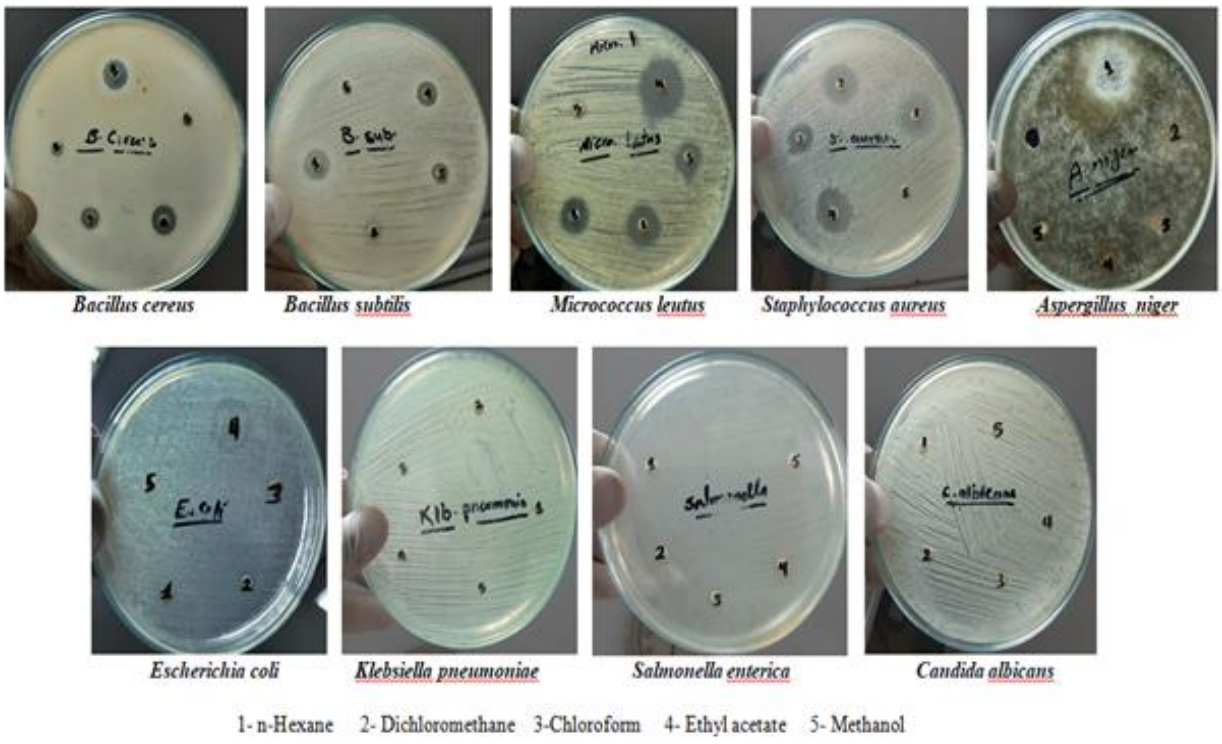

Figure (11): Antimicrobial activities of different fractions obtained by the successive extraction of the cell free MS fermentation broth with different solvents of different polarities.

