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Characterization and Biological Evaluation of Phenazin-1-Caboxylic Acid Produced by Locally Egyptian Isolate *Pseudomonas aeruginosa* OQ158909

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ABSTRACT

In the present study, the crude secondary metabolites, previously extracted from the fermentation medium of the locally isolated *Pseudomonas aeruginosa* OQ158909 strain were subjected to silica gel column chromatography using optimized solvent systems. The most biologically active fraction was further purified via high-performance liquid chromatography (HPLC) and identified as phenazine-1-carboxylic acid (PCA) through a comprehensive analytical approach involved ultraviolet (UV) spectroscopy, Fourier transform infrared (FTIR) spectroscopy, mass spectrometry (MS), and proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. The biological activities of the purified PCA were evaluated, revealing a potent antimicrobial effect against a diverse array of human pathogens. The inhibition zones recorded against Gram-positive bacteria ranged from 16 to 28 mm, while those against Gram-negative bacteria ranged from 13 to 36 mm. PCA also exhibited antifungal activity, with inhibition zones of 16 mm and 17 mm against *Aspergillus niger* and *Candida albicans*, respectively. Furthermore, PCA demonstrated significant antioxidant activity, scavenging 75.2% of DPPH free radicals at a concentration of 100 µg/mL, with an IC_{50} value of 40.4 µg/mL. This efficacy surpassed that of butylated hydroxyanisole (BHA) and vitamin C, which served as standard positive controls in the antioxidant assays.In addition, PCA exhibited pronounced anti-inflammatory effects. Treatment with 100 µg/mL of PCA resulted in an 82.54% reduction in lipopolysaccharide (LPS)-induced nitric oxide (NO) production and nearly complete suppression of inducible nitric oxide synthase (iNOS) expression. PCA also displayed significant cytotoxic effects against various human cancer cell lines. The highest efficacy was detected against the HePG2 cell line, with an IC_{50} of 45.5 µg/mL and a selectivity index exceeding 2. Moreover, 100 µg/mL of PCA inhibited 74% of PACA cells and 50% of PC3 and MCF7 cells. Lastly, PCA showed remarkable wound healing potential, achieving nearly complete wound closure at a concentration of 100 µg/mL within 24 hours.

Keywords: Antimicrobial activity; Antioxidant activity; Anti-inflammatory effect; Natural compounds; Phenazine-1-caboxylic acid; *Pseudomonas aeruginosa*; Secondary metabolites.

INTRODUCTION

The exploration of microorganisms as sources of therapeutically useful compounds, specifically bioactive secondary metabolites, for use in human medicine, is currently imperative (Vicente *et al.*, 2003; Liu *et al*., 2010; Kato *et al*., 2012). These metabolites have proven to be rich and renewable source of new human drugs. Despite the identification of thousands of microbial secondary metabolites, they remain a virtually inexhaustible reservoir for the discovery of novel antimicrobials, antivirals, antitumor agents, and agricultural compounds. Furthermore, numerous secondary metabolites such as benzylpenicillin, cephalosporins, and erythromycin, serve as precursor structures for the development of synthetic and semi-synthetic derivatives with enhanced pharma-cological properties (Vicente *et al.*, 2003). Among these secondary metabolites, phenazines represent a significant group of pigmented molecules that encompasses over 150 naturally occurring and synthesized nitrogen-containing aromatic compounds. These compounds are exclusively produced by bacteria and play significant roles in the ecological fitness and pathogenicity of the bacterial strains that synthesize them (Turner and Messenger, 1986; Blankenfeldt and Parsons, 2014; Cha *et al*., 2015). The phenazine parent compound (Figure 1) is a dibenzopyrazine with the molecular structure of $C_{12}H_8N_2$ (Yu *et al*., 2018). In contrast, phenazine-1-carboxylic acid (PCA) is a phenazine derivative where the hydrogen at the C-1 position is replaced by a carboxylic group (Figure 2). Phenazine natural prod-ucts are a class of nitrogen-containing heterocyclic compounds produced by microorganisms. PCA is produced by various species within the orders Pseudomonadales and Streptomycetales. Recently, it has been extensively utilized in agricultural applications due to its potent inhibitory effects against several soil-borne fungal phytopathogens (Huang *et al*., 2024; Deng *et a*l., 2024; Niu *et al*., 2017; Xiong *et al*., 2017). PCA has been registered in China as a certified biofungicide under the trade name "Shenqinbactin," recognized for its high fungicidal efficiency, low toxicity to humans and animals, environmental friendliness, and ability to improve crop production (Xiong *et al*., 2017).

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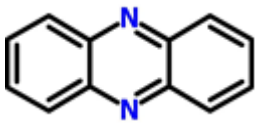

Figure (1): Phenazine compound (PHZ), (Yu *et al*.,2018)

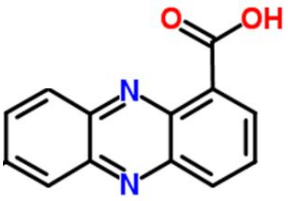

Figure (2): Phenazine-1- carboxylic acid (PCA), (Yu *et al*., 2018)

PCA and its analogues have demonstrated significant antifungal activities. For instance, phenazine-1-carboxylic hydrazine, an analogue of PCA, exhibits potent fungicidal activities against pathogens such as *Botrytis cinerea*, powdery mildew, *Rhizoctonia solani*, *Fusarium oxysporum*, *Phytophthora capsici*, and *Sclerotinia sclerotiorum* (Xiong *et al*., 2017). Additionally, phenazine-1-carboxylic esters have shown excellent fungicidal activities against *Magnaporthe oryzae* (Niu *et al*., 2017; Xiong *et al*., 2017). PCA itself is active against several soil-borne fungal pathogens, including *Gaeumannomyces graminis*, *Phytophthora capsici*, and *Colletotrichum orbiculare* (He *et al*., 2008; Yu *et al*., 2018). Furthermore, PCA has been identified as an antifungal compound with high activity and has been applied as a plant growth promoter and germination booster (Trung *et al*., 2020; Mahdi *et al*., 2023). Additionally, PCA exhibits antioxidant and anti-inflammatory effects, which can be harnessed for therapeutic applications in treating various diseases and promoting wound healing. The growing interest in PCA has led researchers to explore diverse microbial sources capable of producing this compound. The ability to isolate and characterize strains that synthesize PCA can enhance our understanding of its biosynthesis and expand its availability for industrial and medical applications. Therefore, the aim of this study is to purify and characterize phenazine-1-carboxylic acid (PCA) from the crude extract of secondary metabolites produced by the locally isolated *Pseudomonas aeruginosa* OQ158909 strain. This will be achieved using silica gel column chromatography, thin-layer chromatography (TLC), high-performance liquid chromategraphy (HPLC), and identification techniques including UV spectroscopy, Fourier-transform infrared spectroscopy (FTIR), mass spectrometry (MS), and proton nuclear magnetic resonance (^{1}H-NMR). Additionally, the study will evaluate the biological activities of the purified PCA, focusing on its anti-microbial, antioxidant, anti-inflammatory, cytotoxic, and wound healing effects, thereby assessing its potential applications in medicine and other relevant fields.

MATERIALS AND METHODS

The tested strains were obtained as courtesy of the Biological Assessment of Natural and Microbial Products Unit, Lab. 174, Chemistry of natural and microbial products department, National Research Centre, Dulbecco's modified Eagle's medium (DMEM, Corning, USA).

Partial purification of phenazine compound using silica gel column chromatography

The secondary metabolites of the *Pseudomonas aeruginosa* strain OQ158909, grown in a specific medium, were extracted using ethyl acetate. The purification of the compounds present in the ethyl acetate extract was performed via silica gel column chromatography. A glass column measuring 4.5 cm in diameter and 65 cm in height was packed with 50.0 g of silica gel (Silica gel 60, 0.063-0.200 mm, 70-230 mesh ASTM, Merck, Germany) suspended in n-hexane to a height of 45 cm and was subsequently washed with 100.0 mL of n-hexane. The concentrated ethyl acetate extract (3.0 g) was homogeneously mixed with 3.0 g of the silica gel suspension to prepare metabolite-silica gel slurry, which was air-dried before being loaded into the column. The column was eluted with 600.0 mL of n-hexane, followed by the same volume of three solvent systems in different ratios of n-hexane to ethyl acetate as follows: S1: n-hexane/ethyl acetate 3:2 (v/v); S2: n-hexane/ethyl acetate 3:3 (v/v); S3:n-hexane/ethyl acetate 2:3 (v/v). Finally, the column was eluted with 600 mL of ethyl acetate. Thirteen fractions of 200.0 mL each were successively collected. Samples from each fraction were then spotted on TLC plates and eluted using ethyl acetate/n-hexane 3:2 (v/v) as a solvent system. Separated spots, with similar Rf values, were pooled and tested for their antimicrobial activities against the different, human pathogenic bacteria and fungi using disc diffusion method (Kennedy *et al*., 2015; Radwan *et al*., 2021).

