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SECONDARY METABOLITES OF ANTAGONISTIC BACTERIAL STRAIN *Cytobacillus firmus* TCP1 EXERT ANTIBACTERIAL AND ANTICANCER ACTIVITY AGAINST LUNG CANCER A549 CELLS

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Marine bacteria are abundant producers of many different kinds of bioactive substances. In this study, we isolated the antagonistic marine bacterial strain *Cytobacillus firmus* TCP1 from soil samples collected from the shoreline and evaluated the antibacterial and anticancer effects of its crude secondary metabolites. The potent antagonistic bacterial isolate *C. firmus* was identified using 16S rRNA sequencing analysis. The secondary metabolites from strain *C. firmus* were extracted by centrifugation with ethanol, and the agar well diffusion method was used to evaluate them for antibacterial activity against respiratory tract bacterial pathogens. This study demonstrated that the metabolic extract of the TCP1 effectively inhibited the growth of respiratory tract bacterial pathogens. Moreover, it established concentration-dependent cytotoxicity on A549 lung cancer cells. The administration of metabolic extract of the TCP1 showed an effective inhibitory effect on colony formation of A549 cells, increased lipid peroxidation, and decreased the antioxidant activity in A549 cells. The results showed that the metabolic extract of the TCP1 showed effective antibacterial and anticancer activity. Therefore, upon additional clinical testing, the secondary metabolites of strain *C. firmus* TCP1 may be exploited to create potent antibiotics and anticancer drugs for lung cancer.

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1. INTRODUCTION

Approximately 10^6 bacterial cells per milliliter are in the seawater column, indicating that the marine environment is complex and home to various living forms. Bacteria and marine fungi are important sources of novel and abundant biologically active compounds. They coexist close to delicate aquatic organisms. They produce biologically active secondary metabolites that, by themselves or when combined with related micro plants, can help them survive in their harsh environment because they lack precise structural defense systems (based on chemical protection) [1]. Recently, several metabolic substances produced by marine bacteria and fungi have significantly increased [2,3], increasing academic and industrial research attention.

"Many bioactive substances from marine microbes have been reported recently, including the active compound Pyron from *Pseudomonas* [4], Agrochelin from *Agrobacterium"* [5], Koromicin from *Pseudoalteromonas* [6] and Pelagiomicin from *Pelagiobacter variabilis* [7], Tiomarinol from *Altromonas* [8], Loloatin from *Bacillus* [9], Marinopyrroles from *Streptomyces* [10].

"The most prevalent bioactive compounds from terrestrial and marine Bacillus spp. are polyketides, macro-lactones, fatty acids, lipoamides, isocoumarin, lipopeptides, and polypeptides. These compounds have a variety of biological activities, including antioxidant, antibacterial, antifungal, antifouling, and, most importantly, anticancer activity" [11-13]. The marine *Bacillus* sp derived Halobacillin effectively induced cell death in MCF-7 cells [14]. "The metabolic products Bacillistatins 1 and 2 isolated from *Bacillus silvestris* exhibited cytotoxicity on the various human cancer cell lines, according to" [15]. In the current investigation, we used 16S rRNA gene sequencing to identify the species of antibiotic-producing bacteria that were isolated from soil samples collected from the coastline. It was discovered that the isolated bacteria belong to the genus *Cytobacillus*. This study investigated the antibacterial and anticancer activity of secondary metabolites isolated from the marine bacterial strain.

2. MATERIALS AND METHODS

2.1 Sample Collection

The seashore soil samples were collected from the tide region of Thandavarayan Cholanganpettai (11°24'45.5"N 79°48'50.2"E) coastal region in Cuddalore district, Tamilnadu, India. At a depth of 5–10 cm, soil samples weighing about 10 and 30 g were collected in sterile plastic bags. After obtaining the removal of stones and plant remains, the soil samples were sieved through a 0.5 mesh sieve. Soil samples were immediately brought into the lab and kept there for future use in a refrigerator.

2.2 Isolation of Marine Bacteria

A soil sample was employed to isolate the bacteria using the dilution spread plate method [16]. One gram of soil was diluted in 10 ml of 50% seawater (1:1 v/v seawater (30 ppt): distilled water). An aliquot of 0.1 mL samples from the 10-5 dilution was then put into Nutrient agar plates with various concentrations of NaCl (10, 20, 30, 40, and 50%) and incubated for 24 hours at 37 °C. After incubation, the plates were checked for visible colonies on the surface of the agar plates. The total number of colonies was counted using a microbiological colony counter. Further, the morphologically distinct bacterial colonies found on nutrient agar (NA) plates containing 20% NaCl were picked and purified. The purified bacterial isolates were stored in NA slants containing 20% NaCl at 4°C. the collected bacterial isolates were designated based on the isolation source viz., TCP1, TCP2, TCP3, TCP4, TCP5, TCP6.

