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Evaluation of antibacterial and anticancer properties of secondary metabolites isolated from soil *Bacillus* spp focusing on two strains of *Bacillus licheniformis* and *Bacillus siamensis*

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Abstract

Background *Bacillus* strains are well recognized for their inherent production of bioactive compounds that exhibit antibacterial and anticancer properties. This study aims to evaluate the antimicrobial and anticancer effects of the secondary metabolite isolated from *Bacillus licheniformis* and *Bacillus siamensis strain*.

Material and method We developed and purified a new soil-derived Bacillus strain to study its metabolites on cancer cells and bacteria. After evaluating the antimicrobial effects of the selected strains' secondary metabolites by well diffusion, growth conditions and temperature optimised using liquid-liquid extraction, secondary metabolites isolated, and active compounds identified using GC-MS. Evaluation of PC-3 and HPrEpC cytotoxicity. AV/PI staining and comet assay assessed necrosis and apoptosis. Real-time PCR measured apoptotic gene expression. Finally, the scratch test measured cell movement.

Results *Bacillus* strain metabolites exhibit dual-purpose antimicrobial and anticancer properties. *Bacillus licheniformis* isolate 56 and S2-G12 isolate 60 demonstrated the greatest antibacterial activity. Among all *Bacillus* isolates, isolates 56 (*Bacillus licheniformis*) and 60 (*Bacillus siamensis* strain) had the highest antibacterial activity. Crude extracts obtained from strains 56 and 60 decreased PC-3 cell viability in a dose-dependent manner. At 200 µg/mL, the survival rate of cells treated with strain 56 and 60 crude extract was 23% and 25%, respectively ($p < 0.001$). The treatment of PC-3 cells with strains 56 and 60 crude extract led to considerable apoptosis (46.2% and 50.09%, respectively) compared to the control group. After treatment with the crude extract from strains 56 and 60 at an IC_{50} concentration, a significant number of PC-3 cells showed comet formation, indicating DNA fragmentation. Metabolites extracted from strain 56 and 60 enhanced *caspase 3*, *caspase 8*, and *Bax* genes expression and reduced *Bcl-2* expression ($p < 0.001$). Cell migration was also prevented.

Conclusion Our findings show that the secondary metabolites of *B. licheniformis* and *B. siamensis* have antibiotic and anticancer properties. However in vivo studies are necessary to confirm these findings.

Keywords Anti-cancer activity, Prostate cancer, *Bacillus* strains, Secondary metabolite

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Introduction

Throughout history, humans have relied on natural products as a means to preserve optimal health and safeguard against a multitude of ailments. Natural products have been identified as a very abundant and valuable resource for developing new pharmaceuticals [1]. Presently, it has been shown that around 65% of the drugs licensed for use in medical sectors have been derived from natural sources. The natural products database has a vast collection of about 210,000 physiologically active molecules, exhibiting a significant degree of chemical variety [2]. Natural products have gained considerable recognition within scientific communities due to their superior therapeutic potential compared to synthetic products [3]. Additionally, compounds derived from natural sources exhibit a greater degree of structural diversity when compared to synthetic compounds, thereby playing a crucial role in the discovery of novel drugs [3]. Microorganism-derived compounds are essential in treating infectious diseases and cancer [4]. Bacteria and fungi are the most suitable candidates for synthesizing bioactive substances between microorganisms, as they have been present on Earth for billions of years and have developed numerous biosynthetic pathways using novel mechanisms to synthesize secondary metabolites [5]. Identifying bioactive molecules derived from microorganisms encompasses many stages, such as isolation, clarification of their structure, and establishing the metabolic pathway responsible for producing secondary metabolites. Secondary metabolites have garnered significant attention due to their ecological and biogeochemical impacts and prospective applications in medicine and biotechnology [6].

Soil is a microorganism-rich resource that supports the diversity of microorganisms differently according to geographical location and environmental conditions [7]. Soil microorganisms are integral constituents of forest biomes and have a crucial role in soil aggregation, nitrogen fixation, and nutrient cycling via the breakdown of lignin and cellulose [8]. Many medications have been sourced from soil microorganisms classified under the genera *Bacillus*, *Streptomyces*, *Micromonospora*, *Penicillium*, and *Cephalosporium*. Every year, a significant number of bioactive compounds, over 500, are identified [9]. Notably, about 60% of these compounds are sourced from soil bacteria. Members of the genus *Bacillus* are often seen in soil environments and can synthesize diverse beneficial secondary metabolites. These compounds have shown efficacy against various severe pathological conditions [10]. A significant portion of research efforts are directed toward investigating soil microbial communities and the variety of soil bacteria, particularly on *Bacillus* colonies [10]. The most notable species of *Bacillus* include *Bacillus lichaniformis* (*B. lichaniformis*), *Bacillus subtilis* (*B. subtilis*), *Bacillus circulans* (*B. circulans)*, *Bacillus amyloliquefacience* (*B. amyloliquefacience*), *Bacillus polymixa* (*B. polymixa*), *Bacillus pumilus* (*B. pumilus*), and *Bacillus cereus* (*B. cereus*). These species often synthesize isocoumarins, lipopeptides, polyketides, aminoglycosides, aminopolyols, phospholipids, phosphonoligopeptides, and terpenoids, which have a wide range of clinical features, including anticancer activity [10].

The metabolites derived from soil bacterial strains that exhibit anticancer properties have the potential to serve as a viable and safe substitute for manufactured pharmaceuticals [11]. The existing cancer treatment modalities, including radiation therapy, surgical intervention, chemotherapy, hormone therapy, and immunotherapy, often fail to achieve comprehensive cancer remission [12]. The administration of these therapy modalities is associated with notable adverse effects in human subjects, including but not limited to the occurrence of blood clots, alopecia, discomfort, anemia, tiredness, thrombocytopenia, gastrointestinal disturbances such as diarrhea and constipation, neurological problems, as well as the potential for severe and life-threatening infections. Furthermore, the safety and effectiveness of the presently available anticancer medicines are constrained. The efficacy of cancer treatment is contingent upon its ability to selectively eradicate cancer cells while minimizing or eliminating adverse effects on normal cells. This highlights the critical need to find novel bioactive chemicals that may be used safely and effectively to treat cancer [13, 14].

Prostate cancer (PCa) ranks as the second most prevalent form of malignancy among males globally, behind lung cancer. In the year 2018, there were a recorded 1,276,106 newly diagnosed cases of prostate cancer, resulting in 358,989 deaths. These fatalities accounted for about 3.8% of all cancer-related deaths in the male population [15]. The primary treatment modality for recurrent prostate cancer is androgen deprivation therapy. Nevertheless, with time, these individuals will inevitably acquire resistance and transition into a condition known as castration-resistant prostate cancer (CRPC) [16]. In the presently endorsed therapeutic regimens, the administration of docetaxel in conjunction with prednisone yielded a survival extension of 2.8 months [17]. Similarly, sipuleucel-T demonstrated a survival prolongation of 4.1 months, while abiraterone acetate exhibited an additional 4 months of survival [17]. Moreover, using enzalutamide resulted in a substantial increase in lifetime, with a prolongation of 4.8 months, specifically in individuals with metastatic castration-resistant prostate cancer (mCRPC) [18]. Nevertheless, these therapies are subject to various limitations and have yet to substantially increase the patient's lifespan beyond a few months. The current investigation extracted secondary metabolites from *B. licheniformis* and *B. siamensis* strains. The chemical exhibited lethal effects by inducing cell apoptosis,

inhibiting cell migration, causing cell cycle arrest, facilitating DNA damage, and promoting necroptosis in prostate cancer cell lines.