The human pathogens, listed in Table (1), were obtained courtesy of the Biological Assessment of Natural and Microbial Products Unit, Lab 174, Department of Chemistry of Natural and Microbial Products, National Research Centre. The pathogens were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Corning, USA).

Purification and identification of the biologically active phenazine compound

High Performance Liquid Chromatography (HPLC)

The selected BCD sub-fraction was filtered through a 0.2 µm syringe filter (Millipore) and further purified using preparative HPLC (Yong Lin, Korea) with an Agilent-ZORBAX RX-SiL column (250 × 9.4 mm). The HPLC profiles were monitored at 254 nm and 280 nm using a UV/Vis detector (YL9120, Yong Lin). The solvent system used for elution was ethyl acetate/n-hexane (1:1, v/v) at a flow rate of 4.0 mL/min, operated via an isocratic pump (YL9112, Yong Lin). Fractions were collected based on their retention times. The antimicrobial activities of the purified fractions were subsequently tested against the selected human pathogens (Table 1).

Characterization of the pure compound

Physical characterization

The solubility of the pure isolated crystals was indiv-

idually tested using different solvents including water, chloroform, methanol, ethyl acetate, n-hexane, dimethyl sulfoxide (DMSO) and dichloromethane. The solubility of the pure crystals was recorded for each solvent.

Table (1): Pathogenic bacterial and fungal strains used in antimicrobial evaluation assay.

Pathogenic test strains	ATCC Number
Bacillus subitilis	6633
Enterococcus faecalis	19433
Staphylococcus aureus	25923
Staphylococcus epidermidis	12228
Escherichia coli	25922
Helicobacter pylori	43504
Klebsiella pneumonia	10031
Candida albicans	10231
Aspergillus niger	16888

Chemical characterization

The crystals, dissolved in chloroform, were subjected to different chemical characterization methods, including ultraviolet-visible (UV-vis) spectrophotometry scanning, Fourier transform infrared (FTIR) spectroscopy, mass spectroscopy (MS) and nuclear magnetic resonance (NMR) analyses. All the characterization techniques were performed following the methods reported by Kennedy *et al*., (2015).

Ultraviolet spectrophotometry scanning

The purified crystals (10.0 mg) were dissolved in 20.0 mL of ethyl acetate and subjected to UV-Vis spectrophotometer (JASCO V-730 spectrophotometer, Japan) over the wavelength range of 200 - 500 nm. The maximum absorption peaks were recorded and compared with reference results.

Fourier transforms infrared (FTIR) spectroscopy

FTIR (Perkin Elmer, USA) was performed in order to identify the functional groups and hence the chemical structure of the pure compound. Prior to the analysis, a sample of the bioactive crystalline compound was mixed with potassium bromide (KBr) and compressed using hand press to acquire a tablet (Amer and Awad, 2020). The tablet was then dried under vacuum in a vacuum oven, overnight at 60 °C, to remove any moisture or excess solvent before the measurement. The spectrum was obtained using the absorbance mood of analysis over the wavelength range of 400 to 4000 cm^{-1}, acco-rding to the method reported by Kennedy *et al*., (2015).

Mass spectroscopy analysis

Mass spectrometry is an analytical chemistry technique used to elucidate the chemical structures of bioactive compounds. This method involves the ionization of the compound to be analyzed, generating charged molecules or molecular fragments, and measuring their mass-to-charge ratios. For this experiment, the pure sample was dissolved in a 5 mm sample tube using dimethyl sulfoxide (DMSO) as the solvent. The mass spectrometric analysis was performed on an appropriate mass spectrometer, such as a JEOL JMS-700 or similar model, although the specific model should be accurately identified.

Nuclear magnetic resonance (1H-NMR) analysis

Nuclear magnetic resonance analysis of the pure bioactive crystalline compound was performed using the typical analysis conditions for a 1H-NMR spectrometer (JEOL EX- 400 MHz, Japan) with a spectral width of 400 MHz in order to determine the presence of the characteristic 1H-Nuclear magnetic resonance signals and compared with reference results.

In Vitro evaluation the biological activity of Phenazine-1-Carboxylic Acid (PCA)"

Antimicrobial activity of PCA

The antimicrobial activity of phenazine-1-carboxylic acid (PCA) was evaluated against selected human pathogenic pathogens (Table 1) using the well diffusion method (El-Anssary *et al*., 2021). To prepare the inocula, fresh overnight broth cultures of the test microorganisms were cultivated in nutrient broth medium and incubated at 37°C for 24 hours. Each pathogenic strain's broth was adjusted to approximately 0.5 McFarland standard (1.5×10^8 CFU/mL). An inoculum size of 25 µL was used for each tested pathogenic strain to inoculate 20 mL of sterile nutrient agar medium plates. After solidification, wells with an internal diameter of 0.9 cm were created in the nutrient agar medium layer. The antimicrobial activities of the PCA samples were assessed by adding 100 µL aliquots of the PCA solution in DMSO (50 mg/mL) into each well. Following a one-hour refrigeration period to enhance diffusion, the Petri dishes were incubated at 37°C for 24 hrs. Finally, the zones of inhibition (ZI) were measured in millimeters (Elboraey *et al*., 2021).

Antioxidant activity of PCA

The free radical scavenging activity of the phenazine-1-carboxylic acid was measured using 1, 1-diphenyl-2-picryl-hydrazil (DPPH) according to the method of Shimada *et al*. (1992). The phenazine-1-carboxylic acid was tested in different concentrations ranging from 100 µg/mL, as a starting concentration, diluted to reach 25 µg/mL, in order to detect the IC50, which is the most potent active concentration able to scavenge 50 % of the DPPH free radicals. Briefly, 0.1 mM solution of DPPH in methanol was prepared. Then, 1 mL of this solution was transferred to different test tubes to which different concentrations of the phenazine-1-carboxylic acid (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 mins. 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:

$$A\ (\%) = [(A0-A1)/A0]\ X100$$

Where, A0 is the absorbance of the control (blank) sample; A1 is the absorbance of the tested phenazine-

1-carboxylic acid sample and A (%) represents the percentage of antioxidant activity.

Anti-inflammatory effect of PCA

The inoculation of Dulbecco's modified Eagle's medium (DMEM, Corning, USA) with murine macrophage RAW264.7 cells (ATCC®) was incubated under anaerobic conditions in 5 % CO2 incubator. The cells were washed with phosphate buffer after that, 96-well microtiter plastic plates were inoculated with 0.5 x 106 cells/mL of RAW 264.7 cell and incubated overnight. Then, the non-induced triplicate wells were filled with the medium containing the sample vehicle (DMSO, 0.1% v/v). The inflammatory group of triplicate wells in full culture media with 0.1% DMSO; v/v was administered with 100 ng/mL of Lipopolysaccharide (LPS). Different concentrations of Phenazine-1-carboxylic acid sample (6.25, 12.5, 25, 50 & 100 µg/mL) dissolved in DMSO and diluted in culture media containing lipopolysaccharide (LPS) (Final concentration of DMSO=0.1%, v/v) was used in groups of triplicate amounts. Caffeic acid phenecyl ester (CAPE, 5.0 µM) was used as an anti-inflammatory positive control. By using Griess assay to determine the NO in all tested wells after incubation for 24.0 hours (Eloutify *et al*., 2023). Equal volumes of culture supernatants and Griess reagent were mixed well and incubated at room temperature for 10 minutes to form the colored diazonium salt which absorbance was detected at 540 nm using a Tecan Sunrise™ microplate reader (Austria). The % of NO inhibited by the Phenazine-1-carboxylic acid sample was determined using the Alamar Blue™ reduction assay, then calculated relative to the LPS-induced inflammation group normalized to cell viability. The concentration that inhibits 50 % of LPS-induced NO release was statistically calculated on GraphPad Prism software using curve fit to non-linear regression analysis. The Phenazine-1-carboxylic acid sample was then subjected to the Western blotting test to detect the inhibition of the inflammation marker protein expression (iNOS).

Cytotoxic activity of PCA

Cell viability was evaluated using the MTT assay, based on the mitochondrial-dependent reduction of yellow MTT to purple formazan, as described by Wood *et al*. (2015). All procedures were conducted under aseptic conditions in a biosafety level II laminar flow cabinet (Baker, SG403INT). Cells were grown in DMEM-F12 medium supplemented with 1% antibiotic-antimycotic mixture and 1% L-glutamine. Cultures were incubated at 37°C under 5% CO_2.