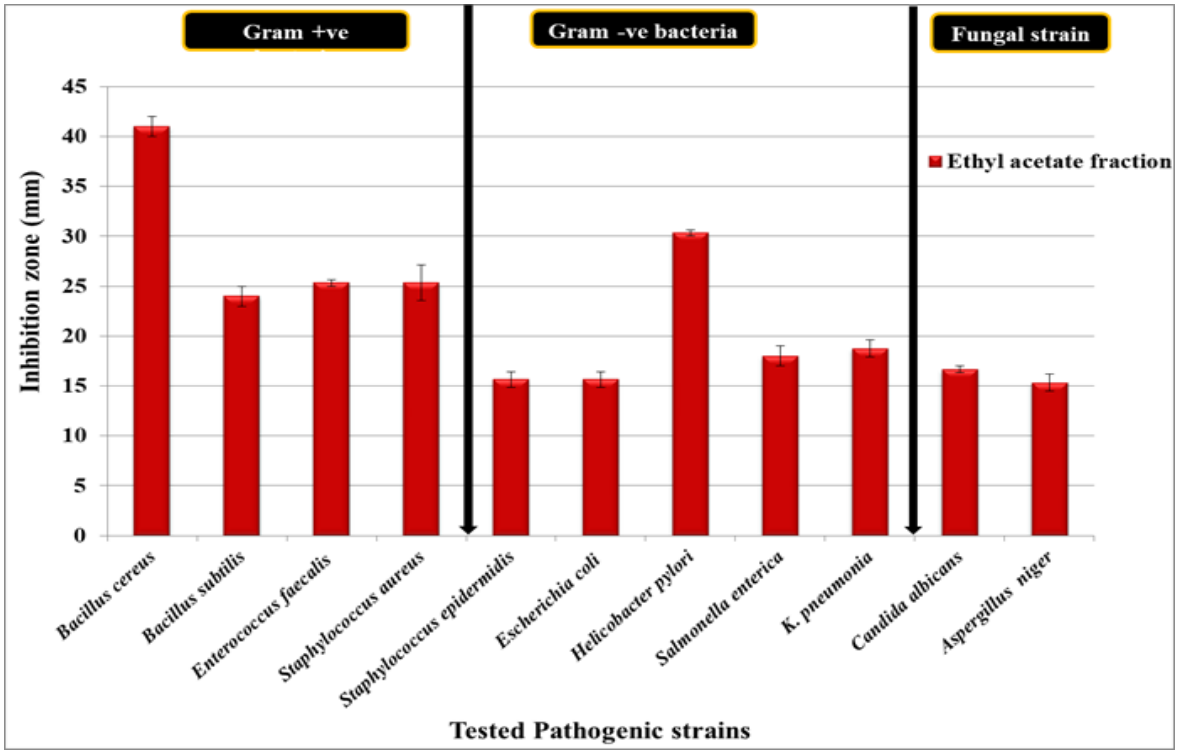

Figure (12): Antimicrobial activities of the ethyl acetate fraction, Data is represented in means ±SE.