Results

Isolation and screening of *Bacillus* spp

Based on the results obtained from the previous study, 70 soil samples were collected from different areas of Shahrekord city. After preparation, the screening was carried out on each of them separately. 467 colonies were obtained from cultivating soil samples in a nutrient agar medium. The number of colonies isolated from moist soil was 251, from dry soil 54, and from semi-moist soil 162. Finally, 9 isolates were selected to evaluate the antimicrobial activity against one or more microorganisms. The number of isolated bacteria showed that most were isolated from wet and semi-humid soils. The statistical analysis of the data showed that according to the *p*-value less than 0.05, there is a significant relationship between the moisture content of the soil and the number of isolated bacteria. 9 isolates suspected to produce antimicrobial metabolites were reported from soils with pH between 7.5 and 8, which indicates a greater abundance of bacteria in neutral and alkaline pH. Statistical analysis of the results obtained from measuring the pH of different soils and counting the number of gram-positive colonies obtained from each soil sample showed a significant relationship between alkaline pH and the increase in the number of colonies.

Antimicrobial activity of *Bacillus* spp

The results obtained at this stage based on the agar diffusion method showed that among the 9 isolates obtained, 6 isolates have strong antimicrobial activity against *S. aureus* (1189 PTCC), *B. cereus* (1154 PTCC) and *E. coli* (1399 PTCC). While 2 isolates (5 and 11) showed relatively weaker antimicrobial activity. The analysis of the acquired findings demonstrates that the supernatant derived from the 72-hour culture of all 6 isolates exhibits its superior growth inhibition compared to the metabolites generated from the 24-hour culture. According to the obtained results, all the isolates can produce antimicrobial metabolites after 72 h, while this activity may not be observed in 24 h for some isolates. The supernatant derived from the 72-hour culture of strains 56 and 60 exhibited a maximum clean area when examined against pathogens (Fig. 1).

Optimizing the production of antimicrobial metabolites

All nine isolates were cultured on a medium with varying pH levels ranging from 5 to 9. According to the obtained results, none of the isolates showed antimicrobial activity at pH 5, indicating that acidic conditions are not suitable for the antimicrobial activity of the obtained isolates (Table S1). All 9 isolates at pH 8 could inhibit the growth of pathogen strains (Table S1). All isolates showed more antimicrobial activity in close to neutral and slightly alkaline conditions, so the optimal pH for isolates 36, 60, and 63 was 7 and for isolates 5, 16, 11, 43, 56, and 59, 8. Also, after 24 h, the production of antimicrobial metabolites of each isolate at different temperatures was investigated by measuring the clear area around the wells (Table S2). The strains 36, 59, 63, and 56 exhibited an optimal temperature of 37 °C after 72 h against S. aureus. In contrast, strains 5, 11, 16, and 43 displayed an optimal temperature of 40 °C, whereas isolate 60 showed an optimal temperature of 30 °C. The optimal activity temperature against *B. cereus* was determined to be 40 °C for strains 5, 11, and 43, 37 °C for strains 56, 59, 63, 36, 16, and 33 °C for strain 60. Various compounds such as glucose, lactose, and peptone were used to investigate the effect of carbon and nitrogen sources on the production of antimicrobial metabolites by these isolates. According to the results, the largest aura of non-growth was obtained in the

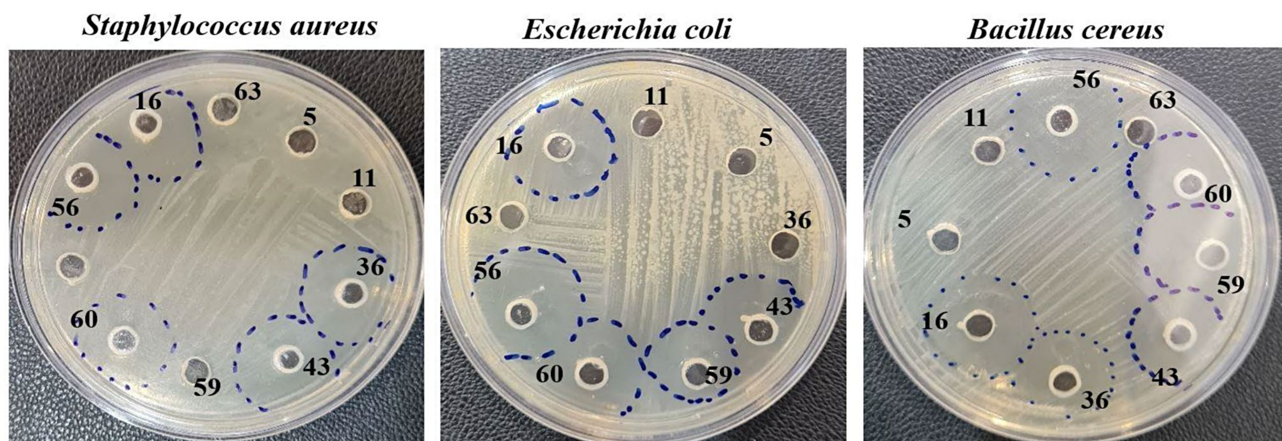

Fig. 1 Antibacterial activity of isolates collected from soil against specific bacterial pathogens by well diffusion method

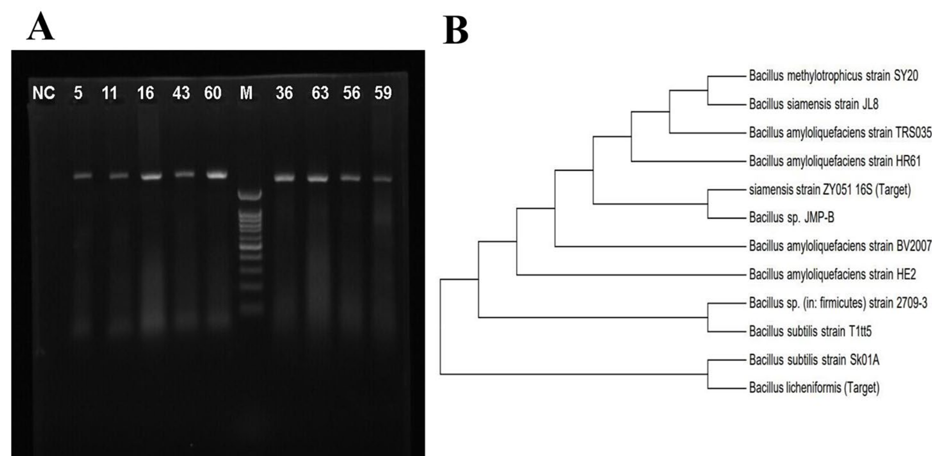

Fig. 2 **(A)** Electrophoresis of polymerase chain reaction products related to *16srRNA* gene. A *16srRNA* gene fragment longer than 1100 bp was detected in each of the isolates. M: DNA marker 1 kb. **(B)** Phylogenetic evaluation of specimens (*B. licheniformis* strain ZBA-YS3 and *B. siamensis* strain S2-G12)

presence of lactose (21 mm). In general, in the presence of glucose and lactose as different carbon sources, the ability of the strains to produce antimicrobial metabolites does not show a noticeable change. Strains 56, 59, 60, and 63 had more inhibition zones in the presence of lactose than glucose. Isolates 5, 6, 11, and 43 showed higher antimicrobial activity in the fact of glucose than lactose.

Molecular detection of gram-positive isolates producing antimicrobial metabolites

A polymerase chain reaction was performed using universal primer *16srRNA* to identify the selected gram-positive strains. 1400 bp band was observed on 1% agarose gel to determine the accuracy of DNA extraction (Fig. 2A). The sequence obtained via DNA sequencing was then analyzed by multiple sequence alignment with the closely related sequences revealed in the BLAST search (Fig. 2B). The alignment results show a significant alignment between the putative DNA sequences of *B. licheniformis* strain ZBA-YS3 and *B. siamensis* strain S2-G12 with the *16s rRNA* regions of *B. methylotrophicus* strain SY20, *B. siamensis* strain JL8, *B. amyloliquefaciens* strain TRS035, *B. amyloliquefaciens* strain HR61, *Bacillus* sp. JMP-B, *B. amyloliquefaciens* strain BV2007, *B. amyloliquefaciens* strain HE2, *Bacillus* sp. (in: firmicutes) strain 2709-3, *B. subtilis* strain T1tt5, and *B. subtilis* strain Sk01A. Finally, isolates 56 (*B. licheniformis*) and 60 (*B. siamensis* strain S2-G12), which had the most antibacterial properties, were selected to perform other tests.