Cells were seeded at 10^4 cells/well in 96-well plates and incubated for 24 hrs. They were then treated with phenazine-1-carboxylic acid (PCA) at different concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56, or 0.78 µg/mL. After 48 hrs, MTT was added, and plates were incubated for an additional 4 hrs. The reaction was stopped by adding 10% SDS, and absorbance was measured at 595 nm using a microplate reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA). Doxorubicin served as a positive control. Statistical analysis was performed using an independent t-test in SPSS 11. DMSO solvent was used to dissolve PCA 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:

$$V\% = \frac{absorption of\ PCA}{(absorption\ of\ negative\ control - 1)} X\ 100$$

The IC_{50} and IC_{90} values were also calculated using Probit analysis in SPSS 11. Selectivity index (SI) was calculated as IC_{50} against normal cells / IC_{50} against cancer cells.

Wound healing activity of PCA

The wound healing activity of pure phenazine-1-carboxylic acid (PCA) was evaluated using the *in vitro* scratch assay. Human dermal fibroblast adult cells (BJ.1) were obtained from the National Research Center (NRC), Cairo, Egypt, and cultured in a humidified incubator at 37°C with 5% CO2. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; GIBCO, USA) supplemented with 1% Penicillin/Streptomycin (PS; GIBCO, USA) and 10% fetal bovine serum (FBS; GIBCO, USA) in T-75 flasks until 90% confluence before subculturing.

For the assay, BJ.1 cells were seeded at a density of 1×10^5 cells/mL in 24-well plates and allowed to reach confluence over 24 hours. A small linear scratch was created along the confluent monolayer using a sterile P200 pipette tip (Harishkumar *et al*., 2013). Cells were rinsed with PBS to remove debris before being treated with PCA at a concentration of 100 µg/mL. Meanwhile, images of the outcome results were captured using a digital camera connected to an inverted microscope (Olympus, Japan) at ×40 magnification (scale bar = 500 µm) at 0 hours before treatment and after 24 hrs. Image analysis was performed using Image J software (NIH, Bethesda, MD).

The distance travelled by cells from the wound edge to the wound area was calculated and compared to control plates without PCA treatment. An increase in the percentage of the closed area indicated enhanced cell migration. Alternatively, wound closure rate can be expressed as the percentage of area reduction over time, calculated as follows:

$$Wound\ Closure\ Rate = \left(1 - \frac{At = \Delta h}{At = 0h}\right) X100$$

Where, At=0h is the area of the wound measured immediately after scratching (t=0 h), At=Δh is the area of the wound measured hrs after the scratch is performed.

Statistical analysis

Statistical analyses were performed to evaluate the significance of the results obtained. For antimicrobial, antioxidant and anti-inflammatory activities, ANOVA test was performed followed by post hoc t-tests. In the MTT assay measuring cell viability, an independent t-test was utilized in SPSS version 11 to compare treated samples with the negative control group. The IC_{50} and IC_{90} values were calculated using Probit analysis to evaluate the cytotoxic effects of phenazine-1-

carboxylic acid (PCA) on both cancerous and normal cell lines. In the wound healing assay, the percentage of wound closure was determined and compared between treated and control groups using one-way ANOVA followed by post hoc t-tests. Statistical significance was established at a *p*-value of ≤ 0.05 for all analyses. Data are presented as means ± standard error (SE).

RESULTS

Partial purification of the ethyl acetate extract

Fractionation of the concentrated ethyl acetate extract using silica gel open column chromatography (Figure 3) yielded thirteen sub-fractions: A, B, C, D, E, F, G, H, I, J, K, L, and M. Thin-layer chromatography (TLC) analysis for all fractions revealed that sub-fractions B, C, and D, detected under UV light, had similar Rf values (Figure 4A-B). In mean time the biological activities of the separated sub-fractions (A-M) were evaluated against most common human pathogens which showed similar results (Figure 5A-B). These sub-fractions were combined and coded as BCD for further purification using HPLC analysis.

Purification of phenazine sub-fraction BCD using preparative HPLC-analysis

Sub-fraction BCD, which was selected as the most potent antimicrobial active phenazine compound, was mixed and subjected to further isolation and purifyc-ation using preparative HPLC under the standard analysis conditions mentioned in the material and method section. The preparative HPLC-analysis Isola-tion chart (Figure 6) showed different peaks eluted at different retention times. All 4 eluted sub-fractions were recuperated and tested for purity by TLC profiling and visualized under UV light as well as by their spraying with the anisaldehyde - sulfuric acid coloring reagent. The antimicrobial activities, of each of these fractions, were confirmed.

Characterization of the pure compound

The BCD sub-fraction was retrieved and subjected to different spectroscopic techniques, including UV, FTIR, MS and ^{1}H-NMR, for the structure elucidation of the pure compound.

Ultraviolet spectrophotometer scanning analysis

The UV-spectrophotometric scanning analysis of the purified compound revealed distinct absorbance peaks at 258 nm and 362 nm, indicating characteristic electronic transitions within the molecular structure (Figure 7). These absorption maxima suggest the presence of conjugated π-electron systems, typical of phenazine derivatives. Furthermore, mass spectrum-etric (MS) analysis of phenazine-1-carboxylic acid (PCA) exhibited a prominent molecular ion peak at m/z 224.85, corresponding to its molecular weight. The high intensity of this peak suggests the stability and predominance of the ion in the spectrum. Additionally, the structural representation of PCA confirms its molecular framework, including the phenazine core and carboxyl functional group. The 3D molecular model further illustrates the spatial arrangement of atoms, offering insights into the compound's electronic distribution and steric properties. These results strongly support the structural identification and molecular characterization of PCA using UV-spectrophotometry and mass spectrometry, reinforcing its chemical identity and purity.

Fourier transforms infrared (FTIR) spectroscopy

The infrared spectrum of the purified compound (Table 2 and Figure 8) showed three major absorption bands. The first band was detected at the wavelength of 1421 cm^{-1} and represented the stretching vibration of the C=C bond of the aromatic ring. The second band was detected at the wavelength of 1436 cm^{-1} and representing the stretching vibration of the C=N bond of the aromatic ring. The third base peak was detected at the wavelength of 1616.06 cm^{-1} and was charac-teristic for the C=O bond of the carboxylic acid group.

Mass spectrometer analysis

In order to determine the molecular weight of the purified compound, the MS-Spectroscopy analysis was

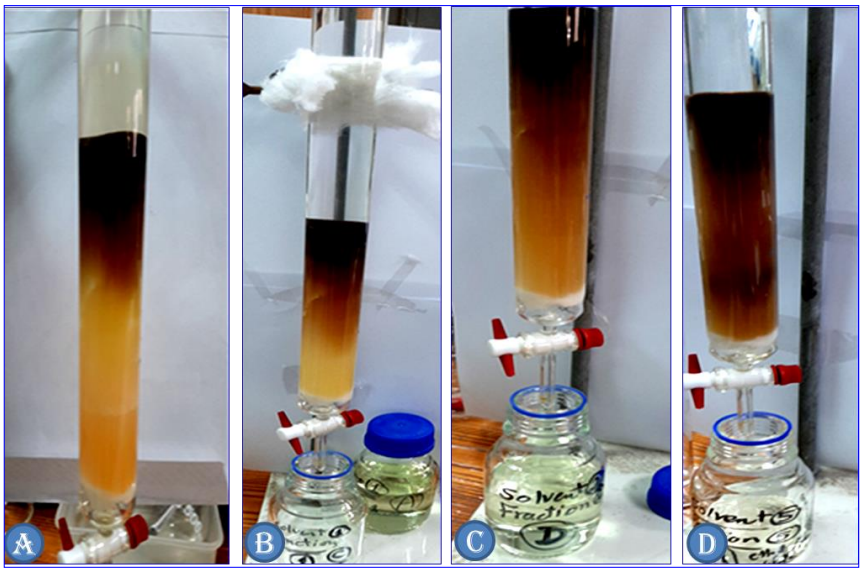

Figure (3): Fractionation and Purification of ethyl acetate extract using open column chromatography.

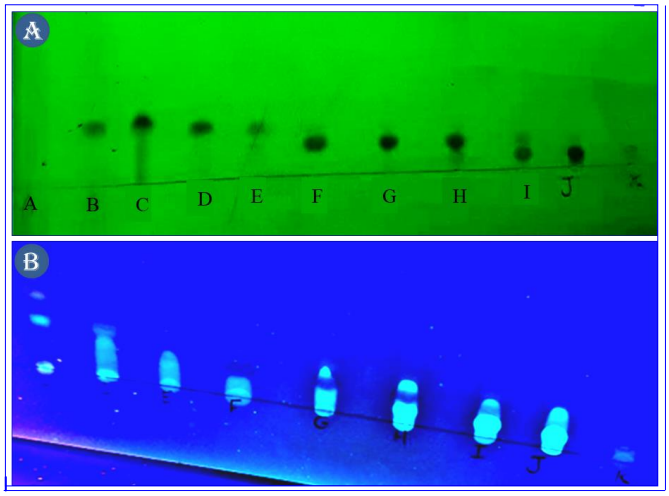

Figure (4): TLC-profile of ethyl acetate sub-fractions. A, spot virtualization was done using short range UV lamp; B, spot virtualization was done using long range UV lamp.