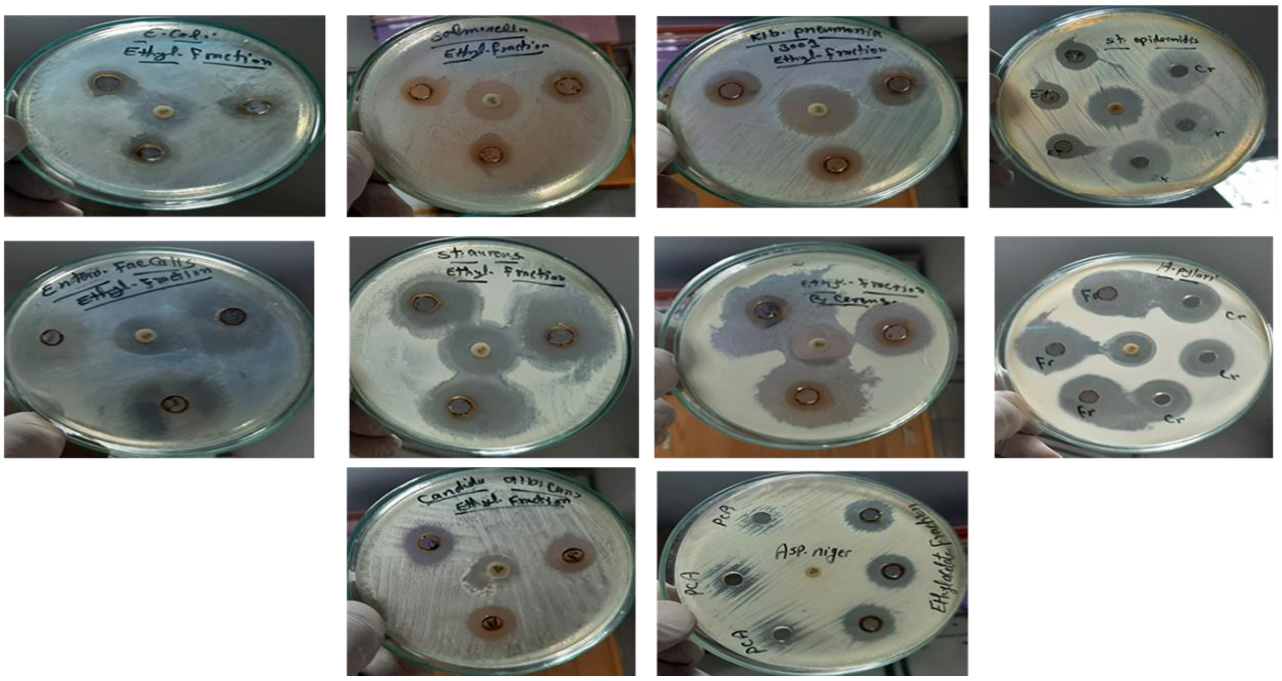

Figure (13): Antimicrobial activities of the ethyl acetate fraction using agar well diffusion method.