GC-MS identification of bioactive volatile chemicals

The GC-MS analysis of the crude extracts of the 56 and 60 strains that had extremely significant antibacterial activity in comparison to the other strains tested enabled us to discover a total of 7 volatile chemicals. These compounds exhibit antimicrobial properties and other

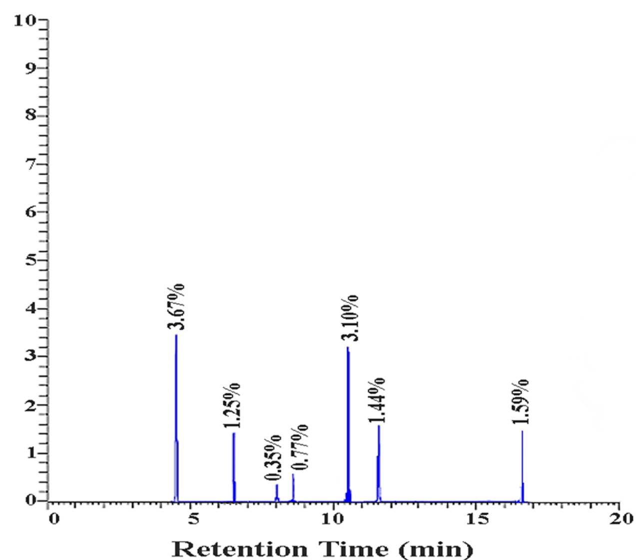

Fig. 3 The presented data includes a chromatogram obtained through the use of gas chromatography-mass spectrometry (GC-MS) for the analysis of *B. licheniformis* and *B. siamensis* extracts. The highest values in this graph correspond to the values used in Table 2

notable biological activities, including anticancer, antioxidant, and anti-inflammatory effects (Fig. 3; Table 1).

Cytotoxicity assay

Assessment of survival via MTT

The cytotoxicity of crude extract samples derived from *B. licheniformis* (strain 56) and *B. siamensis strain S2-G12* (strain 60) was assessed using an MTT test against PC-3 human prostate cancer cells and human primary prostate epithelial cell (HPrEpC). Figure 4 demonstrates that the crude extract of strain 56 exhibited less cytotoxicity than strain 60, while 25 µg/ml of strain 56 was required to begin toxicity with cell viability of nearly 80%. In contrast, the crude extract derived from strain 60 exhibited

Table 1 A total of seven volatile chemicals were detected and identified using gas chromatography-mass spectrometry (GC-MS) in the crude extract obtained from *B. Licheniformis* and *B. siamensis* strain S2-G12

RT (time)	Area (%)	Molecular weight (g/mol)	Molecular formula	Compound name	Bioactivity property
4.52	3.67	200.3178	$C_{12}H_{24}O_2$	Dodecanoic acid	Anticancer activity, Antimicrobial [19]
6.6	1.25	434.15	$C_{14}H_{10}Mo_2O_4$	Molybdenum	Anticancer activity, Antimicrobial [20]
8.02	0.35	88.1051	C4H8O2	Butanoic acid	Antimicrobial [21]
8.77	0.77	340.32	$C_{32}H_{68}O_4Ti$	Titanium (IV) butoxide	Antimicrobial, Anticancer, Anticoagulant [22]
16.76	1.59	390.564	C24H38O4	Di-n-octyl phthalate	Anticancer, Anti-inflammatory [23]
10.52	3.10	508.7	C34H36O4	Cyclopenta[d]anthracene-8,11-diol, 3-isopropyl-1,2,3,3a,4,5,6,6a,7,12-decahydro-, dibenzoate	Antimicrobial, Anticancer [24]
11.34	1.44	323.41	$C_{18}H_{17}N_3OS$	Acetamide, N-[4-[2-[(3-methylphenyl)amino]-4-thiazolyl]phenyl]-	Anticancer activity [25]

RT: Retention time

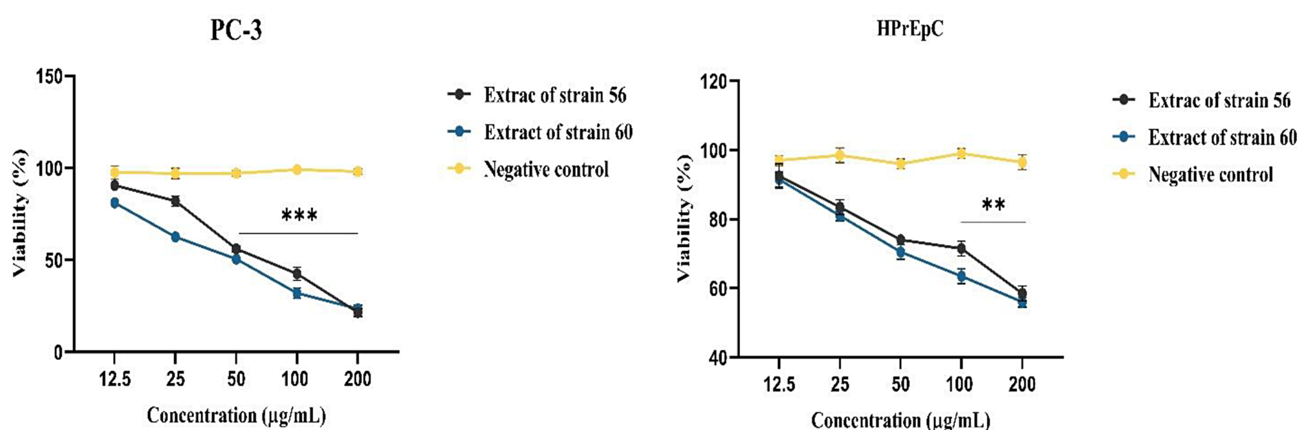

Fig. 4 Evaluation of cytotoxicity using MTT method in different concentrations of supernatants obtained from strains 56 and 60 at PC-3 and HPrEpC cell lines. The results are repeated 3 times and mean ± SD. ***p* < 0.01, ****p* < 0.001

significant toxicity even at a relatively low concentration of 25 µg/ml, resulting in a cell viability of just 60%. At 200 µg/ml, the survival rate of cells treated with strain 56 and 60 crude extract was 23% and 25%, respectively ($p<0.001$). Also, at a concentration of 100 µg/ml, the survival rate of cells treated with the crude extract of strains 56 and 60 was reported as 45% and 34%, respectively ($p<0.001$). In concentrations of 50 and 25 µg/ml, the survival rate of cells treated with crude extract of strain 56 was reported as 55% and 80%, while the survival rate after treatment with crude extract of strain 60 was reported as 50% and 63%, respectively. At a concentration of 12.5 µg/ml, cell survival after treatment with crude extracts of strains 56 and 60 was reported to be above 80%. This study showed that almost half of the cells died at a concentration of 50 µg/mL of both extracts. Therefore, the IC_{50} value was 50 µg/ml. At the 200 µg/ml concentration of the extracts of both selected strains, the highest cell death was observed (Fig. 4). In addition, the MTT test results showed that the treatment of normal cell line with the crude extract of strains 56 and 60 in concentrations of 100 and 200 µg/mL leads to a decrease in survival. After 24-hour treatment with crude extracts of strain 56 and 60 at a concentration of 200 µg/mL, the survival of HPrEpC cell line decreased by 60% and 55%, respectively ($p<0.01$) (Fig. 4).