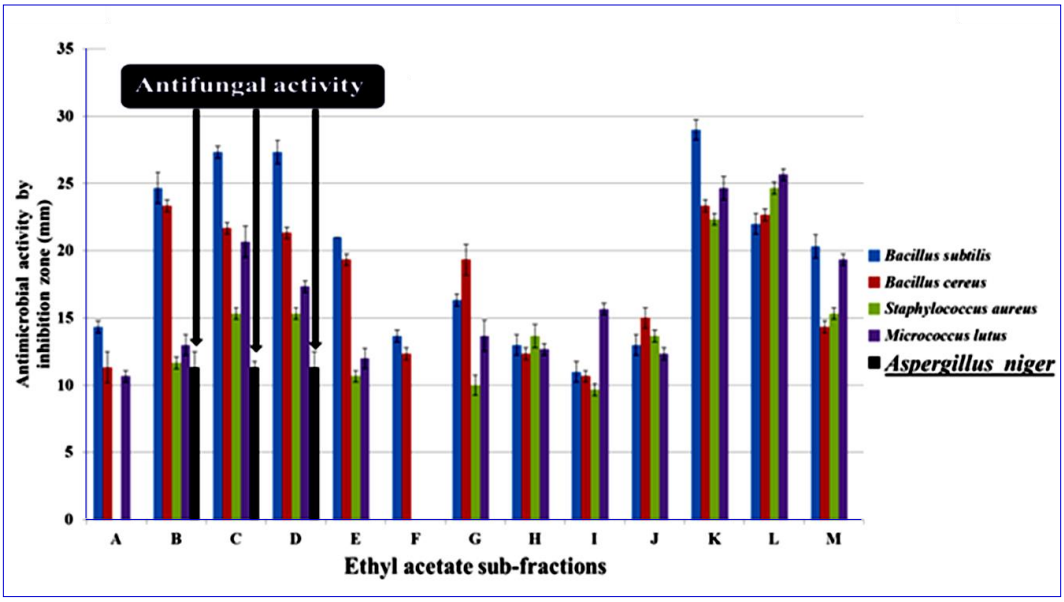

Figure (5A): Antimicrobial activities of ethyl acetate sub-fractions against most common human pathogenic microorganisms, data are represented in means ±SE.

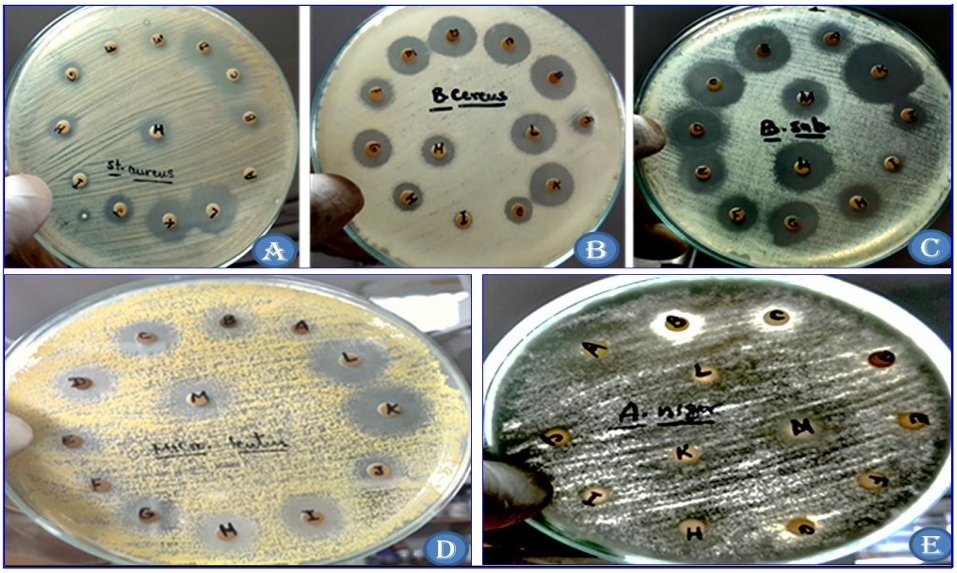

Figure (5B): Antimicrobial activities of ethyl acetate sub-fractions against most common human pathogens using agar plates disc diffusion method. The pathogens include: A, *S. aureus*; B, *B. cereus*; C, B. *subtilis;* D, *Micrococcus luteus* and E, *A. niger.*

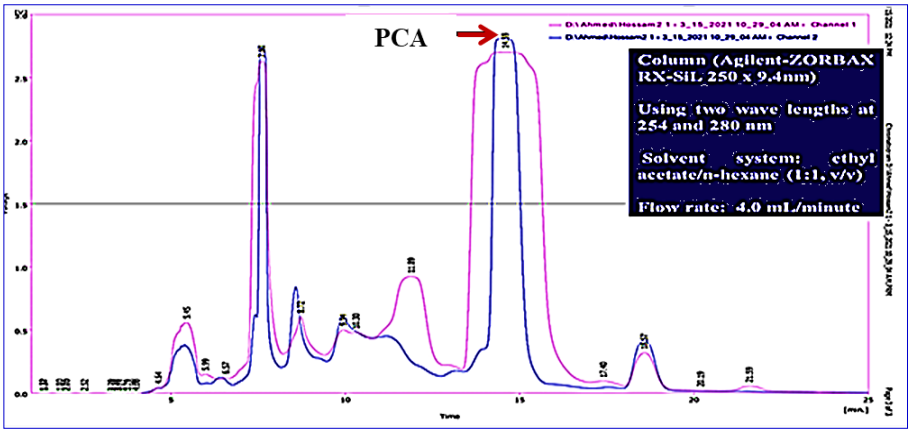

Figure (6): HPLC profile of acetyl acetate fraction: Chromatographic separation and detection of Phenazin-1-Caboxylic Acid (PCA).

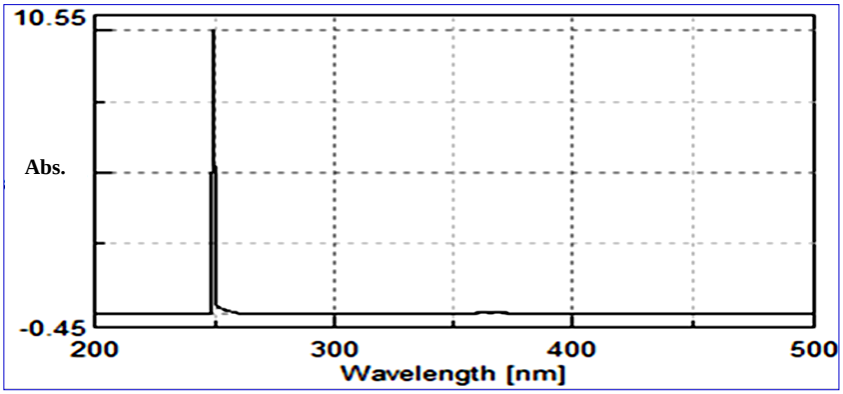

Figure (7): Characterization of isolated purified PCA compound using UV-Visible Spectroscopy.

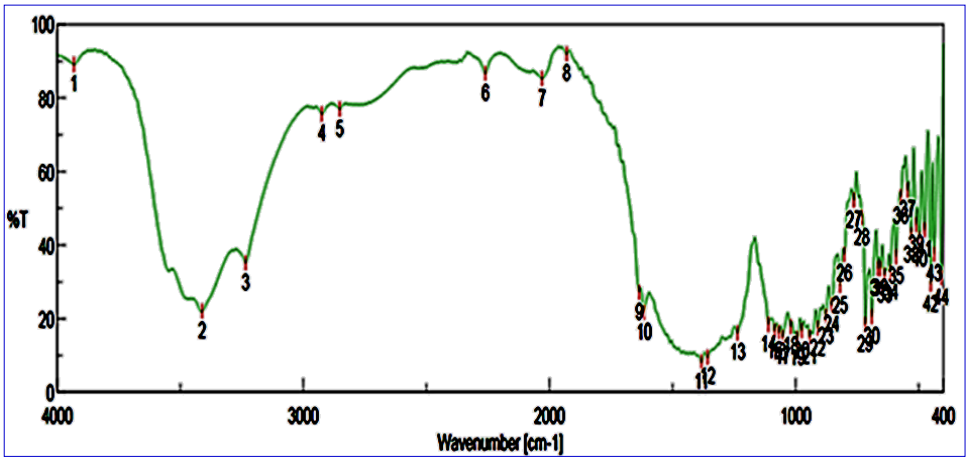

Figure (8): Fourier Transform Infrared (FTIR) spectral analysis of purified PCA: Molecular fingerprinting of structural and functional groups.

performed. The obtained low-resolution ESI-MS spectrum, illustrated in figure (9), showed a molecular ion peak (M+H) at *m/z* 224.85 which represents the MW of the studied compound.