2.3 Collection of Test Bacterial Pathogens

The respiratory tract pathogenic bacterial cultures *Haemophilus influenzae* (ATCC- 49247), *Streptococcus pneunoniae* (ATCC - 19615), *Streptococcus pyogens* (ATCC - 49619), *Pseudomonas aeruginosa* (ATCC - 9027) were obtained from the American Type Culture Collection (ATCC), USA, for the study. The acquired cultures were maintained in NA slants.

2.4 Screening for Antagonistic Activity

Using the cross-streaking method, isolated bacterial strains were evaluated for their antagonistic behaviour toward bacterial pathogens. The bacterial pathogens were cross-streaked next to the test bacteria on Muller-Hinton agar (MHA) plates, which had been streaked with a bacterial test strain. The plates were then incubated at 37 °C for 24 hours. To provide the active organism sufficient time to create the metabolites that penetrate the agar media. After incubation, the plates were checked for visible bacterial pathogen growth inhibition.

2.5 Identification of Antagonistic Bacterial Strain

Using a complete DNA extraction kit (OMEGA BioTek, Norcross, GA, USA) in accordance with the manufacturer's instructions, the genomic DNA of antagonistic bacteria, TCP1, was obtained. After isolating the genomic DNA, it was amplified at the 16S rRNA V4 region using polymerase chain reaction using universal primers 27F (50-AGAGTTTGATCCTGGCTCAG-30) and 1492R (50-GGTTACCTTGTTACGACTT-30) (PCR). The nucleotide sequences of strain TCP1 were discovered and deposited in GenBank (Ass.No: ON873785). The phylogenetic tree was created using a neighbour-joining technique after the nucleotide sequences of the strain were compared to known sequences in the NCBI database using MEGA 6.0.

2.6 Extraction of Secondary Metabolites

The bacterial isolate TCP1 was chosen for further investigation based on its antagonistic behaviour toward bacterial pathogens. A 2000 mL conical flask containing 1000 mL of Nutrient Broth medium with optimum nutrient levels was used to prepare the inoculum of the chosen bacterial isolate. At 30°C, inoculated flasks were incubated for 48 hours while shaken at 200 rpm. After incubation, the culture broth was carefully separated from the supernatant and centrifuged at 10,000 rpm for 20 minutes. In addition, the supernatant was mixed with 100% cold ethanol in a ratio of 13 (v/v) and maintained at 4 °C for 24 hours to precipitate secondary metabolites. Secondary metabolite pellets were dried at 60 °C after being recovered by centrifugation and purified by washing with Milli-Q water.

2.7 Assessment of Antibacterial Activity

A loopful of cells was subcultured to the nutrient broth and incubated for 24 hours at 37 °C to create the active young cultures for the experiment. To match the turbidity to the 0.5 Mc-Farland standard, or around 1×10^6 CFU/ml, the cultures were suspended in the sterile nutritional broth for 24 hours. The test bacterial cultures were swabbed in the well-diffusion assay over the Muller Hinton agar (Kirby-Bauer Method). Then, different amounts of crude secondary metabolite extract from the antagonistic bacteria (100, 150, and 200 µg) and positive control (the common antibiotic ciprofloxacin, 20 µg) were added to the wells. All plates were incubated for 24 hours at 37 °C. The plates were examined after incubation to determine any obvious growth inhibition around the wells. By measuring the inhibition zone, the growth inhibition's diameter was calculated.

2.8 Cell Line

The lung adenocarcinoma A549 cells were procured from National Centre for Cell Science (NCCS), Pune, India. DMEM was used to keep and cultivate the cells, and they were then incubated at 37 ºC with 5% CO_2 and 95% air (humidified environment). The crude secondary metabolite extract from the competing bacteria was dissolved in dimethyl sulfoxide (DMSO) to treat cells.