Examination of cell apoptosis and cell cycle arrest

The three primary kinds of cell death are apoptosis, autophagy, and necrosis. The PC-3 cells have been treated with 50 µg/ml crude extract of strain 56 and 60 for 24 h and stained with Annexin V and PI using flow cytometry to measure the percentage of apoptotic and necrotic cells. Q_1 exhibited necrotic cells, whereas Q_2 indicated late apoptotic cells. Q_3 revealed early apoptotic cells, whereas Q_4 revealed healthy cells. The treatment of PC-3 cells with the crude extract of strains 56 and 60 resulted in 46.2% and 50.09% apoptosis, respectively, which was

significant compared to the control group (Fig. 5). Also, the amount of cell necrosis in the treatment with crude extracts of strains 56 and 60 was reported as 6.10% and 7.61%, respectively (Fig. 5A and B). In addition, we investigated the changes in the cell cycle while treating the PC-3 cell line with a crude extract generated from *Bacillus* spp (strains 56 and 60) at a concentration of 50 µg/mL. The flow cytometry method was used to analyze the cell cycle alterations throughout a 24-hour treatment period. The PC-3 cell line, which lacks androgen receptors, was halted in the G0/G1 phase due to the influence of a secondary metabolite derived from strains 56 and 60. This appears in Fig. 5C. The findings of this investigation also confirmed the elevation of apoptosis in the prostate cancer cells in question. The cell cycle analysis revealed that PC-3 cells in the treated group with strain 56 and 60 had the most reduced rate of progression into the G2/M phase.

The results showed that 90% ($p<0.05$) and 80% ($p<0.01$) of PC-3 cells remained in the G0/G1 phase in the groups treated with crude extract of strain 56 and 60, respectively.

Apoptosis morphological analysis

In order to confirm apoptosis, we used a single-cell gel electrophoresis technique, often called the "comet assay," to quantify the extent of DNA damage at the cellular level. Figure 6 displays the fluorescent pictures of the cell comets captured using a fluorescence microscope. The photos were analyzed using the openComet plugin inside the ImageJ program. The aforementioned studies include a diverse range of data and quantify the mobility of DNA. A considerable proportion of PC-3 cells, upon treatment with the crude extract derived from strains 56 and 60 at an IC_{50} level of 50 µg/mL for 30 min, exhibited a distinctive comet formation indicative of DNA fragmentation. In contrast, when examined under a fluorescence microscope, the control cells did not display any observable comet formation.

Real-time PCR

In order to investigate the potential anticancer properties of the crude extract derived from strains 56 and 60 against PC-3 cell line, the transcription levels of *Bax*, *caspase3*, *caspase 8*, and *Bcl-2* genes were quantified using the reverse transcription polymerase chain reaction (RT-PCR) technique. The proteins *Bax* and *Bcl-2* are members of the *Bcl-2* family, a group of proteins that play a role in regulating the process of apoptosis through the mitochondrial pathway. *Bax* is known to promote apoptosis, whereas *Bcl-2* acts as an inhibitor of apoptosis. The results of this study showed that after 24-hour treatment of PC-3 cells with the crude extract obtained from strains 56 and 60, *Bax* gene expression significantly increased compared to the control group ($p<0.05$) (Fig. 7A). Furthermore, our findings have shown that the crude extract obtained from strain 56 and strain 60 has a downregulating effect on *Bcl-2* expression ($p<0.01$), while simultaneously upregulating the expression of caspase 3 and caspase 8, in comparison to the control group. ($p<0.001$) (Fig. 7A).

Scratch assay

The dissemination of cancer cells to distant sites in the body, known as metastasis, is a prominent feature of late-stage cancer and a significant contributor to the overall

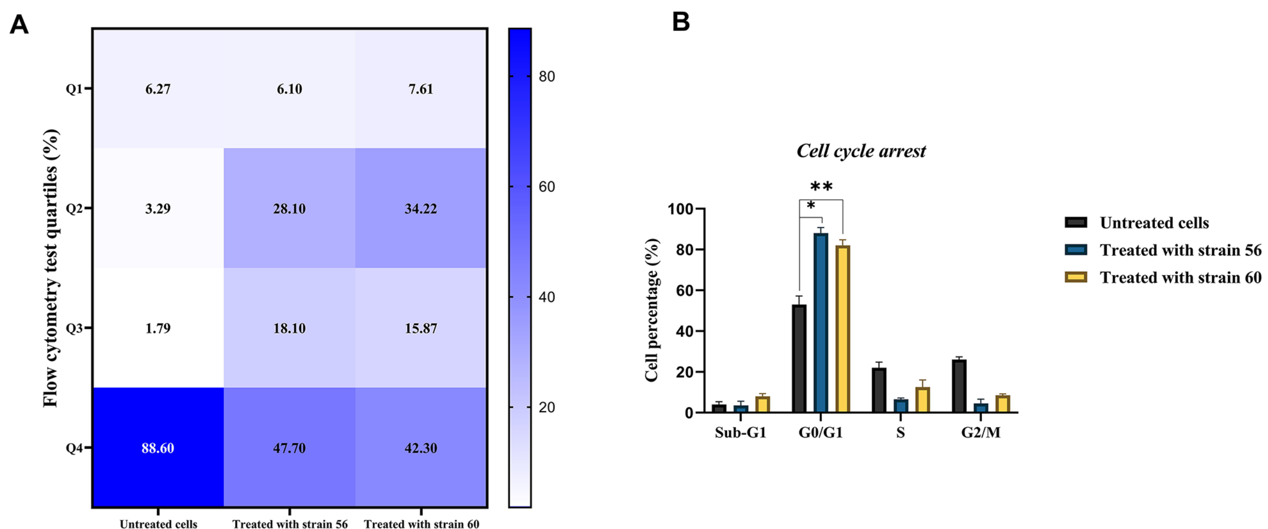

Fig. 5 The results obtained from examining cell apoptosis using annexin-V/Propidium iodide staining and flow cytometry with 50 µg/ml crude extract of strain 56 and strain 60 (**A**). The findings provide the percentage of cells undergoing early apoptosis, late apoptosis, and necrosis. (**B**) Evaluation of cell cycle arrest of PC-3 cell line after treatment with crude extract of strain 54 and 60. The results are repeated 2 times and mean ± SD. **$p<0.01$, *$p<0.05$