Proton Nuclear Magnetic Resonance (^{1}H-NMR)

The Proton nuclear magnetic resonance (CDCl3, 400 MHz,) results, illustrated in figure (10) and table (3) revealed the presence of eight protons as follow: one proton singlet at δ 15.58, characteristic for the carboxylic acid proton, and seven methine protons at δ 8.97 (dd, J = 7.2, 1.2 Hz, 1H, H-2), δ 8.53 (dd, J = 8.8,1.2 Hz, 1H, H-4), δ 8.28 (dd, J = 8.0, 1.6 Hz, 1H, H-3), δ 8.34(dd, J = 8.0, 1.6 Hz, 1H, H-8)and δ 8.05–7.95 (m, 3H, H-6, H-7 and H-9). This detailed analysis aids in understanding the compound's structural features and connectivity which prove that the compound is Phenazin-1-Caboxylic Acid (PCA).

Biological activities of PCA

Antimicrobial activity of PCA

The purified PCA exhibited outstanding broad-spectrum activity against a wide range of human pathogens either Gram-positive or Gram-negative bacteria, in addition to pathogenic fungal strains known for their ability to resist common antimicrobial drug. The recorded results (Figures 11 and 12) showed that the inhibition zones against several Gram-positive patho-gens ranged from 16 to 25 mm.

An inhibition zone of 16 mm was observed against *Bacillus subtilis*, a bacterium responsible for various gastroenteritis-related illnesses, including vomiting, nausea, intestinal irritation, and bowel infections. The compound also exhibited excellent antibacterial activity against both *Staphylococcus epidermidis* and *Staphylococcus aureus*, with inhibition zones of 23 mm and 25 mm, respectively. These strains are highly antibiotic - resistant pathogens that cause numerous hospital-acquired infections, including wound infections, abscesses, pulmonary infections, and bacteraemia.

Table (2): Fourier Transform Infrared (FTIR) Spectroscopic analysis of purified PCA: Identification of key functional groups (-C=O, -C=N, -C=C) using 0.5% potassium bromide tablets.

Functional groups	FTIR Spectra of purified compound (PCA)
-C=O	1616.06
-C=N	1436
-C=C	1421

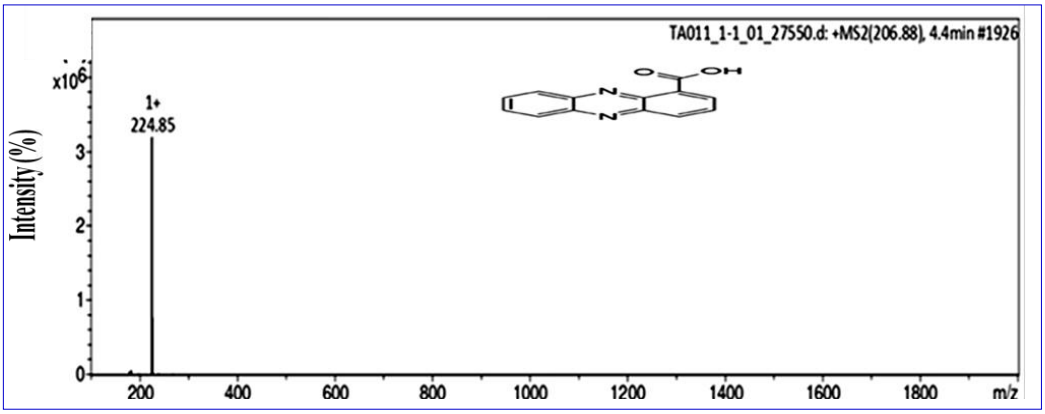

Figure (9): Electrospray mass ionization chromatogram of the purified compound. Mass Spectrometric analysis of purified PCA: Molecular ion peak identification and structural confirmation.

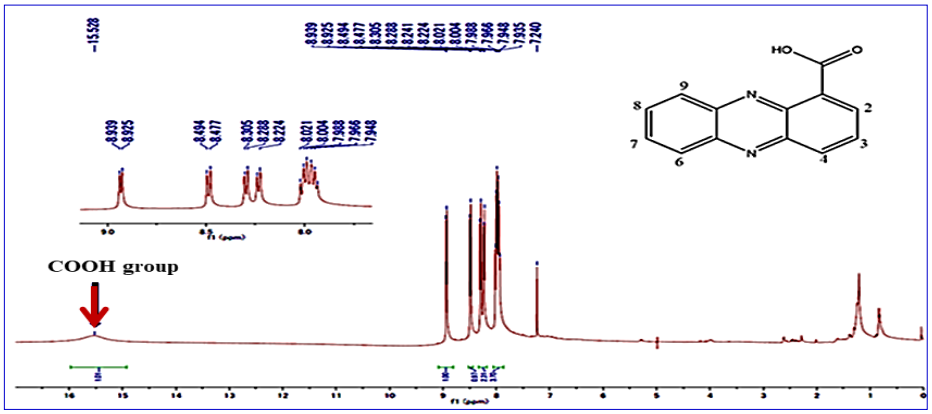

Figure (10): ^{1}H-Nuclear magnetic resonance chromatogram of the purified compound.

Table (3): Proton nuclear magnetic resonance (^{1}H-NMR) signals of purified PCA: Chemical characterization.

Proton	δH (ppm)	Multiplicity (J in Hz)
H-2	8.97	dd (*J* = 7.2, 1.2)
H-3	8.28	dd (*J* = 8.0, 1.6)
H-4	8.53	dd (*J* = 8.8, 1.2)
H-6	8.05–7.95	m
H-7	8.05–7.95	m
H-8	8.34	dd (*J* = 8.0, 1.6)
H-9	8.05–7.95	m
COOH	15.58	s

δH (ppm): Chemical shift in parts per million (ppm). Multiplicity:s = singlet, d = doublet, dd = doublet of Doublets,m = multiplet. J (Hz): Coupling constant in Hertz (Hz).

However, great antibacterial effects of this compound were also recorded against Gram-negative bacteria with inhibition zones ranging between 13-36 mm. The latter inhibition zone was obtained against *Helicobacter pylori* which is the main cause of stomach ulcers that mainly develop into stomach cancer. The PCA compound was also able to significantly inhibit the growth of *K. pneumonia* which is responsible for sever diseases such as *pneumonia*. Moreover, the recorded results showed that the PCA has a potent fungicidal effect against both tested human pathogenic fungal strains *Aspergillus niger* and *Candida albicans* with distinctive inhibition zones of 16 and 17 mm respectively. (To our knowledge, until now, almost all the published reports only recorded the antimicrobial effect of PCA on phytopathogenic fungi (Mavrodi *et al*., 2013, Jain and Pandey, 2016 and Devnath *et al*., 2017), however, these results exclusively proved the

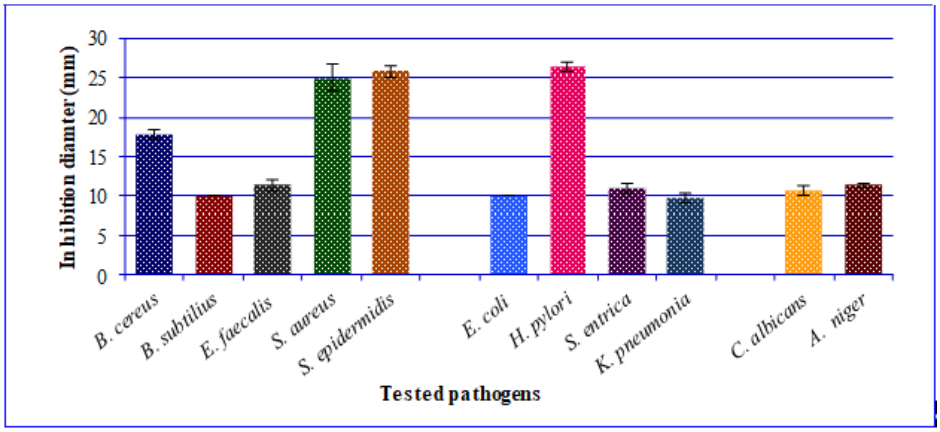

Figure (11): Antimicrobial activities of PCA against different human pathogens, Data are represented in means ±SE.

