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Antagonistic, antibiofilm antioxidant and anticancer activity of secondary metabolites of isolated bacteria from mangrove soil

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Abstract

Different antagonistic and antioxidant medications are mostly derived from bacterial metabolites generated by mangrove species. The antagonistic bacterial strain isolated PP1 bacteria was isolated from soil samples, and its crude secondary metabolites were evaluated for antagonistic, antioxidant, and cytotoxic characteristics. Using ethanol centrifugation, the secondary metabolites of the PP1 strain were isolated, and their antagonistic action toward clinical bacterial pathogens was evaluated. Additionally verified MCF-7 breast cancer cell anticancer activity in a concentration-dependent manner. These findings imply that the isolated bacterium PP1's secondary metabolites may be useful as antioxidant and antagonistic substances. It is crucial to keep in mind that more research, such as clinical trials, would be required to thoroughly assess the efficacy and safety of using these metabolites in either people or animals.

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Introduction

In mangrove ecosystems, there are several microbial communities, including bacteria, fungi, archaea, and protozoa, and physical and chemical variables affect their activity and abundance. Because of their particular geographic circumstances, mangrove habitats offer a home for a variety of microbial species (Xu *et al.*, 2014). Diazotrophs, phosphate solubilizers, cellulose decomposers, nitrifiers and denitrifiers, sulphur oxidizers, and iron oxidizers are among the bacterial species that may be found in mangrove environments (Holguin *et al.*, 2001). Microbiological ecologists are interested in mangrove ecosystems because of their complexity and dynamic circumstances. They want to learn more about these ecosystems.

They are frequently found in tropical and subtropical climates and are distinguished by their capacity to flourish in environments with high salt levels (Gonzalez-Acosta *et al.*, 2006). These activities, which are carried out by a variety of microorganisms including bacteria, fungi, archaea, and protozoa, are crucial for the ecosystem's health and efficiency. These bacteria are involved in the heterotrophic food chain and actively degrade the leaves and wood of terrestrial mangrove trees. Estuarine systems' microbial activity levels are complex, varied, and impacted by a number of elements, including physical and chemical circumstances (Alongi, 1994).

Cancer is a chronic condition caused by the growth of abnormal cells in human tissues, making it the second most deadly disease in the world with an annual increase in incidence. The high cost and possibility for toxicity of several synthetic drug-based chemotherapeutic preventative medicines have limited their use in the treatment of cancer.

Today, research into isolating anticancer drugs from plants is rapidly expanding. According to the current study, most secondary metabolites found in a wide range of plant families showed a unique emphasis on their potential to be developed into anticancer medicines (Jain and Jain, 2011; Bhanot *et al.*, 2011).

Breast cancer is the most often diagnosed condition and the main cause of cancer mortality in women, accounting for 23% of all cancer diagnoses and 14% of cancer deaths in this population. Research in this field is therefore essential to reducing the financial and psychological burden (Gunduz and Gunduz, 2011). It has been clear in recent years that breast cancer is actually a group of molecularly distinct tumours that arise from the breast's epithelial cells rather than a single disease (Done, 2011).

The objective of the current study was to identify the efficient bacterial strain from the mangrove estuary area that produces beneficial compounds. Additionally, we wanted to evaluate its anticancer, antibiofilm, antagonistic, and antibiofilm activities.

Materials and methods

Sample collection

Soil samples were collected from the Parangipettai mangrove estuary region (11°30'12.3"N 79°46'30.5"E) in Cuddalore district, Tamilnadu, India. About 10-30 g of samples were collected at a depth of 5-10 cm in sterile plastic bags. Soil samples were sieved through a 0.5 mesh sieve and immediately brought into the lab and stored in a refrigerator for future use.

Isolation of marine bacteria

Using the dilution spread plate method developed by Warcup (1950), the bacteria were recovered from a soil sample. 10 ml of 50% salt water and 1 g of dirt were combined (1:1 v/v seawater (30 ppt): distilled water). The 10-5 dilution aliquot of 0.1 mL samples were put into Nutrient agar plates with various amounts of NaCl (10, 20, 30, 40, and 50%), and they were then incubated for 24 hours at 37 °C. After incubation, the plates were checked to see if any colonies were visible on the agar plates' surface. Furthermore, using nutritional agar (NA) plates containing 30% NaCl, different morphological bacterial colonies were recovered and purified. The bacterial isolates were purified and stored on NA slants with 20% NaCl at 4 °C. The collected bacterial isolates were labelled PP1, PP2, PP3, PP4, and PP5 based on the source of isolation.