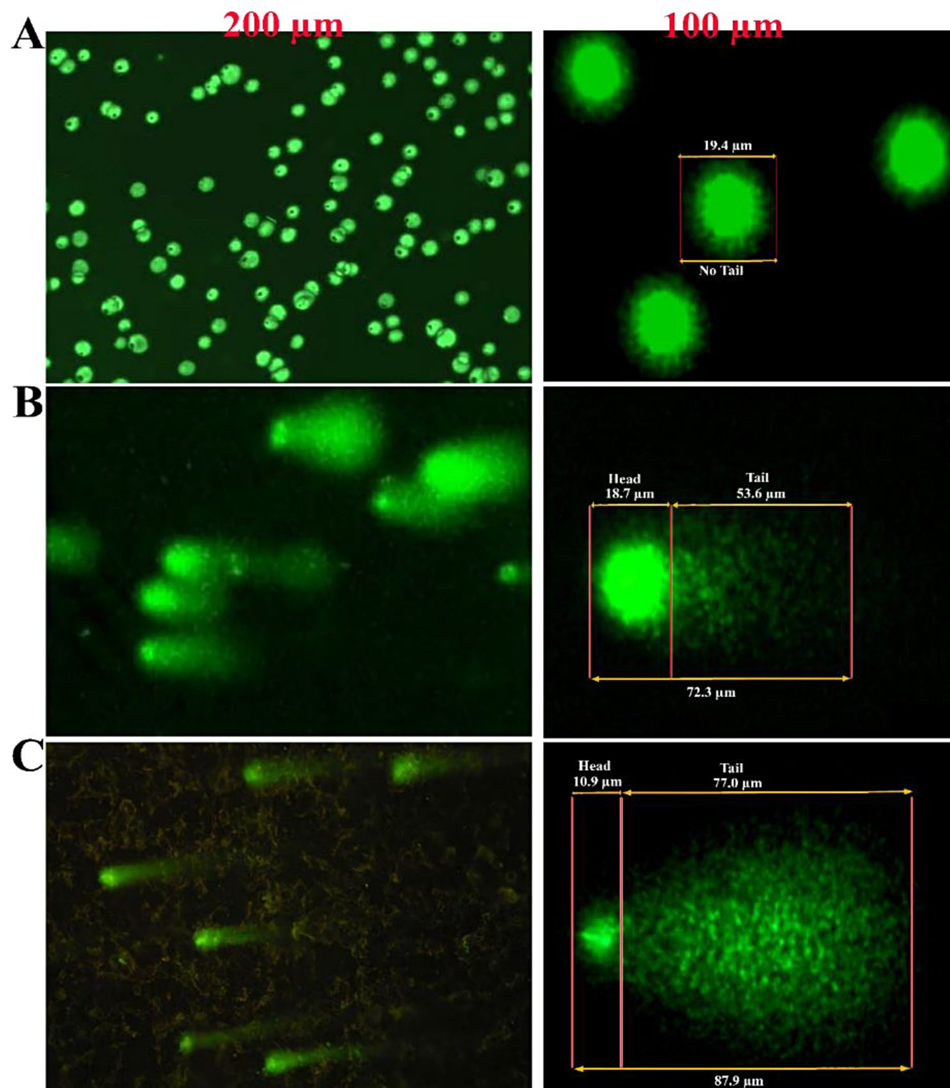

Fig. 6 Comet assay of the control (**A**), the supernatant 56-treated (**B**) and the supernatant 60-treated (**C**) cancer cells. The cancer cells were treated with supernatants for 30 min. After the treatment, cancer cells were stained by ethidium bromide

mortality seen in cancer patients. The scratch test is a frequently used technique for assessing the in-vitro cell migration of cancer cells. The crude extract from strains 56 and 60 significantly reduced wound closure compared to the control cells, which exhibited a very active migration of cells treated alone with a culture medium. The study observed that the cell migratory mobility was measured at 2.12 µm for the control group, 9.52 µm for the group treated with crude extract of strain 56, and 80.14 µm for another group also treated with crude extract of strain 60 (Fig. 7B).

Discussion

Over many centuries, Mother Nature has been recognized as a valuable repository of chemically varied and physiologically active chemicals that possess several therapeutic qualities, including antineoplastic activity [26]. Natural chemicals from animal, plant, and microbial origins have gained significant attention in several scientific and industrial sectors, particularly pharmaceutical medication development [26]. The bacterial kingdom has made significant contributions to the medical well-being of human beings, acting as a valuable source of bioactive secondary metabolites [27]. Dr. William Colley pioneered the first exploration of using bacteria as a therapeutic approach for cancer management [27]. The researcher used a mixture of culture supernatants derived from *Streptococcus pyogenes* and *Serratia marcescens* to provide therapeutic interventions to individuals afflicted with sarcoma [28]. The current work used the PC-3 cell line as a cellular model to examine cell death mechanisms and apoptosis activation in prostate cancer cells. Also, this study aimed to determine the antimicrobial effect against pathogens and the cytotoxicity effect.

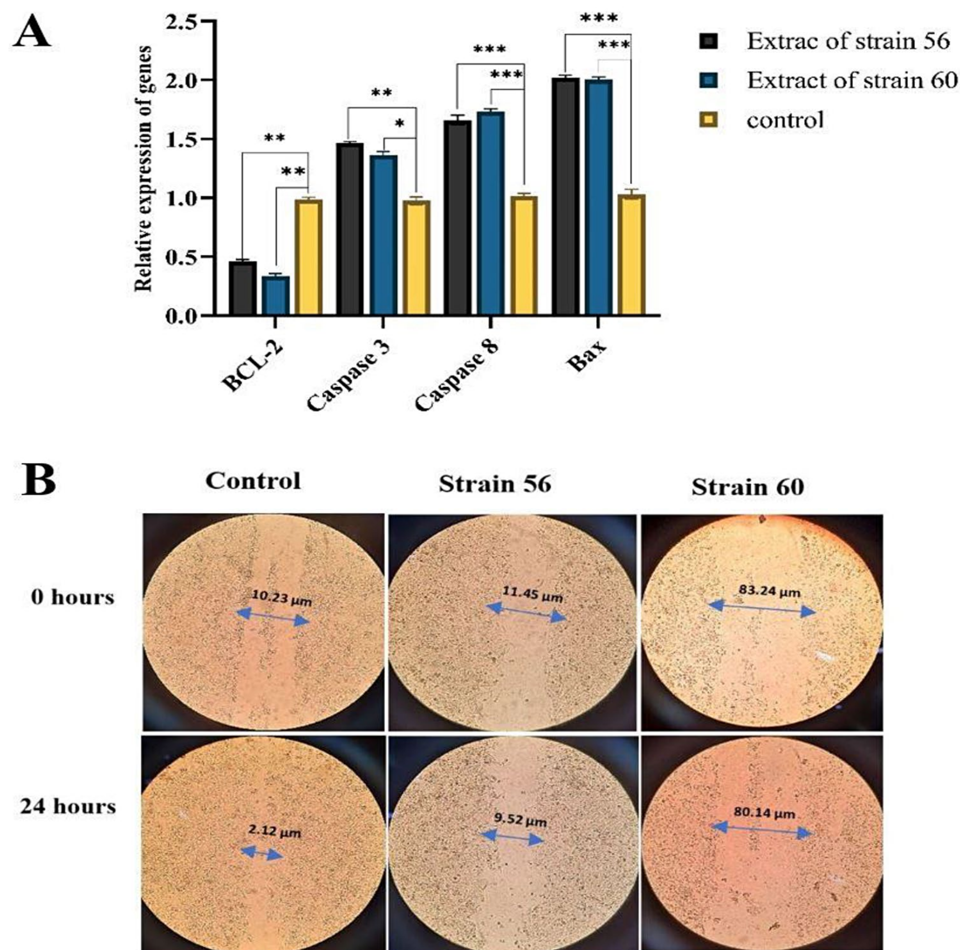

Fig. 7 **(A)** Real-time PCR analysis of the expression of apoptosis-regulating genes in groups treated with crude extract of strains 56 and 60. The results are repeated 3 times and mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. **(B)** Microscopic pictures of a scratch test were conducted on the PC-3 cell line, which was treated with the crude extract of strain 56 and 60

In this study, among 467 strains isolated from soil, 9 strains that were able to produce antimicrobial metabolites were selected. The identification of the 9 isolates was conducted by analyzing their phenotypic and genotypic features. The microorganisms under consideration exhibit a Gram-positive phenotype, characterized by their possession of spore-forming bacillus-shaped morphology. Furthermore, these microorganisms react positively to the catalase and oxidase enzymatic activities. The isolates were subjected to partial sequencing of the *16 S rRNA* gene, revealing a significant similarity with known sequences associated with the *Bacillus* genus. The nine obtained isolates were tested against *B. cereus*, *E. coli*, and *S. aureus*. The agar well diffusion test results showed that almost all 9 strains can produce secondary metabolites with antimicrobial properties. Also, different carbon and nitrogen sources were used to optimize the growth conditions. The results showed that the isolated strains in the presence of lactose and alkaline conditions have the highest potential of producing antimicrobial metabolites. Furthermore, the study's findings indicate that strains 56 (*B. licheniformis*) and 60 (*B. siamensis* strain S2-G12) exhibit the most significant inhibitory zone for pathogens.