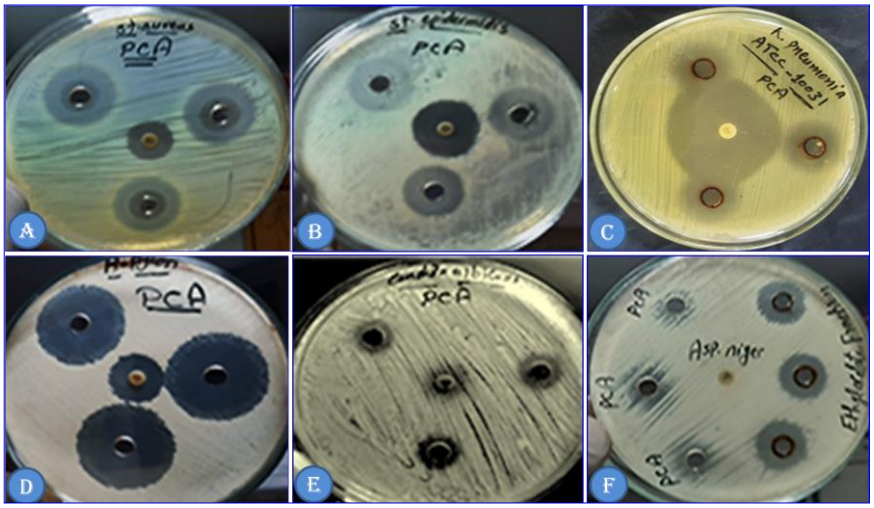

Figure (12): Antimicrobial activities of PCA using well diffusion method on agar plates against different human pathogens, in comparison to standard Gentamicin control discs (central position).

high antimicrobial activity of PCA against many human pathogens and therefore consequently confirmed that this compound can be applied for the treatment of many human diseases in addition to its current effective uses in biological crop control.

Antioxidant activity of PCA

PCA exhibited strong antioxidant activity, as 100 µg/mL of the purified compound effectively scavenged 75.2% of DPPH free radicals in solution. The recorded IC_{50} value of 40.4 µg/mL indicates potent antioxidant potential, particularly when compared to the significantly higher IC_{50} values of 53.9 ± 3.1 µg/mL and 42.80 ± 1.5 µg/mL observed for butylated hydroxyl-anisole (BHA) and vitamin C, respectively, which were used as standard positive controls (Figure 13). Conspicuously, this finding is of considerable significance, as, to the best of our knowledge, no prior studies have reported on the antioxidant properties of PCA.

Cytotoxic activity of PCA

PCA exhibited significant cytotoxic effects on tested cancer cell lines (Table 4). Interestingly, PCA demonstrated the lowest IC_{50} value of 45.5 µg/mL against the HePG2 cancer cell line, with a selectivity index (SI) of approximately 2.2. However, at a concentration of 100 µg/mL, PCA inhibited approximately 74% of PACA cells and around 50% of both PC3 and MCF7 cancer cell lines. These findings suggest that PCA has potential as a cytotoxic agent, rather than a hormonal one since its mode of action does not involve hormonal pathways but rather direct cytotoxic effects on cancer cells. This is evidenced by its ability to inhibit cell as indicated by its ability to inhibit cell viability in various cancer cell lines (e.g., HePG2, MCF7, PACA2, and

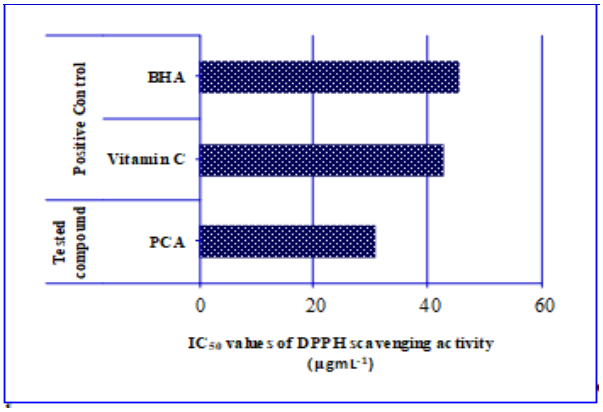

Figure (13): Evaluation of the antioxidant activity of phenazine-1-carboxylic acid (PCA) based on IC50 values for DPPH scavenging, in comparison to Vitamin C and Butylated hydroxyanisole (BHA).

PC3). Therefore, PCA is more accurately described as a cytotoxic agent rather than a hormonal one.

Anti-inflammatory activity of PCA

The results of the anti-inflammatory effect of PCA on murine macrophages (Figure 14) demonstrated a strong, concentration-dependent inhibition of lipopolysaccharide (LPS)-induced nitric oxide (NO) release in RAW264.7 macrophages. Remarkably, treatment with 100 µg/mL of PCA effectively reduced NO production by 82.54%, compared to the standard positive control, caffeic acid phenethyl ester (CAPE, 5 µM), which exhibited an inhibition percentage of 82.09%.

Furthermore, the half-maximal inhibitory concentration (IC50) of PCA, statistically determined using GraphPad Prism software, was found to be 15.92 µg/mL. Additionally, Western blot analysis (Figure 15) corroborated these findings, demonstrating a dose-dependent reduction in the expression of inducible nitric oxide synthase (iNOS) despite the presence of 100 ng/mL of LPS. At a concentration of 100 µg/mL, PCA nearly abolished iNOS expression, suggesting that it effectively inhibits the gene responsible for iNOS synthesis. In general, results suggest that PCA effectively suppresses iNOS expression in LPS-stimulated macrophages, indicating its strong anti-inflammatory potential. The dose-dependent inhibition of iNOS aligns with previous findings from the NO inhibition assay, further supporting PCA's role in modulating inflammatory pathways. To date, no previous studies have reported on the anti-inflammatory effects of PCA, which highlighting the novelty of these findings.

Wound healing activity of PCA in vitro

As shown in Figures (16-17), the purified PCA demonstrated significant ($p \leq 0.01$) and efficient wound healing potential. Within 24 hrs, the wound closure rate increased substantially from 18.5% in the absence of PCA to approximately 93% when treated with 100 µg/mL of PCA. This increase indicates a transition from minimal spontaneous healing to a nearly complete cellular regeneration *in vitro*, with a progressive and significant enhancement in wound closure observed at increasing PCA concentrations. Notably, a substantial improvement was recorded at 50 µg/mL, suggesting a threshold concentration for significant wound-healing efficacy. These findings imply that the PCA compound exerts a promotive effect on wound healing in a dose-responsive manner, although further mechanistic studies are required to elucidate the underlying biologyical pathways involved in this enhanced wound closure. In Figure (17), the observed results demonstrate a concentration-dependent enhancement of fibroblast migration and wound closure upon PCA treatment. PCA at 100 ppm exhibited the highest wound-healing efficacy, showing near-complete wound closure within 24 hrs at 100 ppm exhibited the highest wound-healing efficacy, showing near-complete wound closure within 24 hrs. These findings suggest that PCA possesses strong pro-healing properties and may facilitate fibroblast-mediated tissue repair. Further quantitative analysis and mechanistic studies are necessary to elucidate the underlying molecular pathways involved in PCA-induced wound healing.

Table (4): Cytotoxic activity of phenazine-1-carboxylic acid (PCA) against various cancer cell lines in comparison to negative control and doxorubicin as a positive control

Sample Code	IC_{50} (µg/mL)	IC_{90} (µg/mL)	Activity (%) at 100 µg/mL	IC_{50} of Doxorubicin, (µg/mL)
PACA (Pancreatic cancer)	72.9	116.5	73.6	28.3
PC_3 (Prostate cancer)	97.6	150.4	47.5	23.8
MCF7 (Breast adenocarcinoma)	77.9	133.2	50.3	26.1
$HePG_2$ (Hepatocellular carcinoma)	45.5	72.9	3.2	21.6
Controls				
DMSO (Solvent Control)	-	-	1	-
Negative Control	-	-	0	-

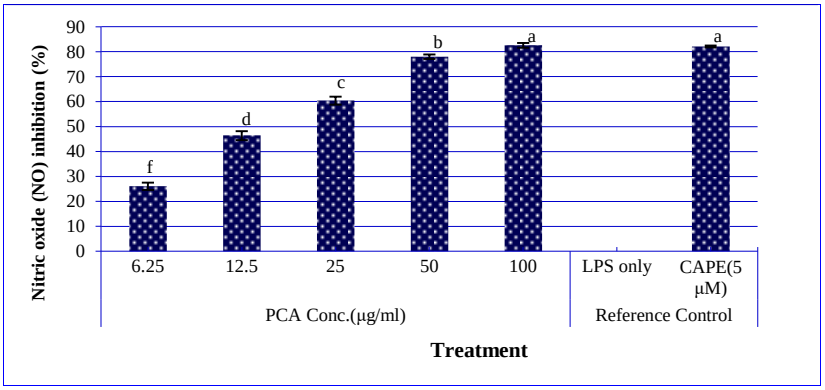

Figure (14): Anti-inflammatory activity of phenazine-1-carboxylic acid (PCA), showing its effect at different concentrations on nitric oxide (NO) inhibition in LPS-stimulated RAW264.7 macrophages. Data are presented as mean ± SE. Columns with different superscript letters indicate statistically significant differences at $p \leq 0.05$ based on Duncan's Multiple Range Test.