Collection of test bacterial pathogens

The pathogenic bacterial cultures *Escherichia coli (MTCC- 1610), Klebsiella pneumoniae (MTCC-* 661*), Staphylococcus aureus (MTCC-* 1430*), Streptococcus pneunoniae* (MTCC - 655), *and Staphylococcus epidermidis (MTCC- 3382)* were obtained from the Microbial Type Culture Collection (MTCC), Chandigarh, India for the study. The acquired cultures were maintained in NA slants.

Screening for antagonistic activity

The antagonistic behaviour of isolated bacterial strains toward bacterial pathogens was examined. The bacterial pathogens were cross-stretched alongside the test bacteria on Muller-Hinton agar (MHA) plates, and the pathogens were streaked on the same plates. The plates were then kept at 37 °C for a further 24 hours. After incubation, the plates were checked for any evident signs of bacterial pathogen growth suppression.

Extraction of secondary metabolites

We chose bacterial isolate (PP1) for this study because of its capacity to prevent the development of other bacterial infections (antagonistic behavior). They employed Nutrient Broth, a nutrient-rich medium, to produce the inoculum of this isolate and incubated it at 30°C for 48 hours while shaking it at 200 rpm. The culture broth was removed from the supernatant after incubation, and any solid particles were then spun off using a centrifuge. The researchers added cold ethanol to the supernatant and left it to remain at 4 °C for 24 hours in order to separate the secondary metabolites generated by the bacterial strain. The resultant pellets underwent drying, Milli Q water purification, and analysis. Chemical substances known as secondary metabolites are created by bacteria but serve purposes other than growth and reproduction. These substances exhibit a variety of biological actions that make them interesting for prospective uses in medicine and other fields.

Biofilm inhibition assay

The antibiofilm experiment's objective was to assess how well metabolic extracts prevented the growth of biofilm (Christensen *et al.*, 1985). For the biofilm inhibition test, 96 flat-bottom polystyrene titer plates containing 96 wells were utilized. 10 liters of an overnight pathogenic bacterial culture were initially added to wells containing 100 L of Muller Hinton broth (MHB), which is a nutrient-rich broth.

After that, the wells were filled with PP1 metabolic extract at varying doses (50, 100, 150, and 200 µg), and they were incubated at 37 °C for 24 hours. In order to remove any germs that could have been floating about after incubation, the contents of the well were removed and washed with 0.2 mL of pH 7.2 phosphate-buffered saline (PBS). Crystal violet (0.1% w/v) was used to dye the sessile bacterium adhesion after sodium acetate (2%) was employed to fix it. Most of the discoloration was removed by the deionized water wash, which was then let to dry. Additionally, dried plates were cleaned in 95% ethanol prior to being analysed at 600 nm using a microtitre plate reader (Thermo). The formula below was used to compute the percentage of biofilm inhibition.

$$\text{Percentage of biofilm inhibition} = \frac{\text{Abscontrol} - \text{Abssample}}{\text{Abscontrol}} \times 100$$

DPPH free radical- scavenging activity

Using the DPPH free radical test, the antioxidant capacity of a metabolic extract from strain PP1 was assessed. In this test, the sample is combined with a stable DPPH radical solution, and the absorbance at 517 nm is measured following the reaction.

According to Brand-Williams *et al.* (1995), a formula based on the change in colour of the solution was used to compute the scavenging activity percentage (AA%). For comparison, a control solution containing ascorbic acid was also made. 0.3 mL of the DPPH radical solution, 3 mL of ethanol, and 0.5 mL of the sample were added to a reaction mixture in order to conduct the experiment.

Additionally, a blank combination was created using 0.5 mL of the sample and 3.3 mL of ethanol. The DPPH radical solution was prepared by dissolving 0.5 mM DPPH in methanol.

$$\text{Radical scavenging activity (AA\%)} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abscontrol}} \times 100$$

Hydrogen peroxide scavenging activity

The bacterial metabolic extract's capacity to scavenge hydrogen peroxide was assessed using the conventional procedure suggested by Govindarajan *et al.* (2003). In PBS (Phosphate Buffer Saline), hydrogen peroxide (10 mM) solution was suspended (0.1 M, pH 7.4). 50, 100, 150, and 200 µg of various extract concentrations were swiftly combined with 2 ml of hydrogen peroxide solution. The absorbance at 230 nm was measured using a UV spectrophotometer (Shimadzu, UV-160A). Alcohol (3.5 mL) and a hydrogen peroxide solution were combined to create the ascorbic acid control solution.