Consequently, these two strains were chosen for further experimental investigations. The GC-MS study yielded findings indicating that the ethyl acetate extracts obtained from strains 56 and 60 contain diverse ingredients, including fatty acids, important trace minerals, metal-organic chemicals, phenols, and terpenes. Notable compounds identified include Dodecanoic acid, Molybdenum, and Titanium (IV) butoxide (Table 1). These biologically active substances include antimicrobial, antibacterial, antifungal, antioxidant, antitumor, antihyperglycemic, analgesic, anti-inflammatory, antipyretic, nematicide, and lubricant properties [29]. The findings obtained in this study were corroborated by Tarek R. Elsayed, who previously documented that *Actinomycetes*, known for their complex morphology and physiology, exhibit a significant focus on secondary metabolism. This

highlights the prominent role of secondary metabolism in the developmental hierarchy of bacteria [30]. Also, the studies conducted by Nas et al. showed that the secondary metabolites extracted from *Bacillus* ssp. by the ethyl acetate and chloroform method have more than 56 active compounds, including tert-butyl phenol compounds, fatty acid methyl esters due to the methylation procedure, hydrocarbons, aldehydes, benzoquinones, pyrrols, and terpenes. The literature documents that these compounds possess a broad range of biological and pharmacological uses [31].

The analysis of our findings revealed a reduction in the necrosis rate and an elevation in the cell apoptosis rate after the incubation of PC-3 cells with the crude extract derived from strains 56 and 60. The morphological analysis was consistent with the findings obtained from apoptosis in PC-3 cells. The investigation into DNA fragmentation has provided more evidence that the sample fractions induced damage to the DNA of cancer cells. The encouraging outcome of nuclear staining served as the foundation for further examination of the same fractions by DNA fragmentation analysis. The results demonstrated that the control DNA had an intact structure, but the DNA subjected to fractions 56 and 60 displayed patterns indicative of damage and fragmentation.

Consistent with the findings of the current study, Lin et al. used physical and chemical techniques to isolate marine sediments in their investigation. The researchers conducted a screening of marine actinomycetes, namely *Streptomyces* spp, which exhibited cytotoxic action. They used fermentation methods to scale up the production of the extract. The metabolites of the crude extract of Lu01-M were acquired using ethyl acetate as the solvent. The research demonstrated that Lu01-M effectively inhibits cell growth in human prostate cancer PC-3, DU145, and LNCaP cells, with IC_{50} values of 1.03 ± 0.31, 2.12 ± 0.38, and 1.27 ± 0.25 μg/ml, respectively. Lu01-M exerted harmful effects via many pathways, involving induction of cell apoptosis, necroptosis, autophagy, ER stress, and suppression of colony formation and cell migration. Furthermore, Lu01-M caused cell cycle arrest specifically in the G2/M phase and resulted in DNA damage [16].

In 2022, Wang et al. demonstrated that 5'-epiquisetin had cytotoxic properties against four specific prostate cancer cell lines, namely LNCaP, 22Rv1, DU145, and PC-3 cells. 5'-Epiequisetin is a compound derived from tetramic acid that has been obtained from the sea sponge *Fusarium equiseti*. 5'-epiquisetin suppressed the growth of prostate cancer cells (LNCaP, 22Rv1, DU145, and PC-3) and normal prostatic cells (WPMY-1) to different extents, depending on the dosage, as shown by the MTT experiment. Notably, it had the most impact on PC-3 cells, with an IC_{50} value of 4.43 ± 0.24 μM. The selective cytotoxicity index (SI) values of Eeq for PC-3, DU145, 22Rv1, and LNCaP cells were 4.55, 1.64, 1.38, and 0.57, respectively. These results indicate that 5'-epiquisetin exhibits the highest selectivity towards PC-3 cells. Subsequent research shown that it has a substantial impact on the regulation of clonal colony formation, apoptosis, and migration of PC-3 cells. The PC-3 cell line, which is a kind of prostate cancer cell, lacks the androgen receptor. Therefore, the results indicate that 5'-epiquisetin does not achieve its anti-prostate cancer actions via influencing the androgen receptor signaling pathway. Subsequently, it must exercise its effects via other channels [32].

Apoptosis is a process of active, programmed cell death controlled by numerous genes. Exogenous or endogenous signal molecules can initiate apoptosis. The extrinsic route can be conveyed to the cell via the death receptor, facilitating the activation of *caspase-8*, *caspase-3*, and the cleavage of PARP [33]. The intrinsic apoptotic pathway affects the mitochondria by activating *Bax* and *Bcl-2* proteins. This leads to alterations in the permeability of the mitochondrial membrane, ultimately resulting in the subsequent activation of *caspase-9*, followed by *caspase-3*. *Caspase-3* is the principal protein involved in the execution of apoptosis. *Caspase-3* can interact with several substrates, primarily PARP molecules [34]. The results obtained from our present study demonstrate that the crude extract derived from strains 56 and 60 has inhibitory effects on PC-3 cells via the induction of apoptosis in an in vitro condition. This study showed that the crude extract obtained from strains 56 and 60 activates the intrinsic apoptotic pathway. In particular, it has been observed that the levels of *Bax* and *caspase 3* are increased, but the expression of *Bcl-2* is decreased. In addition, the expression level of *caspase 8* also increased, which probably indicates the activation of the external pathway of cell apoptosis. The flow cytometry analysis revealed that the extract derived from strains 56 and 60 has the ability to trigger apoptosis and halt the cell cycle at G0/G1 phase in PC-3 cancer cells. Finally, apoptosis occurs as a result of G0/G1 phase arrest. In cancer, the expression of pro-apoptotic factors like Bax is inhibited, while anti-apoptotic proteins like Bcl-2 are increased, leading to the stimulation of uncontrolled cell proliferation. Therefore, manipulating the pro-apoptotic proteins involved in the apoptotic pathways to induce apoptosis in cancer cells is a very successful approach for treating cancer [35]. Our investigation revealed that the organic extracts obtained from strain 56 and 60 exhibited cytotoxic effects on PC-3 cancer cells, with IC_{50} values (half-maximal inhibitory concentration) of 50 μg/ml. Thus, when compared to *Bacillus* species that have been investigated before, ethyl acetate extracts have the ability to cause notable effects at lower doses. Also, this study evaluated the migration of PC-3 cells after treatment

with bacterial supernatants. According to the studies, most PCs are curable, but metastatic forms are associated with lower survival [27]. According to the findings of the current investigation, crude extracts of strains 56 and 60 reduced the migration and invasion potential of PC-3 cells. The outcomes of the current investigation were consistent with the results reported in prior studies about actinomycetes. Elmallah and colleagues emphasized that secondary metabolites produced from marine actinomycetes have the potential to downregulate survivin and XIAP, resulting in increased sensitivity of MDA-MB-231 and HCT116 cells to cell death [36].

The researchers discovered five novel β-resorcylic acid derivatives, namely 14-hydroxyasperentin B (1), β-resoantarctines A-C (3, 5, 6), and 8-dehydro-β-resoantarctine A (4). These compounds were obtained from the ethyl acetate extract of the fungus *Penicillium antarcticum* KMM 4685, which was found in association with the brown alga *Sargassum miyabei*. Additionally, a known compound called 14-hydroxyasperentin (also known as 5'-hydroxyasperentin) (2) was also isolated. The results showed that compounds 3, 4 and 5 showed significant cytotoxicity in LNCaP, DU145 and 22Rv1 cells. However, they did not show any action on PC3 and VCaP cells even at a concentration of 100 μM. Leshchenko et al. employed PC-3 and DU145 cells, which lack a full androgen receptor (AR) and hence do not react to hormone therapy. PC3-DR cells are resistant to both docetaxel and hormone treatments. 22Rv1 and VCaP cells are likewise unresponsive to hormone therapy because they have the AR V7 (AR-V7) splice variant, which causes ligand-independent activation of the AR pathway. and hormone-sensitive LNCaP cells, which express unaltered wild-type AR (complete AR) [37]. PC-3 cells are unique among prostate cancer cell lines like LNCaP and 22Rv1 because they do not have functional androgen receptors and do not produce prostate-specific antigen (PSA). This lack of androgen receptor expression and PSA production in PC-3 cells is clinically important because it indicates that these cells are not dependent on androgen for growth [38]. In confirmation of the results obtained from this research Kumar and colleagues showed that the supernatants obtained from the *B. cereus* and *B. pumilus* isolated from soil have a cytotoxic effect on cancer cells by destroying DNA and inducing apoptosis [39].