2.9 Cytotoxicity of Secondary Metabolite Extract of TCP1 on A549

The cytotoxicity of crude secondary metabolite extract of TCP1 on A549 cells was evaluated by employing an MTT assay. The A549 cells (1×10^5) were seeded in 96 wells plates and grown for 24 h in the humified incubator. After incubation, the old medium was replaced with the new medium, and the cells were then exposed to various doses of crude secondary metabolite extract of TCP1 (50, 60, 70, 80, 90, 100, 110, 120, and 130 µg) for 24 hours. Each well was then filled with 100 L of MTT reagent (5 mg/mL in PBS), and the plate was left in the dark for 4 hours. The resultant formazan was dissolved in 100 L of DMSO. Furthermore, an ELIZA plate reader was used to measure the absorption of dissolved formazan at a wavelength of 595 nm (Tecan Multimode Reader, Austria). Calculations were made based on the test sample concentrations, which revealed 50% cell death.

2.10 Biochemical Assay

"The crude secondary metabolite extract of TCP1 was administered to the A549 cells in doses of 80, 109.1, and 140 µg. The cell suspension was then collected using trypsin EDTA and subjected to the biochemical test. The TBARS was assessed in metabolite extract of TCP1 administered A549 cells using the method described by" Ohkawa et al. [17]. By measuring the state of the antioxidants SOD, CAT, and GPx, the antioxidant activities in A549 cells were evaluated in accordance with the methodology described earlier [18-20]. For all assays, the untreated cells were kept as a control.

2.11 Cell Colonization Assay

The A549 cells (1000 cells/well) were seeded in 24-well plates and incubated for 24 hours. The cells were exposed to different concentrations of the crude

secondary metabolite extract of TCP1 (80, 109.1, and 140 µg) in a humified environment for 24 hours. The cells were then gently washed with cold PBS solution and fixed with ethanol (70%) at 20 ºC for the same time. Additionally, the cells were kept at room temperature for 30 minutes while being exposed to 0.01% (wt/vol) crystal violet. The cells were then checked for cell colonization using a light microscope. The well was left untreated as a control.

3. RESULTS

3.1 Isolation of Bacteria

The collected soil sample was serially diluted and inoculated on nutrient agar plates containing different concentrations of NaCl. The maximum number of colonies (29.54±1.42 CFU/g x 10^5) were found on the NA plates containing 20% NaCl, followed by the 10% (23.4±0.69 CFU/g x 10^5) and 30% (19.24±1.25 CFU/g x 10^5). Very least or no visible colonies were observed on the NA plates containing 40% and 50% NaCl (Table 1). Six morphologically distinct bacterial colonies were isolated, purified, and stored for further study. The growth of isolated bacteria TCP 1 on solid media is shown in Fig. 1A.

3.2 Antagonistic Activity of Isolated Bacteria Strain

To determine whether the isolated bacterial strains were antagonistic to the tested bacterial pathogens. Among the tested bacterial strains, isolate TCP1 and TCP4 showed significant growth inhibitory activity against more than 3 examined bacterial pathogens. Other isolates showed inhibitory activity against one bacterial pathogen or non (Fig. 1B). Further, we selected TCP1 for further studies.

3.3 Identification of Antagonistic Bacteria

The nucleotide sequences of the isolated antagonistic bacterial strain were examined for similarities with known sequences using the BLAST search tool. Over 98% identity was found between the derived sequences and the known *Cytobacillus* sp. sequence. The strain's genus and species names were also determined as *C. firmus* (Fig. 2).

3.4 Antibacterial Activity of the Crude Metabolic Extract

The metabolic extract of the TCP1 strain was examined for the bactericidal activity again the respiratory tract pathogens on solid MHA plates. The significant growth suppression activity was established by the crude secondary metabolites of isolate TCP1 according to the concentrations treated. The maximum growth inhibitory activity of the metabolic extract of strain TCP1 was observed against *Haemophilus influenzae* followed by *Pseudomonas aeruginosa* and *Streptococcus pneumoniae*. However, the metabolites extract of strain TCP1 exhibited lesser inhibitory activity against *Streptococcus pyogens* than other tested bacterial pathogens. The zone of inhibition exhibited by the antibiotic Ciprofloxacin was 18 to 20 mm (Fig. 3) and (Table 2).