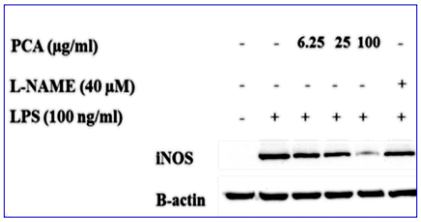

Figure (15): Effect of different concentrations of PCA on inflammation marker protein expression (iNOS) using Western blotting technique.

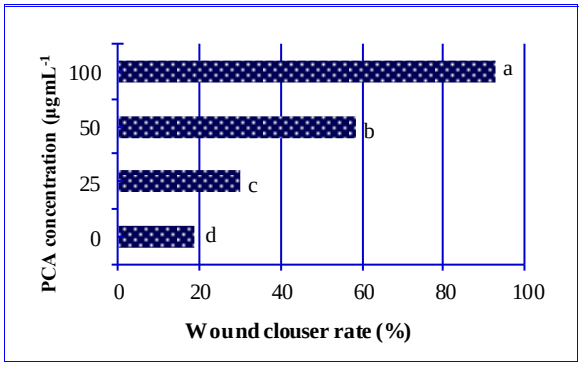

Figure (16): Wound closure rate (%) in response to different concentrations of phenazine-1-carboxylic acid (PCA). Column with different superscript letter are significantly different at $p \leq 0.05$.

DISCUSSION

The study was performed on the ethyl acetate extract of the fermentation medium of *Pseudomonas aeruginosa* OQ158909 which was locally isolated by the research team in a previous work. The crude extract was fractionated using silica gel column chromateography and the most bioactive secondary metabolite was purified and characterised, using different techniques, as PCA. The purification step was performed using HPLC technique. The fraction, which was represented by the most distinctive peak obtained at the retention time of 15 minutes, included the bioactive compound responsible for the majority of the antimicrobial activities of the tested sample and was therefore selected for further study. Compound characterization was achieved through a series of experiments including UV, FTIR, MS and 1H-NMR. The results of the UV spectrophotometer scanning analysis suggested that the isolated compound was PCA since, according to previously reported studies by Jain and Pandey (2016), the PCA, dissolved in ethyl acetate, showed characteristic absorption peaks at 260 nm and 362 nm which are highly similar to the wavelengths at which the peaks of the purified compound were detected.

The UV-spectrophotometric and mass spectrometric analyses provide critical insights into the structural characteristics and purity of phenazine-1-carboxylic acid (PCA). The observed UV absorption peaks at 258 nm and 362 nm are consistent with the presence of an extended conjugated π-electron system, which is a hallmark of the phenazine core structure. These results align with previous reports on phenazine derivatives, indicating strong electronic transitions that contribute to their unique photophysical properties (Yu et al., 2022).

The mass spectrometric analysis further validates the molecular identity of PCA, as evidenced by the prominent molecular ion peak at m/z 224.85, which corresponds to its theoretical molecular weight. The high intensity of this peak suggests the stability of the molecular ion, reinforcing the compound's structural integrity. Additionally, the inclusion of a 3D molecular model provides a more comprehensive understanding of PCA's spatial conformation, which may have implications for its biological activity, solubility, and interaction with biomolecules. Taken together, these findings confirm the successful isolation and structural characterization of PCA. The data strongly supports its chemical purity and reinforce its potential applicability in various biomedical and pharmaceutical contexts. Further studies, such as nuclear magnetic resonance (NMR) spectroscopy and infrared (IR) analysis, could provide additional molecular insights, particularly regarding functional group interactions and electronic distribution.

The results of FTIR spectroscopy were consistent with those of UV spectrophotometric analysis, as the observed peaks corresponded to the characteristic infrared absorption bands of phenazine-1-carboxylic acid (PCA), as reported by Cheng *et al*. (2016). The mass spectrometry (MS) analysis revealed a molecular ion peak (M+H) at m/z 224.85, closely matching the molecular weight of PCA (224 Da) reported by Wu *et al*. (2020), and Blankenfeldt & Parsons (2014). Furthermore, the ^{1}H-NMR spectral data aligned with the characteristic chemical shifts previously documented for PCA by Yu *et al*. (2022).

The structural characterization results align with existing literature, confirming that with existing literature (Blankenfeldt and Parsons, 2014; Che & Wu *et al*., 2020; Yue *et al*., 2022) confirms that the purified compound is phenazine-1-carboxylic acid (PCA), a well-known secondary metabolite of *Pseudomonas aeruginosa* (Jain and Pandey, 2016).To assess its potential biological activities, the purified compound was tested for antimicrobial efficacy. It exhibited broad-spectrum antimicrobial activity against multiple Gram-positive and Gram-negative bacterial strains, as well as various fungal species, including highly pathogenic human pathogens. Notably, the observed inhibition zones were substantial, despite the well-documented resistance of these strains to conventional antibiotics. These findings highlight the potential of PCA as a promising candidate for the development of next-generation antimicrobial agents.

Effects of pure PCA on different human cancer cell lines were also promising, especially hepatic, prostatic, pancreatic and breast carcinoma cell lines. The suggested mechanism for the effect of PCA on these cancer cell lines could be referred to the fact that PCA, as all phenazines compounds, has the ability to trigger the formation of toxic reactive oxygen species by directly reducing molecular oxygen, since Karuppiah *et al.*, (2016), reported that the antitumor activity against prostate cancer cells takes place through reactive oxygen species production (ROS) and mitochonderial-

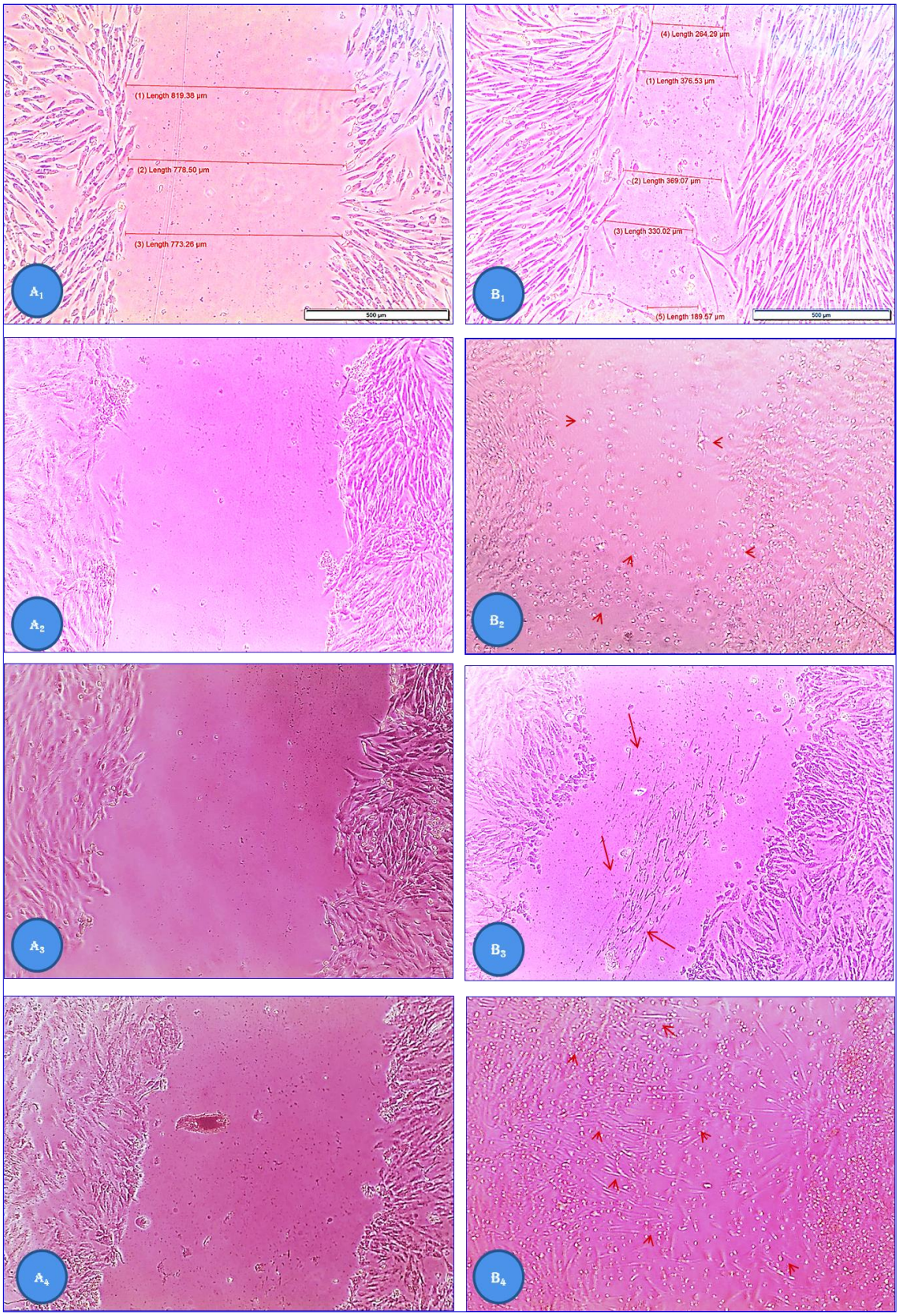

Figure (17): *In vitro* scratch assay assessing the wound-healing potential of phenazine-1-carboxylic acid (PCA) in BJ-1 dermal fibroblast cells over 24 hours. Representative images show the scratch area at 0 hours (A_1, A_2, A_3, A_4) and after 24 hrs (B_1, B_2, B_3, B_4) under different treatment conditions. (A_1-B_1) Untreated control; (A_2-B_2) PCA treatment at 25 PPM; (A_3–B_3) PCA treatment at 50 PPM; (A_4-B_4) PCA treatment at 100 PPM. Scale bar = 500 µm; magnification = 40×.