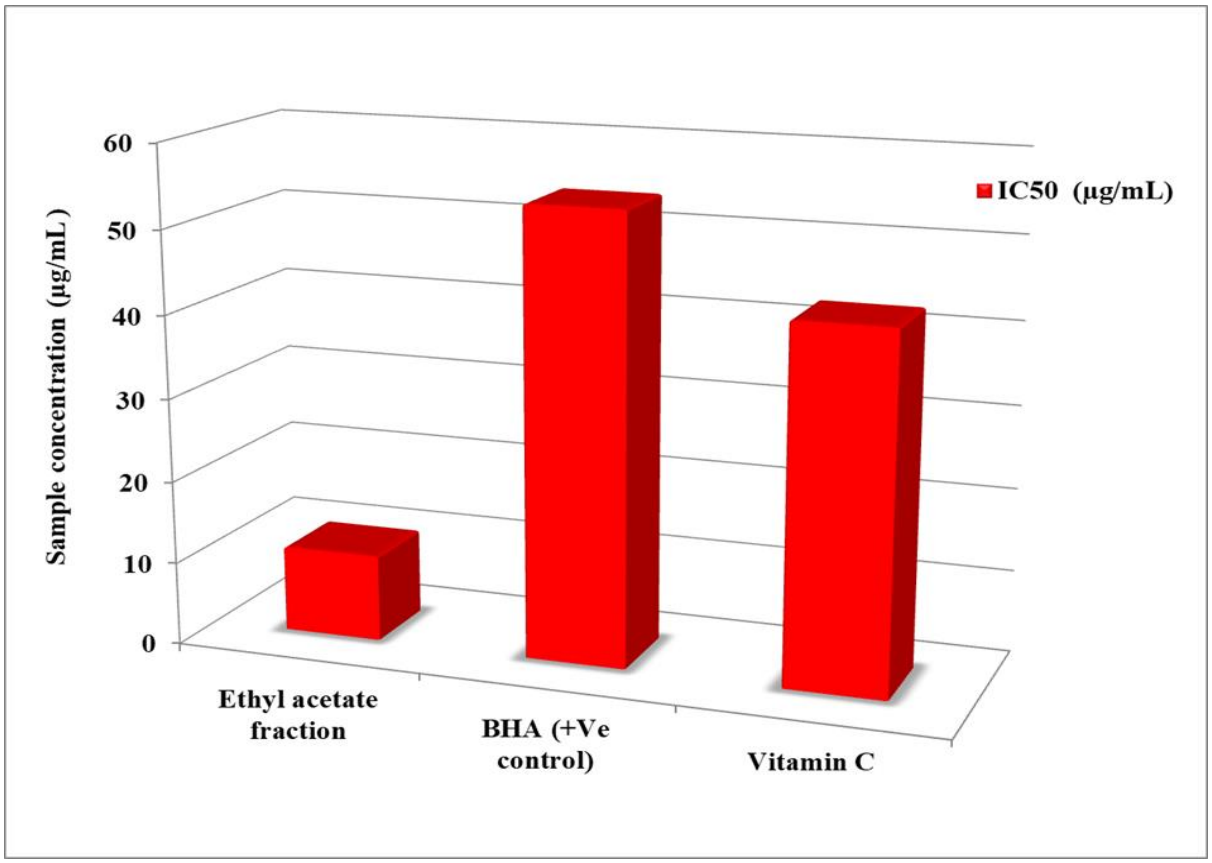

Figure (14): Antioxidant activity of the ethyl acetate fraction.

3.7.3. Cytotoxicity

The ethyl acetate fraction has also been found to exert different levels of cytotoxic activities against various cancer cell lines, including human Caucasian breast adenocarcinoma (MCF_7), pancreatic cancer ($PACA_2$); human hepatocellular carcinoma ($HePG_2$) and prostate cancer (PC_3) cell line. The cytotoxic $HePG_2$ cancer cell line was considerable with an IC_{50} of only 5.1 µg/mL and an exceptional SI of about 20. This IC_{50} result was proved to be four folds stronger compared to that of doxorubicin positive control that required in a much higher IC_{50} of 21.6 µg/mL. Moreover, IC_{90} only 24.6 µg/ml was also recorded against this cell line while 100 µg/ml were able to result in a complete (100 %) destruction of the tested cancer cells (Figure 15). On the other hand, when the fraction was tested against MCF_7 cancer cell line, an IC_{50} of the 27.2 µg/mL and a superb SI of 3.7 were obtained.

This IC_{50} result was considered highly significant if compared to the more or less equal IC_{50} of doxorubicin positive control of 26.1 µg/mL. The fraction also recorded an IC_{90} of 49.8 µg/mL against the MCF_7 cancer cell line and 100 µg/mL of the fraction also led to 100 % death of the tested cell line. The cytotoxicity of this fraction was therefore believed to be mediated through the induction of apoptosis, a process of programmed cell death by activating caspase-3 and -9, two key enzymes involved in the apoptotic pathway at the G0/G1 phase **(Wood *et al.*, 2015 & Kuo *et al.*, 2019)**. These findings suggest that the ethyl acetate fraction, containing phenazine compoun ds produced by *P.aurginosa* OQ158909, has great potentials as a natural source of anticancer agent.