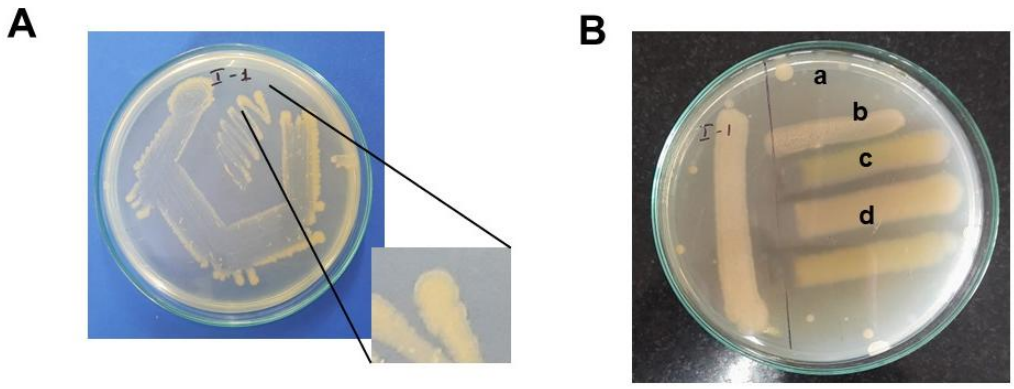

Fig. 1. (A) growth of Isolated bacterial strain *C. firmus* TCP1 on nutrient agar plates. (figure in selection) colony morphology of Isolated bacterial strain *C. firmus* TCP1 on nutrient agar plates. (B) Antagonistic activity of bacterial isolate TCP1 against the human respiratory bacterial pathogens (a) *Pseudomonas aeruginosa (b) Haemophilus influenzae* (c) *Streptococcus pyogens,* (d), *Streptococcus pneunoniae, (*on solid agar medium

Table 1. Total colony forming units in a collected soil sample (CFU/g x 10^5)

NaCl concentration (%)				
10	**20**	**30**	**40**	**50**
23.4±0.69	29.54±1.42	19.24±1.25	1.61±0.13	0.23±.02

The values are expressed in the mean ± standard deviation of three replicates

Table 2. Antibacterial activity and minimal inhibitory concentration of secondary metabolites of strain PK2

Bacterial pathogens	**Zoon of inhibition (mm)**			
	100 µg	**150 µg**	**200 µg**	**Ciprofloxacin (20 µg)**
Haemophilus influenzae	08.76±0.52	10.43±0.35	18.26±0.13	20.25±0.44
Streptococcus pneumoniae	-	08.85±0.35	12.34±0.53	19.31±0.41
Streptococcus pyogens	-	-	09.46±0.33	19.51±0.51
Pseudomonas aeruginosa	07.93±0.73	08.23±0.41	12.34±0.35	18.56±0.76

-: No zone of inhibition. The values are expressed in the mean ± standard deviation of three replicates

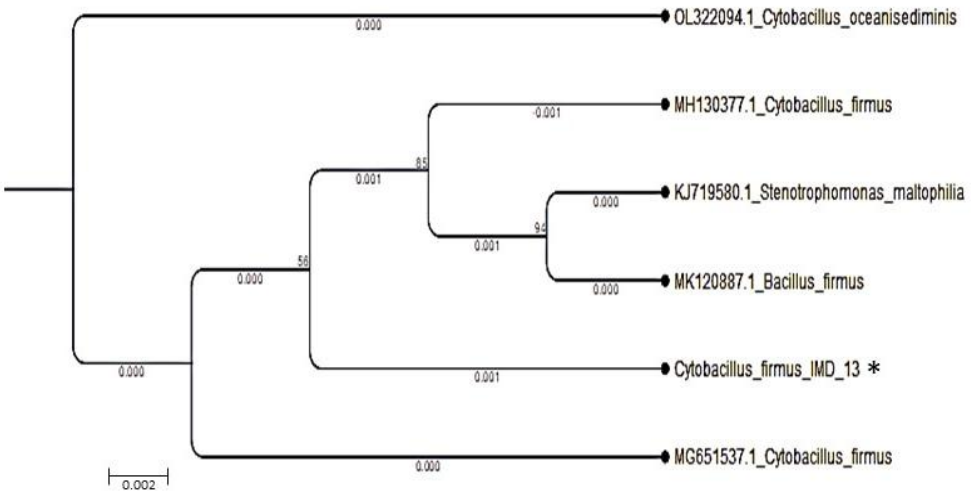

Fig. 2. Phylogenetic classifications of isolated antagonistic bacterial stain TCP1. The tree was constructed by the neighbour-joining method with known bacterial sequences using MEGA 6.0