Cytotoxicity of secondary metabolite extract of PP1 on MCF-7

The cytotoxicity of the crude secondary metabolite extract of PP1 on MCF-7 cells was assessed using an MTT assay. In 96 wells, the MCF-7 cells (110^5) were plated and let to grow for 24 hours in a moist environment. After changing the old medium for the new one during incubation, the cells were then exposed to a range of doses of crude secondary metabolite extract of PP1 (50, 60, 70, 80, 90, 100, 110, 120, and 130 µg) for 24 hours. The plate was placed in the dark for 4 hours after adding 100 L of MTT reagent (5 mg/mL in PBS) to each well. The resultant formazan was dissolved in 100 L of DMSO. Additionally, a 595 nm ELIZA plate reader assessed the absorbance of dissolved formazan (Tecan Multimode Reader, Austria). Calculations based on concentrations of the test sample revealed that 50% of the cells had perished.

Biochemical assay

The 80, 100, and 120 µg of the PP1 crude secondary metabolite extract were given to the MCF-7 cells. The trypsin-EDTA-collected cell suspension was then subjected to biochemical evaluation. The method described by (Kakkar *et al.*, 1984) was used to assess the TBARS in the metabolite extract of PP1-administered MCF-7 cells. By assessing the levels of the antioxidants SOD, CAT, and GPx, the antioxidant activity in MCF-7 cells was evaluated in accordance with the preceding technique reported (Sinha 1972). In all assays, the untreated cells served as the control.

Results

Isolation of bacteria

The soil sample was diluted and applied to nutritional agar plates with various sodium chloride concentrations (NaCl). The plates with 30% NaCl had the most colonies (28.241.62 CFU/g x 10^5) followed by those with 20% NaCl (25.141.32 CFU/g x 10^5) and 10% NaCl (12.320.67 CFU/g x 105). On the plates with 40% and 50% NaCl, there were few or no visible colonies (Table 1). Five distinct bacterial colonies were found, purified, and stored for future research.

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

NaCl concentration (%)				
10	20	30	40	50
15.02±0.64	23.18±0.72	26.42±0.68	3.28±0.72	0.62±0.06

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

Antagonistic activity of isolated bacteria strain

We performed a growth inhibitory experiment to see if the obtained bacterial strains have antimicrobial capabilities against bacterial infections. All of the bacterial pathogens examined showed notable inhibitory effects against the isolate PP1. Inhibitory action was also present in a few of the other isolates against one or more bacterial infections (Fig. 1). In this investigation, we looked for bacteria that produce antibiotics in soil samples taken from the seashore. The PP1 strain was particularly good in preventing the development of bacterial pathogens.

Antibiofilm activity of the crude metabolic extract

In this work, we examined a bacterial strain's (PP1) crude metabolic extract's potential to prevent the

growth of bacterial pathogens' biofilms. Several bacterial pathogens, including *E. coli* and *Streptococcus pneumoniae*, were resistant to biofilm formation when exposed to the crude metabolic extract of strain PP1.

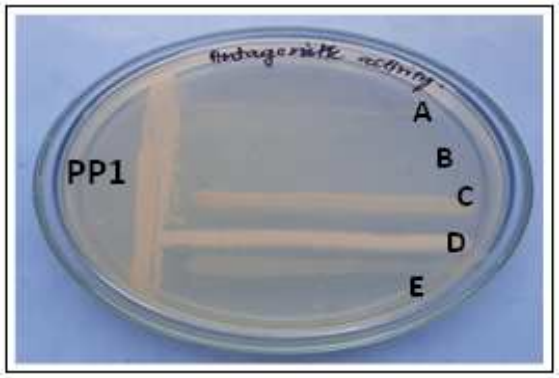

Fig. 1. Antagonistic activity of bacterial isolate PP1 against the clinical bacterial pathogens (A) *Escherichia coli,* (B) *Klebsiella pneumoniae, (C) Staphylococcus aureus, (D) Streptococcus pneumoniae,* and (E) *Staphylococcus epidermidis* on solid agar medium.

This implies that the metabolic extract may have the potential for use as an antibacterial agent to treat illnesses linked to biofilms.