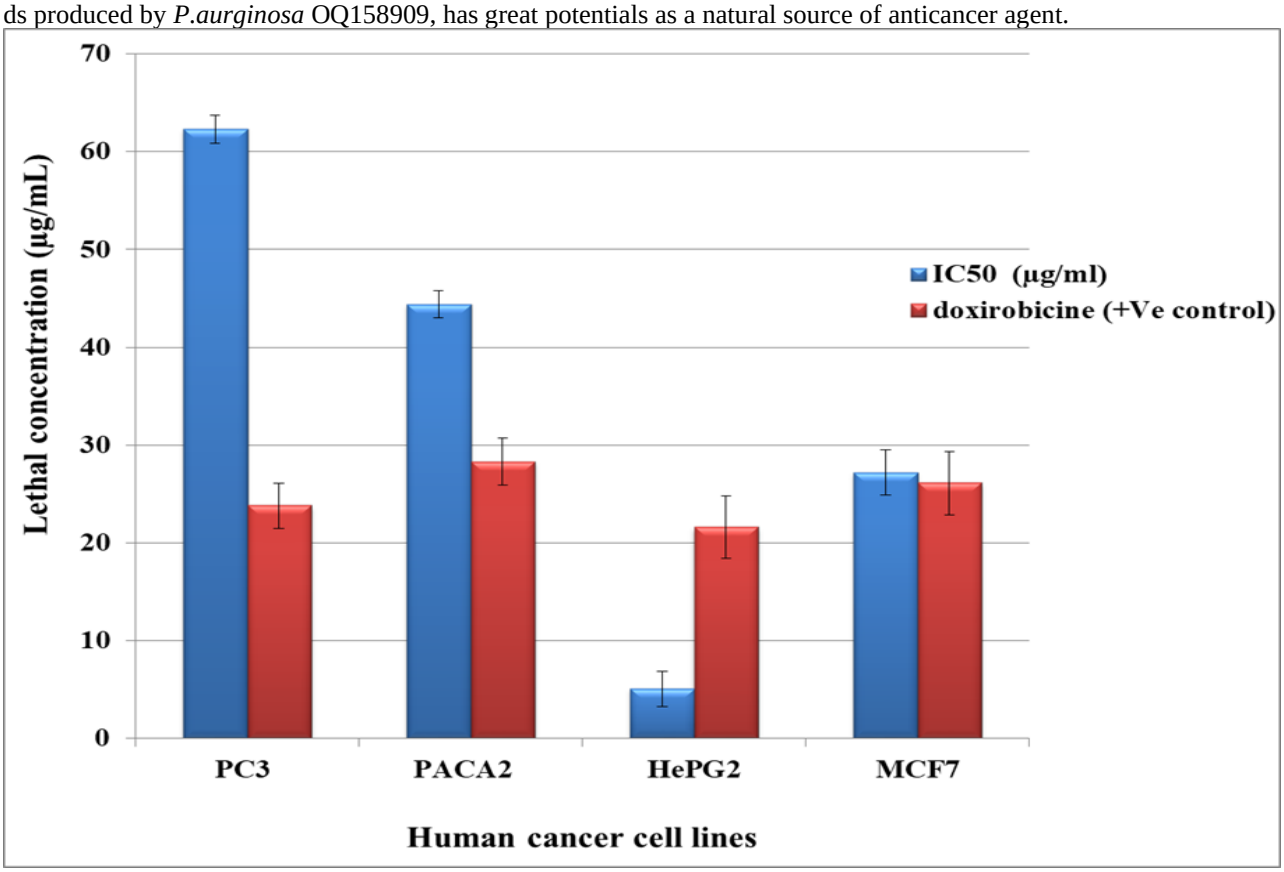

Figure (15): Cytotoxic activities of the ethyl acetate fraction against different cancer cell lines, Data are represented in means ±SE.

Conclusion

In this study, a local *Pseudomonas* strain was isolated from a running water source used for the irrigation of agricultural fields. The isolated strain was identified using 16S-rDNA as *Pseudomonas aeruginosa* OQ158909. The strain was proved to have the ability to produce significant amounts of different phenazine compounds with distinctive antimicrobial, anti-oxidant and anti-cancer effects using a highly economic fermentation medium solely composed of mineral salts.

The antimicrobial activity of the produced phenazine compounds was found to be mainly directed toward Gram positive bacterial such as *Staphylococcus aureus* which causes, among others fatal respiratory diseases and lung infections. Excellent results were also recorded against different pathogenic fungi such as *A. niger* known to be responsible for drastic

pulmonary aspergillosis diseases. The produced phenazine compounds were also able to significantly inhibit the growth of different gram negative bacteria specially those of *K. pneumonia* and *S. enterica* which are both responsible for sever diseases such as pneumonia and salmonellosis. Most of these pathogens are nowadays showing increased resistance to some of the most commonly used antibiotics.

Moreover, the extracted crude phenazine compounds displayed superb antioxidant and anti-cancer effects which, in some cases, surpassed by far the effect of the currently adopted compounds for these purposes.

In a nutshell, the detection of natural compounds that could be easily produced with low cost, is considered as a valuable addition to the naturally produced effective antimicrobial agents, in addition to their use as powerful anti-cancer agent, specially against hepatic carcinoma, which is considered one of the most life threatening and world wide spread cancer disease.

Further researches for the purification of the produced phenazine compounds are however highly recommended.