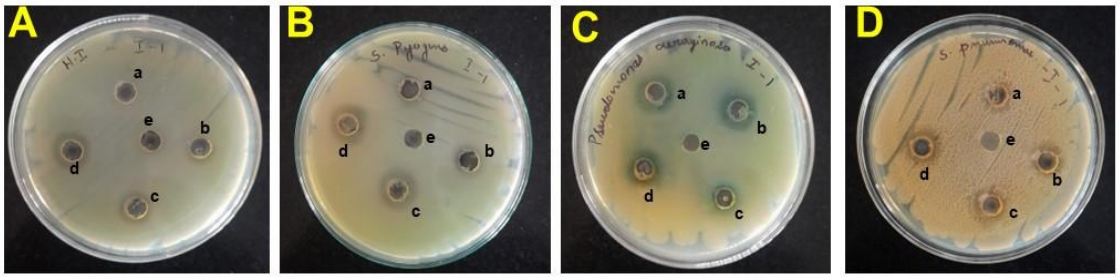

Fig. 3. Antibacterial activity of crude secondary metabolites of TC1 against respiratory bacterial pathogens (A) *Haemophilus influenzae* (B) *Streptococcus pyogens,* (C). *Pseudomonas aeruginosa,* (D) *Streptococcus pneunoniae* on solid MHA plates. (a) 100 µg, (b) 150 µg (c) 200 µg (d) positive control, (e) negative control

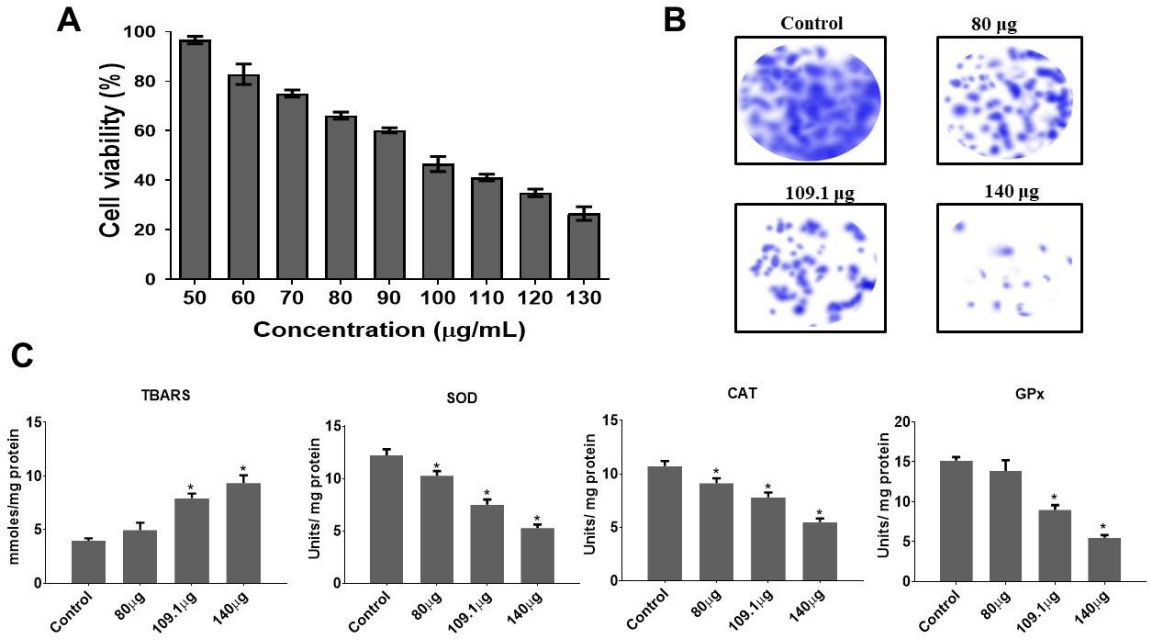

Fig. 4. (A) The cytotoxic effect of secondary metabolites of TCP1 on A549 cells was determined by MTT assay. (B) Images show the effect of secondary metabolites of TCP1 on A549 cells colony formation. The images were taken at 30X magnification and analyzed by ImageJ software. (C) Effect of secondary metabolites of TCP1 on lipid peroxidation (TBARS) and antioxidants such as SOD, CAT, and GPx in A549 cells. The bars reflect the mean ± standard deviation of three experiments

3.5 Cytotoxic Activity and Colony Inhibition of the Crude Metabolic Extract

The MTT assay was used to evaluate the cytotoxic effect that the metabolic extract of TCP1 had on A549 cells. TCP1 metabolic extract administration decreased A549 cell growth in a dose-dependent manner. The metabolic extract of the TCP1's 50% inhibitory concentration in A549 cells was calculated to be 109.10±0.71 µg. As a result, we selected doses of 80, 109.10, and 140 µg for further study (Fig. 4A). Using crystal violet staining, the effect of the metabolic extract of the TCP1 on the colonization of A549 cells was investigated. When supplied in a concentration-dependent way, the metabolic extract of the TCP1 significantly reduced the adhesion of A549 cells compared to untreated cells (Fig. 4B).