It is important to remember that more research is necessary to completely comprehend the mechanisms of action and possible therapeutic uses of this metabolic extract (Fig. 2).

Antioxidant activity crude metabolic extract

DPPH free radical- scavenging activity

The DPPH free radical scavenging activity of strain PP1's crude metabolic extract was concentration-dependent.

The highest inhibition of 47.33% was seen at a concentration of 200 µg, which is much less than the inhibitory activity of the standard (Ascorbic acid), which is 40 µg/ml. It has the potential to give hydrogen (Fig. 3).

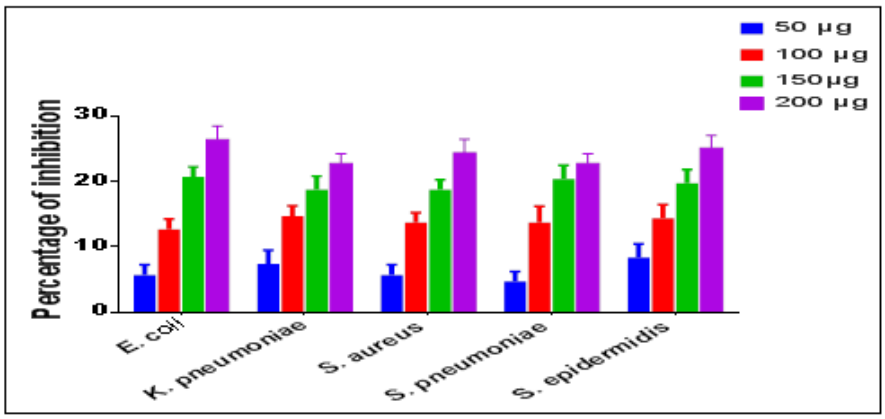

Fig. 2. Biofilm interruption ability of crude secondary metabolites of PP1 on the respiratory bacterial pathogens was tested in 96 well plates. The maximum biofilm inhibition was observed at 200 µg concentration against *E. coli*. Error bars indicate the mean ± standard deviation of three replicates.

Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging activity of strain PP1's crude metabolic extract was concentration-dependent. The maximal inhibition of 36.66% was seen at a concentration of 200 µg, which is much less than the inhibitory activity of the standard (Ascorbic acid), which is 40 µg/ml. It has the potential to donate hydrogen (Fig. 4).

Cytotoxic activity of the crude metabolic extract

The MTT test was used to determine if the metabolic extract of PP1 was cytotoxic to MCF-7 cells. The dose-dependent inhibition of MCF-7 cell growth by the PP1 metabolic extract was observed. The metabolic extract of the PP1's 50% inhibitory concentration in MCF-7 cells was calculated to be 84.32 0.45 µg. As a result, we selected doses of 80, 100, and 120 µg for further investigation (Fig. 5).

Effects on lipid peroxidation and antioxidant status

The key characteristics of oxidative stress-induced apoptosis in cancer cells are decreased antioxidant enzyme levels and increased levels of the lipid peroxidation biomarker TBARS. In cells exposed to the PP1 metabolic extract, the TBARS activity of the lipid peroxidation (LPO) biomarker rose at the utilised concentration. Depending on the doses utilised, the SOD, CAT, and GPx levels in the metabolic extract of the PP1-treated MCF-7 cells were considerably lower as compared to control cells (Fig. 6).

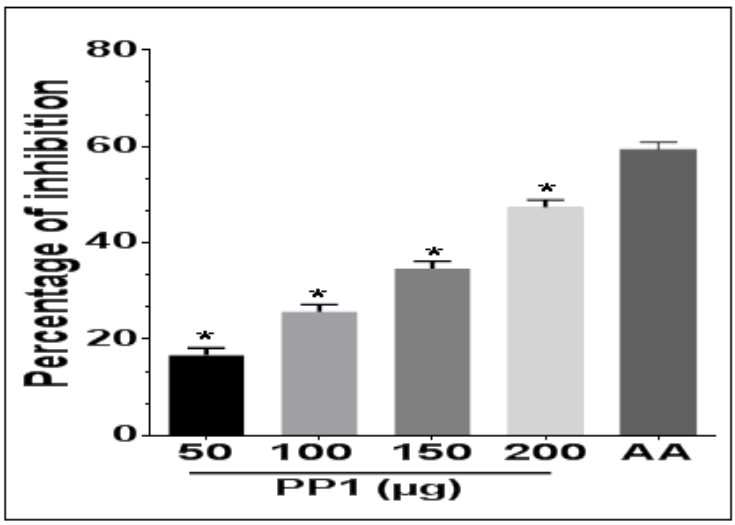

Fig. 3. DPPH radical scavenging activity of secondary metabolites of PP1. The maximum inhibition was observed at 200 µg. Error bars indicate the mean ± standard deviation of three replicates.