Acknowledgements

The authors are grateful to the National Research Centre (NRC) for providing full fund and all facilities to achieve this study. Sincere thanks are also due to the Bioassay-Cell Culture Laboratory (B-CCL) staff, Pharmacology department, Pharmaceutical and Drug industries Institute, National Research Centre, for their technical and scientific support especially in the cytotoxicity section. Finally special greetings must be offered to Prof. Ahmed I. El-Diwany, head of the Biological Assessment of Natural and Microbial Products Unit (BANMPU), Lab. 174, Chemistry of natural & microbial products department, Pharmaceutical and Drug industries Institute, National Research Centre, for providing the standard pathogenic strains that were used in the antimicrobial section.

Conflicts of interest

"The authors declare that there is no conflict of interests regarding the publication of this article".

Funding sources

Full fund was offered from the National Research Centre for Hossam M.Mohamed who is a PhD-candidate.

References

1- Abdul-Hussein, Z. R., & Atia, S. S. (2016): Antimicrobial effect of pyocyanin extracted from Pseudomonas aeroginosa. *European Journal of Experimental Biology*, ***6*(3)**, 1-4.

2- Akula, R., & Ravishankar, G. A. (2011): Influence of abiotic stress signals on secondary metabolites in plants. *Plant signaling & behavior*, ***6*(11)**, 1720-1731.

3- Al-ghazali, L. H., & Omran, R. (2017): Optimization of Medium Composition for Antibacterial Metabolite Production from Streptomyces sp. *OPTIMIZATION*, ***10*(9)**.

4- Amer, M., Eldiwany, A., Elgammal, E., Atwa, N. A., Dawoud, I., & Rashad, F. M. (2021). Nano-exopolysaccharide from the probiotic Weissella paramesenteroides MN2C2: Production, characterization and anticancer activity. *Egyptian Journal of Chemistry*, ***64*(12),** 7123-7133.

5- Arif M, Wang X, Kazi MSK, Ullah Khan S, Saeed S, Khan AM, Khan RA, Afzal M, Nawaz AF, Zia MA, O Elansary H, Shokralla S, Alhalabi A, Gul A, Fiaz S. (2022): Antimicrobial activities of different solvent extracts from stem and seeds of Peganum Harmala L. PLoS One. 2022 Apr **28;17(4)**:e0265206,doi:10.1371/journal.pone.026520 6. Retraction in: PLoS One. 2022 Aug **31;17(8)**:e0273538. PMID: 35482722; PMCID: PMC9049364.

6- Atwa, N. A., El-Diwany, A. I., Daba, G. M., Elmaghraby, I. M., Elmaghraby, M. M., Amer, M. N., & Maany, D. A. (2021). Studies on the antimicrobial activity of a locally isolated lactic acid bacterium and its application on fusarium solani-infected plants. *Egyptian Pharmaceutical Journal*, ***20*(3)**, 207.

7- Boone, D. R., Castenholz, R. W., & Garrity, G. M. (Eds.). (2001). Bergey's manual® of systematic bacteriology (Vol. 2, No. 3). Springer Science & Business Media.

8- Devnath, P., Uddin, M., & Ahamed, K. (2017): Md. Hossain F, Manchur TMA Extraction, Purification and Characterization of pyocyanin produced by Pseudomonas aeruginosa and evaluation for its antimicrobial activity. *Int. Res. J. Biological Sci*, ***6***, 1-9.

9- El-anssary, A. A., Raoof, G. F. A., Saleh, D. O., & El-Masry, H. M. (2021). Bioactivities, physicochemical parameters and GC/MS profiling of the fixed oil of Cucumis melo L seeds: A focus on anti-inflammatory, immunomodulatory, and antimicrobial activities. *Journal of Herbmed Pharmacology*, ***10*(4),** 476-485.

10- Elboraey, A. N., Abo-Almaged, H. H., El-Ashmawy, A. A. E. R., Abdou, A. R., Moussa, A. R., Emara, L. H.,El-Masry, M., H., El Bassyouni, E., G. & Ramzy, M. I. (2021). Biological and mechanical properties of denture base material as a vehicle for novel hydroxyapatite nanoparticles loaded with drug. *Advanced Pharmaceutical Bulletin*, ***11*(1)**, 86.