3.6 Effects on Lipid Peroxidation and Antioxidant Status

The significant characteristics of oxidative stress-induced apoptosis in cancer cells are reduced antioxidant enzymes and increased levels of the lipid peroxidation biomarker TBARS. In cells exposed to the metabolic extract of the TCP1, the lipid peroxidation (LPO) biomarker's TBARS activity rose at the concentration employed. According to the doses administered, the SOD, CAT, and GPx were considerably decreased in the metabolic extract of the TCP1-administered A549 cells compared to control cells (Fig. 4C).

4. DISCUSSION

"The search for new antibiotics from marine harboured microorganisms resulted in the isolation of 10,000 metabolites" [21], many of which are endowed with pharmacological properties. Numerous biological activities have been identified, including antibiotic, antifungal, cytotoxic, and neurotoxic properties. This work utilized the soil samples taken from the Thandavarayan Cholanganpettai coastline region to isolate the marine antagonistic bacterial strain. Significant bactericidal activity was exhibited by the isolated strains against the tested bacterial pathogens. The hostile bacteria were discovered to be *C. firmus*.

Marine *Bacillus* species are potential sources of secondary metabolites with a wide range of structural characteristics, including lipopeptides, polypeptides, macrolactones, fatty acids, polyketides, lipoamides, isocoumarins, and carotenoids. Complex biosynthetic metabolic pathways give rise to the structurally diverse natural compounds produced by marine

isolates. There is great potential in several of these bioactive chemicals to create powerful pharmaceutical and agrochemical products. *Bacillus* strains obtained from particular niches (such as a hydrothermal vent, deep sea, pH > 9.0, and salt lakes) may produce beneficial bioactive chemicals because of their genetic capacity to adapt to extreme settings [22]. The isolate TCP1 showed effective antibacterial activity in the current study against the tested respiratory tract infections. Against all of the test microorganisms, the crude metabolic extract of *C. firmus* strain showed encouraging antibacterial activity. At low concentrations, *Streptococcus pyogens* were resistant to the tested crude extract. The lipopolysaccharide barrier for the hydrophobic chemicals and the limited permeability of the bacteria's outer membrane may be responsible for this resistance [23,24].

"Amicoumacin A and Bacilosarcin B, two anticancer substances discovered in the strain *Bacillus subtilis* B1779, are significantly cytotoxic against human cervical cancer HeLa cell lines" [25]. According to another study [26], "the metabolic fraction of the Indian *Bacillus* cereus strain exhibited cytotoxicity on Hep-2 cells with an IC50 value of 225.4 µg. The *C. firmus* synthesized silver nanoparticles exhibited significant bactericidal and cytotoxicity on cancer cells" [27]. According to the doses utilized in the current study, we found that the metabolic extract of TCP1 exhibited effective cytotoxicity against the A549 cells. Oxidative stress harms the cellular microenvironment when the synthesis and clearance of reactive free radicals are out of balance. "Lipid peroxidation is a severe side effect of oxidative stress brought on by the increased release of free radicals into the intracellular environment" [28]. Cancer cells enter the early stages of apoptosis due to decreased intracellular antioxidant levels and increased lipid peroxidation [29,30]. Based on the concentration employed, the treatment with the metabolic extract of the TCP1 significantly decreased the levels of the antioxidants SOD, CAT, and GPx in our investigation. In addition, it dramatically increased the level of the lipoperoxidation biomarker TBARS. Overall findings indicate that lipoperoxidation and antioxidant activity were stimulated and suppressed by the metabolic extract of the TCP1 to cause cell death in A549 cells successfully.

5. CONCLUSION

The antagonistic bacteria *C. firmus* effectively inhibited a number of bacterial pathogens. The *C. firmus* crude secondary metabolic extract on MH agar plates exhibited effective bactericidal activity against respiratory bacterial pathogens. A crude secondary metabolic extract also demonstrated cytotoxicity activity according to the concentrations used. Moreover, it significantly increases lipid peroxidation and decreases the antioxidants in A549 cells. The results indicate that the crude secondary metabolic extract of *C. firmus*, an antagonistic bacterial strain, has potent antibacterial and anticancer properties in an in-vitro condition. Therefore, following appropriate clinical trials, it could be used to treat infectious diseases and lung cancer.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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