The decanoic acid compound obtained from the bacilli collected in this work is a saturated fatty acid with a linear C10 chain. Research has shown that decanoic acid effectively inhibits the growth of human liver cancer cells by downregulating HGF/c-Met signaling pathways both in vitro and in vivo. decanoic acid was shown to inhibit HGF-induced activation of c-Met and its downstream signaling. decanoic acid induces programmed cell death and suppresses the production of carcinogenic proteins. Furthermore, decanoic acid inhibited tumor growth and lung metastasis in a mouse model of hepatocellular carcinoma [40]. Contrary to prior research that highlighted the cancer-fighting characteristics of the artificial version of these substances, this study specifically examined the metabolites derived from natural sources. Furthermore, there is no substantiated proof of the anticancer properties of secondary metabolites derived from bacilli found in soil, specifically in relation to prostate cancer. Afterwards, methods such as western blotting may be used to assess the potential anticancer characteristics of the separated metabolites.

Conclusion

Our results support the notion that secondary metabolites produced from soil have the potential to serve as new pharmacological substances. The discovery of these compounds may lead to modifications in treatment approaches within the field of cancer. The recent study findings have shown that these metabolites can trigger apoptosis in the human prostate cancer cell line. In addition, secondary metabolites from *Bacillus* species derived from soil have antimicrobial potential against pathogenic strains. To summarize, we acquired secondary metabolites that were extracted from soil bacilli. The results of GS-MS analysis showed that these strains produce secondary metabolites with different biological activities. The discovered chemicals include dodecanoic acid, molybdenum and titanium (IV) butoxide, which have previously been confirmed to have anti-cancer properties. The metabolites derived from *B. lichaniformis* and *B. siamensis* hindered the growth and movement of cells in a prostate cancer cell line by triggering cell death, halting the cell cycle, and causing DNA damage. The results of the present study showed that treatment with the crude extract obtained from *B. lichaniformis* and *B. siamensis* leads to apoptosis of PC-3 cells with a rate of 46.2% and 50.09%, respectively. In addition, the cell cycle was stopped in the G0/G1 phase, and these events were confirmed by increasing the expression of *caspase 3*, *caspase 8*, and *Bax* genes and decreasing the expression of *Bcl-2* gene. The findings suggest that the separated secondary metabolites show promise as a possible treatment for prostate cancer.

Materials and methods

Sample collection

This research is a descriptive cross-sectional investigation that employs specific inclusion and exclusion criteria. The inclusion criterion focuses on the generation of metabolites by *Bacillus* spp, while the exclusion criterion pertains to the impact of these metabolites on apoptosis induction in prostate cancer cells (PC-3). Following the previous research done by Shahniani et al.,

70 soil samples were collected from different parts of Chaharmahal and Bakhtiari provinces in the spring of 2018, and these samples were obtained from a depth of 10 to 15 cm next to the plant roots. Then, the collected samples were transferred to the laboratory to isolate and identify gram-positive bacteria. To determine the phenotype of the bacteria, an analysis was conducted on the macroscopic and microscopic properties of each isolate after their culture and purification. Following the first identification process, the isolate that showed the highest potential for producing antimicrobial compounds with a broader spectrum of effects was determined via molecular identification methods. Genotypic identification of isolated soil isolates was done using *16SrRNA* gene sequence, *16SrRNA* forward and reverse primers, and polymerase chain reaction (PCR) [41]. Initial DNA extraction was conducted using a CinnaGen kit (Iran). Ensured DNA quality by measuring light absorption at 260/280 nm wavelength. To create a 25 µL master mix, mix 18 µL sterile distilled water, 2.5 µL 10X PCR buffer, 0.75 µL MgCl2, 0.5 µL dNTP, 1 µL forward and reverse primers (10 pmol/µL), 0.25 µL polymerase enzyme, and 1 µL template DNA. Final PCR program using thermal cycling equipment (Eppendorf, Germany). It was done at 95 C° for 30 s, 45 s at 56 C°, and 30 s at 72 C°. This process was repeated 30 times. A phylogenetic analysis was performed to compare the similarity of the sequences obtained in this study with the nucleotide databases of GenBank. For this purpose, the BLAST tool was used and the sequences selected from the bacterial strains isolated from the soil were evaluated with 10 sequences from the reference strain. The two stains' sequence was submitted to the National Center for Biotechnology Information (NCBI) Data Bank and assigned accession numbers, which are listed in the Data Availability section.

Measuring the antimicrobial activity of gram-positive isolates

Following the initial discovery of gram-positive bacteria, a further investigation was conducted to explore their potential for creating antibiotic substances. This investigation used the agar well diffusion technique. One loop of selected pure isolates was inoculated into 50 mL of tryptic casein broth (Merck, Germany) culture medium and incubated at 37 °C and 250 rpm in a shaker incubator for 24 to 72 h. After the proliferation of the desired microorganisms, sterile sampling was conducted. 100 ml of the culture medium was centrifuged at 10,000 rpm for 20 min. The resulting supernatant was then collected for antimicrobial activity evaluation. To evaluate the antibacterial activity of the secondary metabolites isolated from the target gram-positive bacteria, standard strains of *Escherichia coli* (*E. coli*) (PTCC1399), *Staphylococcus aureus* (*S. aureus*) (PTCC1189), and *B. cereus* (PTCC1154) were prepared from the Scientific and Industrial Research Organization of Iran, the collection center of industrial and infectious fungi and bacteria. The bacteria were cultured in nutrient broth (Condalab, Spain) for 18–24 h at 37 °C. After that, the bacterial suspension was adjusted to a turbidity comparable to 0.5 McFarland solution ($1–2\times10^8$ CFU/mL, OD_{600}). The aseptic brush was immersed in the solution and, afterward, used to disperse bacteria evenly onto a Muller-Hinton agar (Condalab, Spain) medium, aiming to achieve equal distribution throughout the surface of the plate. Cork borers were used to excise agar wells of 6 mm diameter, which were then filled with 100 µL of filtered supernatants. The plates were then incubated at 37 °C for 24 h. The millimeter-sized inhibition zones surrounding the wells were measured after incubation to assess the antibacterial effect [42].

Optimizing the production of secondary antibacterial metabolites

The purpose of optimization is to obtain the best environmental conditions for the production of antimicrobial metabolites with the highest production rate. Glucose, lactose, maltose, and peptone were used to determine the most suitable carbon and nitrogen sources. The above sources added 1% to the broth's Tryptic Soy Broth (TSB) (Merck, Germany) medium. After the inoculation of the bacteria, the culture medium was incubated at the specified optimal temperature for 72 h. Also, the effect of pH (5–9) and temperature (30, 33, and 40) on optimizing the growth conditions of selected bacteria was investigated. Antimicrobial activity was measured using the agar well diffusion method against pathogenic bacteria *E. coli*, *B. cereus*, and *S. aureus*, and the plates were incubated at 37 °C for 24 h [43].

Extraction of compounds

The selection of the *Bacillus* isolates, which exhibited high activity, was based on the outcomes of primary and secondary screening (strains 56 and 60). These isolates were then subjected to a liquid-liquid extraction technique using several solvents to identify the bioactive metabolites. To the fermented culture filtrate, equal volumes of butanol, diethyl ether, petroleum ether, ethyl acetate, hexane, chloroform, acetone, methanol, and ethanol (bought from Sigma Company) were added and vigorously shaken for 30 min. In this method, each solvent was added separately to the culture medium. The two phases were subjected to vortexing for one hour. Then, a separating funnel was used for liquid-liquid extraction, and the resulting solution was added to it with two phases that did not dissolve in each other. The lower aqueous phase was removed, while the upper organic phase was collected and subjected to evaporation using

a water bath maintained at a temperature of 40 °C. The thoroughly desiccated crude extract was collected and used for further investigations [44].