11- El-Menshawi, B. S., Fayad, W., Mahmoud, K., El-Hallouty, S. M., & El-Manawaty, M. (2010):

Screening of natural products for therapeutic activity against solid tumors.

12- Gahlout, M., Prajapati, H., Chauhan, P., Patel, N., & Solanki, D. (2017): Isolation and screening of pyocyanin producing Pseudomonas spp. from soil. *Int J Adv Res Biol Sci*, ***4*****(4)**, 147-152.

13- Goldufsky, J., Wood, S., Hajihossainlou, B., Rehman, T., Majdobeh, O., Kaufman, H. L., Ruby, E., C. & Shafikhani, S. H. (2015): *Pseudomonas aeruginosa* exotoxin T induces potent cytotoxicity against a variety of murine and human cancer cell lines. *Journal of medical microbiology*, ***64***(Pt 2), 164.

14- Jain, R., & Pandey, A. (2016): A phenazine-1-carboxylic acid producing polyextremophilic Pseudomonas chlororaphis (MCC2693) strain, isolated from mountain ecosystem, possesses biocontrol and plant growth promotion abilities. *Microbiological research*, ***190***, 63-71.

15- Karthik, Y., & Kalyani, M. I. (2023): Occurrence of *Streptomyces tauricus* in mangrove soil of Mangalore region in Dakshina Kannada as a source for antimicrobial peptide. *Journal of Basic Microbiology*, ***63*****(3-4),** 389-403.

16- Krishnaiah, M., de Almeida, N. R., Udumula, V., Song, Z., Chhonker, Y. S., Abdelmoaty, M. M., Nascimento, A., V., Murry, J., D. & Conda-Sheridan, M. (2018): Synthesis, biological evaluation, and metabolic stability of phenazine derivatives as antibacterial agents. *European Journal of Medicinal Chemistry*, ***143***, 936-947.

17- Kuo, C. Y., Weng, T. S., Kumar, K. S., Tseng, Y. H., Tung, T. W., Wang, S. Y., & Wang, H. C. (2019): Ethanol extracts of dietary herb, Alpinia nantoensis, exhibit anticancer potential in human breast cancer cells. *Integrative Cancer Therapies*, ***18*****,** 1534735419866924.

18- Leinberger J, Holste J, Bunk B, Freese HM, Spröer C, Dlugosch L, Kück AC, Schulz S, Brinkhoff T.(**Jun 2021**): High Potential for Secondary Metabolite Production of Paracoccus marcusii CP157, Isolated From the Crustacean Cancer pagurus. Front Microbiol. **28;12**:688754. doi: 10.3389/fmicb.2021.688754. PMID: 34262548; PMCID: PMC8273931.

19- Liandi, A. R., & Cahyana, A. H. (2022). Synthesis and antioxidant activity of benzo [a] pyrano [2, 3-c] phenazine derivative compound via one-pot multicomponent reaction. *Journal of Research in Pharmacy*, ***26*****(4)**, 954-961.

20- Lichtenberg, M., Jakobsen, T. H., Kühl, M., Kolpen, M., Jensen, P. Ø., & Bjarnsholt, T. (2022). The structure–function relationship of Pseudomonas aeruginosa in infections and its influence on the microenvironment. *FEMS microbiology reviews*, ***46*****(5)**, fuac018.

21-Liu, G. (2021): Antioxidant therapy and antioxidant-related bionanomaterials in diabetic wound healing. *Frontiers in Bioengineering and Biotechnology*, ***9***, 707479.

22- McFarland, J. (1907). The nephelometer: an instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines. *Journal of the American Medical Association*, ***49*****(14),** 1176-1178.

23-Mohamed, M. A., & Awad, H. M. (2021). New lipase-producing Streptomyces isolated from halo-alkaline habitat in Wadi El Natrun: polyphasic identification and statistical optimization of enzyme production. *Beni-Suef University Journal of Basic and Applied Sciences*, ***10*****(1),** 1-14.

24- Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods*, ***65*****(1-2),** 55-63.