Discussion

Due to their fast growth, the existence of multi-enzyme complexes, and tolerance to unfavourable environments, bacteria are thought of as powerful and useful enzyme makers (Ladeira *et al.*, 2015). Numerous studies have revealed that bacteria isolated from mangrove habitats exhibit a variety of advantageous traits (Behera *et al.*, 2014a; Kunasundari *et al.*, 2017). Our results provide evidence that various bacterial populations, which have been demonstrated to create secondary metabolites, exist in mangrove soils. For instance, the bulk of the recovered isolates is from genera that have been connected to the synthesis of several secondary metabolites, including antimicrobials and enzymes, in the past (Naik *et al.*, 2013; Pupin and Nahas, 2014).

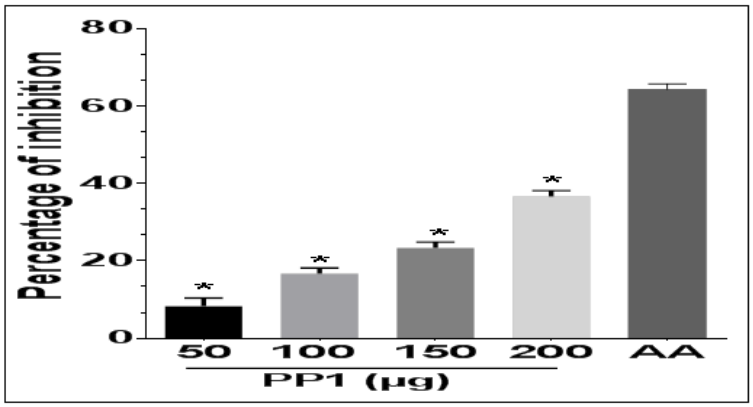

Fig. 4. Hydrogen peroxide scavenging activity of secondary metabolites of PP1. The maximum inhibition was observed at 200 µg. Error bars indicate the mean ± standard deviation of three replicates.

The endophytes of mangrove plant species have grown in prominence as a significant source of beneficial metabolites (Ananda and Sridhar 2002). Therefore, the goal of the current study was to determine the variety of endophytic bacteria in marine halophytic plants. A uniquely adapted group of woody plants called mangroves and mangrove allies can be found between land and water. They are specially adapted with salt-excreting glands, stilt roots, prop roots, pneumatophores, high phenolic content, and higher UV-absorbing chemicals, among other features (Kathiresan and Bingham 2001).

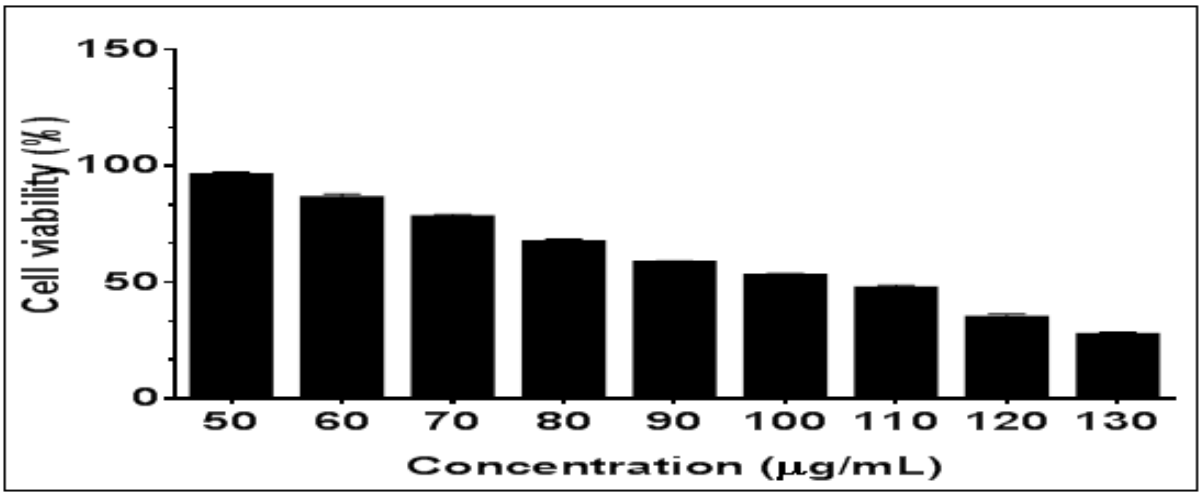

Fig. 5. The cytotoxic effect of secondary metabolites of PP1 on MCF-7 cells was determined by MTT assay.