GC-MS analysis

Gas chromatography-mass spectrometry (GC-MS) was used to analyze the constituents of the crude extract from strains 56 and 60. The starting temperature of the gas chromatography (GC) oven was set to 50 °C and held for 5 min. Following this, the temperature was incrementally raised at a rate of 5 °C per minute until it reached a final temperature of 260 °C. The injector temperature was set to 250 °C, and the helium gas flow rate was set to 1.0 ml per minute with a 10:1 split ratio. The volume of the injected sample was 1 µl. The ion source temperature in the MS system was set to 250 °C, while the voltage applied was 70 eV. The examination was conducted at two separate times [45].

In vitro cytotoxicity study

Cytotoxicity examination

The underlying concept of the MTT test is the enzymatic conversion of tetrazolium salt into formazan, producing a blue-colored compound. The enzyme succinate dehydrogenase facilitates this conversion process. A total of 2×10^6 PC-3 cells (human prostate cancer cell line) and Human primary prostate epithelial cell (HPrEpC) (Institute Pasteur Institute, Iran) were distributed over 96- and 24-well plates and incubated in a carbon dioxide (CO_2) incubator for an entire night. The cells were treated with concentrations ranging from 12.5 to 200 µg/mL of a pure chemical of strains 56 and 60 for 24 h, after which they were exposed to a reagent at a concentration of 0.5 mg/ml. The culture medium was used as a negative control. The plates were incubated for 3 h in aluminum foil. ELISA readers measured absorbance at 570 nm as a standard. The dose-response curves for PC-3 and HPrEpC cell lines indicated the percentage of inhibition and the extract concentration required to inhibit cell growth by 50% (IC_{50}) [46]:

$$\%\text{of cell viability} = [\text{Absorbance of a sample at 570 nm}/\text{Absorbance of control at 570 nm}] \times 100$$

Annexin-V/Propidium iodide apoptosis detection assay

Apoptosis rates in PC-3 cell populations treated with crude extracts of strains 56 and 60 were determined using the Apoptosis Detection Kit (APC)/annexin V (Mab Tag, Iran), following the instructions provided by the manufacturer. The cells were placed on 60 mm plates with a density of 2×10^6 and were exposed to a dose of 50 µg/mL of the crud extract of *Bacillus* spp (strain 56 and 60) for 24 h. The cells were then collected using trypsinization for 3 min, then washed with PBS and finally suspended in the binding buffer included with the kit. The cells that did not receive any treatment were labeled as the untreated control. The cells were treated with APC/annexin V and PI at room temperature for 15 min in a darkroom prior to the addition of binding buffer. Following this, the cells underwent examination using a flow cytometer (BD FACS Calibur), and the inquiry results were calculated using BD Cell Quest Pro Ver.6.0 software [47].

Investigation of cell cycle arrest

The assessment of cell proliferation was conducted using propidium iodide (PI) staining. The DNA content is used to ascertain the phase of the cell cycle, since the amount of DNA present is closely correlated with the binding of PI to DNA. The cells were cultivated in complete media in 6-well plates at a density of 1×10^6 cells per well. Following overnight incubation and three washes with PBS, the cells were exposed to a concentration of 50 µg/mL of the crude extract derived from *Bacillus* spp (strains 56 and 60) for a duration of 24 h in a complete medium. Subsequently, the cells were collected and preserved in 70% refrigerated ethanol for a duration of one night at a temperature of 4 °C. Afterwards, the cells were treated with 450 µL of PI solution (including RNase) in the absence of light for 20 min at room temperature. Finally, the cells were examined using flow cytometry. The experiments were replicated thrice [48].

Comet assay

The comet assay was conducted following the methods described in the relevant literature. The slides were submerged in a lysis solution containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 90 mM sodium sarcosinate, NaOH (pH=10), 1% Triton X-100, and 10% DMSO. Subsequently, the slides were refrigerated at 4 °C for 2 h. DNA was unraveled for 30 min on horizontal slides in an electrophoresis chamber. Next, electrophoresis was performed at 25 V and 300 mA for 20 min. Post-electrophoresis, slides were washed thrice in a neutralizing buffer (400 mM Tris, HCl, pH 7.5). After treating the slides with ethidium bromide (10 µg/ml), a fluorescence microscopy apparatus (Motic, Germany) with a green filter magnified them 100 times [48].

Gene expression analysis of apoptotic and anti-apoptotic genes

The expression levels of apoptotic genes, including *caspase 3*, *caspase 8*, and *Bax*, and the anti-apoptotic gene *Bcl-2* in PC-3-treated cancer cells were verified using Real time PCR. Initially, over 24 h, the cancer cells were treated with a crude extract derived from *Bacillus* spp, using varying doses of 50 µg/ml and 50 µg/ml. Subsequently, the whole RNA was extracted from both the

Table 2 The qPCR primers designed by primer3plus tool

Gene name	Sequence 5′ to 3′	Product size
GAPDH	F: CGAGATCCCTCCAAAATCAA	170 bp
	R: TTCACACCCATGACGAACAT	
Bcl-2	F: GGATTGTGGCCTTCTTTGAG	114 bp
	R: GCCGGTTCAGGTACTCAGTC	
Caspase 3	F: TTTTTCAGAGGGGATCGTTG	151 bp
	R: CGGCCTCCACTGGTATTTTA	
Caspase 8	F: TATGGCACTGATGGACAGGA	232 bp
	R: GCAGAAAGTCAGCCTCATCC	
Bax	F: GACGGCCTCCTCTCCTACTT	106 bp
	R: CTCAGCCCATCTTCTTCCAG	

control and treated cells, and the process of cDNA synthesis was carried out according to the guidelines provided by the manufacturer using a cDNA synthesis kit (Parstous, Iran). Following the cDNA production, the investigation of apoptotic gene expressions was conducted using RT-PCR analysis. The fold difference was normalized using the $2^{-\Delta\Delta ct}$ method. The sequences of the primers used are listed in Table 2. The *GAPDH* gene was used as a reference gene [49].

Assessment of cell migration (scratch test)

PC-3 cells were inoculated onto 6-well plates and allowed to incubate for one night. Subsequently, a linear incision was created inside the cellular monolayer using a delicate scratch instrument or implement. Using caution to preserve consistency in the scratch tool or knife is essential, ensuring uniformity in scratch shape and breadth. Subsequently, following the creation of the scratch, an image was expeditiously taken using a cellular microscope to serve as the baseline time point (0 h). Each well received the growth media with *Bacillus* spp. Crud extract and the cells were incubated at 37 °C, 5% CO_2 for 24 h. Cells treated with blank culture medium were considered as a control group. After incubation, the same region was imaged again using a cell microscope, and the scratch breadth was measured [49].

Statistical analysis

The data was subjected to statistical analysis using GraphPad Prism 5.0, and the results were presented as the mean±standard deviation. The data underwent examination via the use of a one-way analysis of variance (ANOVA). To facilitate comparisons between group pairings, a T-test was used at a significance level (*p*-value) of 0.05.

Abbreviations

mCRPC — Metastatic castration-resistant prostate cancer
PCa — Prostate cancer
E. coli — *Escherichia coli*
S. aureus — *Staphylococcus aureus*
B. cereus — *Bacillus cereus*
GC-MS — Gas chromatography-mass spectrometry
CO_2 — Carbon dioxide
PI — Propidium iodide
ANOVA — One-way analysis of variance
B. lichaniformis — *Bacillus lichaniformis*
B. subtilis — *Bacillus subtilis*
B. amyloliquefacience — *Bacillus amyloliquefacience*
B. methylotrophicus — *Bacillus methylotrophicus*
B. siamensis — *Bacillus siamensis*
B. cereus — *Bacillus cereus*

Supplementary Information

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Supplementary Material 1

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Author contributions

Z.B. Responsible author and presentation of the original idea. AS: Conducting tests related to the article, Writing and editing articles. A.S. and F.M. Writing and editing articles, Conducting statistical analysis and parts of experiments. L.R. Perform statistical analysis and editing.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors declare no competing interests.

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