related apoptotic pathways. Hepatic, prostatic, pancreatic and breast carcinoma are considered some of the most worldwide spread and life-threatening cancer diseases as reported by Karuppiah *et al.*, (2016) and Sana *et al*., (2019). The pure PCA also displayed a superb antioxidant effect with a calculated IC50 that exceeded in its affinity that recorded for the standard positive controls recommended for samples evaluation when performing the common antioxidant tests.

The findings regarding the anti-inflammatory and wound healing effects of purified phenazine-1-carboxylic acid (PCA) are supported by various studies using higher plants (Chaniad *et al*., 2020). The compound demonstrated significant anti-inflammatory effects *in vitro*, with results indicating that even at minimal concentrations, PCA effectively suppressed inflammatory marker proteins, despite the presence of inflammation-inducing agents. Furthermore, PCA exhibited remarkable wound healing activity, as a concentration of just 100 µg/mL was sufficient to accelerate cell regeneration and enhance wound closure by more than five times, leading to noticeable healing within approximately 24 hrs. These observations highlight the potential of PCA as a therapeutic agent for managing inflammation and promoting wound healing, making it a valuable candidate for further research and application in clinical settings.

For would healing potential of PCA, a significant enhancement in wound closure upon treatment with phenazine-1-carboxylic acid (PCA), indicating its potential as a pro-healing agent. The results revealed a concentration-dependent response, where the wound closure rate increased progressively with higher PCA concentrations. Notably, at 100 µg/mL, PCA facilitated an accelerated healing response, achieving approximately 93% wound closure within 24 hours. This suggests a substantial enhancement in cellular migration and proliferation compared to the untreated control, which exhibited only 18.5% closure under the same experimental conditions. The observed improvement in wound closure at 50 µg/mL suggests a threshold concentration beyond which PCA exerts a pronounced effect on cellular regeneration. The significant difference between treated and control groups ($p \leq 0.05$) implies that PCA may modulate key signaling pathways involved in tissue repair, potentially through antioxidant activity, antimicrobial properties, or regulation of cellular proliferation mechanisms. These findings align with previous studies on phenazine derivatives, which have been reported to influence redox balance and promote cellular responses beneficial for wound healing (Liu *et al*., 2023; El-Masry *et al*., 2023).

While these results highlight PCA's promising role in enhancing wound healing, further mechanistic investigations are necessary to elucidate the molecular pathways underlying this effect. Future studies should focus on evaluating its impact on key factors such as reactive oxygen species (ROS) modulation, inflammatory cytokine expression, and extracellular matrix remodeling. Additionally, in vivo validation is required to assess PCA's therapeutic potential in wound healing applications.

CONCLUSION

Phenazine-1-carboxylic acid (PCA) has been produced by microorganisms like *Pseudomonas aeruginosa* which isolated from local Egyptian samples. PCA shown to possess multifaceted bio-activity, with significant antimicrobial effects against common human pathogens, promising cytotoxicity against various cancer cell lines, potent anti-inflammatory properties, and a remarkable capacity to enhance wound healing *in vitro*. Notably, PCA exhibited superior antioxidant activity compared to standard controls like BHA and vitamin C. The novelty of these findings lies in the scarcity of previous studies on PCA's effects against human pathogens and its antioxidant, anticancer, anti-inflammatory, and wound healing properties. These results collectively indicate that PCA is a promising candidate for drug development, particularly in wound healing and oxidative stress-related conditions. Future studies should focus on elucidating the molecular mechanisms underlying these activities and evaluating PCA's potential in clinical and biotechnological applications.

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توصيف وتقييم النشاط البيولوجي لحمض الفينازين-1-كربوكسيليك المنتج بواسطة العزلة المصرية المحلية *Pseudomonas aeruginosa* OQ158909

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تمثل مركبات الايض الثانوي التي تنتجها الكائنات الحية الدقيقة مصدرًا غنيًا للمركبات النشطة بيولوجيًا، والتي تلعب دورًا مهمًا في تطوير العلاجات الدوائية، خاصة المضادات الحيوية والمضادات الفطرية والمركبات المضادة للأكسدة والمضادة للالتهابات. ويعد *Pseudomonas aeruginosa* من الكائنات الدقيقة المعروفة بإنتاج مجموعة واسعة من المركبات الايض الحيوية ذات الفعالية الدوائية، ومن أبرزها مركبات الفينازين، التي أظهرت خصائص دوائية متعددة، بما في ذلك النشاط المضاد للميكروبات والقدرة على مكافحة الإجهاد التأكسدي، فضلًا عن إمكاناتها المضادة للسرطان والتئام الجروح. تهدف هذه الدراسة إلى تنقية وتحديد أحد مركبات الايض الثانوي ذات النشاط الحيوي العالي المستخلصة من وسط تخمير سلالة *Pseudomonas aeruginosa* OQ158909 المعزولة محليًا. تم فصل المستقلبات باستخدام Silica gel column chromatography بأنظمة مذيبات محسّنة، ثم خضعت الفئة الأكثر نشاطًا بيولوجيًا لمزيد من التنقية عبر High-performance liquid chromatography (HPLC). تم تحديد المركب النقي على أنه حمض الفينازين-1-كربوكسيليك (PCA) من خلال التحليل الطيفي المتكامل، الذي شمل الأشعة فوق البنفسجية (UV)، اختبار فورييه لطيف الأشعة تحت الحمراء (FTIR)، مطياف الكتلة (MS)، والرنين المغناطيسي النووي للبروتون (^{1}H-NMR). أظهرت التقييمات البيولوجية أن مركب PCA يمتلك نشاطًا مضادًا قويًا لمجموعة واسعة من مسببات الأمراض البشرية، حيث تراوحت مناطق تثبيط النمو للبكتيريا موجبة الجرام بين 16 و28 مم، بينما سجلت البكتيريا سالبة الجرام مناطق تثبيط تراوحت بين 13 و36 مم. كما أثبت المركب فعالية مضادة للفطريات، حيث بلغت مناطق التثبيط 16 مم ضد *Aspergillus niger* و17 مم ضد *Candida albicans*. إضافةً إلى ذلك، أظهر مركب PCA نشاطًا مضادًا للأكسدة، حيث تمكن من تثبيط 75.2% من الجذور الحرة DPPH عند تركيز 100 ميكروجرام/مللي، مع قيمة IC_{50} بلغت 40.4 ميكروجرام/مللي، متفوقًا على مضادات الأكسدة القياسية مثلBHA وفيتامين C. كما أبدى المركب تأثيرًا قويًا مضادًا للالتهابات، حيث أدى تركيز 100 ميكروجرام/مللي إلى تقليل إنتاج أكسيد النيتريك (NO) المحفّز بالليبوبوليساكاريد (LPS) بنسبة 82.54%، إلى جانب تثبيط شبه كامل لإنزيم أكسيد النيتريك المحفّز.(iNOS) علاوة على ذلك، أظهر مركب PCA تأثيرًا سامًا انتقائيًا ضد عدة خطوط خلوية سرطانية، حيث كان الأكثر فعالية ضد خلايا سرطان الكبد HePG2، مع قيمة IC_{50} بلغت 45.5 ميكروجرام/مللي ومعامل انتقائية تجاوز 2. كما أدى تركيز 100 ميكروجرام/مللي من المركب إلى تثبيط 74% من خلايا سرطان البنكرياس PACA، و50% من خلايا PC3 و MCF7. وأخيرًا، أظهر المركب قدرة ملحوظة على تعزيز التئام الجروح، حيث أدى تركيز 100 ميكروجرام/مللي إلى إغلاق شبه كامل للجروح خلال 24 ساعة، مما يشير إلى إمكاناته الواعدة في مجال الطب التجديدي والعلاج الحيوي.