The succinate dehydrogenase enzyme transforms MTT into formazan salt in live cells. After 4 hours, a stopper reagent was introduced. The stopper reagent, which also lyses the cell membrane, dissolves the formazan salt. To measure the formed formazan salts, a spectrophotometer was employed. The higher the absorbance, the more live cells are present because the CS polymer's approach for drug administration comprises a very high amine group, which enhances the CS affinity through electrostatic interactions with the cancer cell membrane (Ghasemi *et al.*, 2021). We found that at the concentrations utilised in the present study, the metabolic extract of PP1 is effectively cytotoxic against the MCF-7 cells.

When the generation and elimination of reactive free radicals are out of balance, oxidative stress damages the cellular microenvironment.

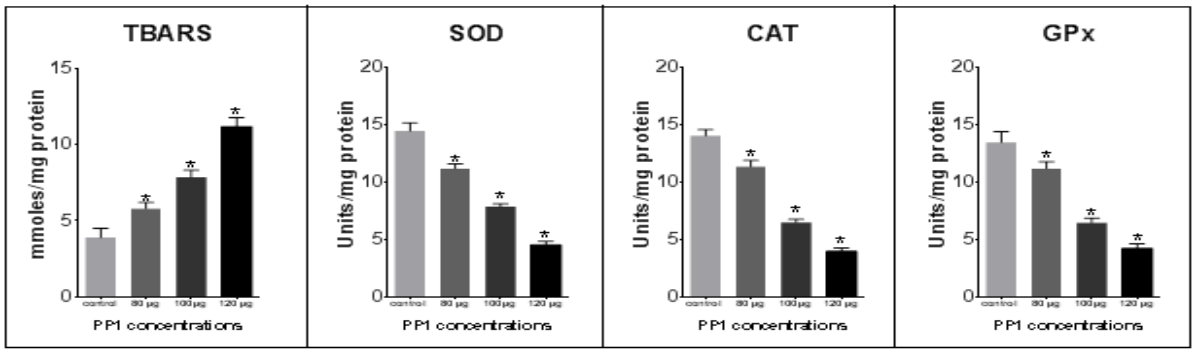

Fig. 6. Effect of secondary metabolites of PP1 on lipid peroxidation (TBARS) and antioxidants such as SOD, CAT, and GPx in MCF-7 cells. The bars reflect the mean ± standard deviation of three experiments.

Lipid peroxidation is a harmful consequence of oxidative stress, which is brought on by a rise in the release of free radicals into the intracellular environment (Simon *et al.*, 2000). Cancer cells undergo the early phases of apoptosis as a result of decreased intracellular antioxidant levels and

increased lipid peroxidation (Shilpa *et al.*, 2012; Karthikeyan *et al.*, 2015). Depending on the dosage employed, the treatment with the metabolic extract of the KD1 significantly decreased the levels of the antioxidants SOD, CAT, and GPx in our investigation. Furthermore, it significantly increased the level of the lipoperoxidation biomarker TBARS. Overall findings indicate that the PP1 metabolic extract efficiently induced cell death in MCF-7 cells by promoting and inhibiting antioxidant and lipoperoxidation activities.

Conclusion

The isolated strain of PP1 used in this work exhibits strong in vitro anticancer and antioxidant activities. This strain's unprocessed secondary metabolic extract exhibited strong bactericidal activity against clinical bacterial pathogens and has the ability to prevent the development of bacterial biofilms. Additionally, it demonstrated the capacity to neutralise DPPH radicals, which are frequently employed to gauge the level of antioxidant activity. These findings imply that the isolated PP1 bacteria's crude secondary metabolic extract may be useful as a therapeutic agent for the treatment of infectious disorders. It is crucial to remember that more research, like clinical trials, would be required to establish the safety and efficacy of this extract in animals.

Conflict of interest

There is no conflict of interest in the current study.

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