25- Mustafa SM, Chua LS, El-Enshasy HA.(2019): Effects of Agitation Speed and Kinetic Studies on Probiotication of Pomegranate Juice with *Lactobacillus casei*. Molecules. Jun **26; 24(13):**2357. doi: 10.3390/molecules24132357. PMID: 31247970; PMCID: PMC6651325.

26 - Naik, S., Bouladoux, N., Linehan, J. L., Han, S. J., Harrison, O. J., Wilhelm, C., Conlan, S., Himmelfarb, S., Byrd, L., A., Deming, C., Quinones, M., Brenchley, M., J., Kong, H., H.,Tussiwand, R., Murphy, M., K., Merad, M., Segre, A., J. & Belkaid, Y. (2015): Commensal–dendritic-cell interaction specifies a unique protective skin immune signature. *Nature*, ***520*****(7545)**, 104-108.

27- Ramakrishna, A., & Ravishankar, G. A. (2011): Influence of abiotic stress signals on secondary metabolites in plants. Plant Signal Behav **6:** 1720–1731.

28- Shimada, K., Fujikawa, K., Yahara, K., & Nakamura, T. (1992): Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *Journal of agricultural and food chemistry*, ***40*****(6)**, 945-948.

29- Sultan, M., Nagieb, Z. A., El-Masry, H. M., & Taha, G. M. (2022): Physically-crosslinked hydroxyethyl cellulose-g-poly (acrylic acid-co-acrylamide)-Fe3+/silver nanoparticles for water disinfection and enhanced adsorption of basic methylene blue dye. *International journal of biological macromolecules*, ***196***, 180-193.

30- Tadijan Mitrović, Ivana & Grahovac, Jovana & Dodic, Jelena & Grahovac, Mila & Dodic, Sinisa & Vucurovic, Damjan & Vlajkov, Vanja. (2017). Effect of agitation rate on the production of antifungal metabolites by Streptomyces hygroscopicus in a lab-scale bioreactor. Acta Periodica Technologica. **48. 231-244.** 10.2298/APT1748231M.

31- Thabrew, M. I., Hughes, R. D., & McFarlane, I. G. (1997): Screening of hepatoprotective plant components using a HepG2 cell cytotoxicity assay. *Journal of pharmacy and pharmacology*, ***49*(11)**, 1132-1135.

32- Tumpa NI, Chowdhury MHU, Asma AA. (Apr 2023): Deciphering the antimicrobial, antibiofilm and membrane stabilizing synergism of *Mikania scandens* (L.) Willd. leaves and stems substantiation through in vitro and in silico studies. Biotechnol Rep (Amst). **16;38:**e00797. doi: 10.1016/j.btre.2023.e00797. PMID: 37124472; PMCID: PMC10131130.

33- VanderMolen, K. M., Raja, H. A., El-Elimat, T., & Oberlies, N. H. (2013): Evaluation of culture media for the production of secondary metabolites in a natural products screening program. *Amb Express*, *3*, 1-7.

34- Wood, S., Goldufsky, J., & Shafikhani, S. H. (2015): *Pseudomonas aeruginosa* ExoT induces atypical anoikis apoptosis in target host cells by transforming Crk adaptor protein into a cytotoxin. *PLoS pathogens*, ***11*(5),** e1004934.

35- Yan, S., Yue, Y., Zeng, L., Su, L., Hao, M., Zhang, W., & Wang, X. (2021): Preparation of graphene oxide-embedded hydrogel as a novel sensor platform for antioxidant activity evaluation of Scutellaria baicalensis. *Frontiers in Chemistry*, ***9*,** 675346.

36- Zhang, W., Chen, L., Xiong, Y., Panayi, A. C., Abududilibaier, A., Hu, Y., Yu, C., Zhou, W., Sun, Y., Liu, M., Xue, H., Hu, L., Yan, C., Xie, X., Lin, Z., Cao, F., Mi, B. & Liu, G. (2021): Antioxidant therapy and antioxidant-related bionanomaterials in diabetic wound healing. *Frontiers in Bioengineering and Biotechnology*, ***9***, 707479.