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Anticancer activity of bacterial extract from a novel strain of *Bacillus cereus* IND 10

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Abstract
Backgrounds: *Bacillus cereus* is an underrated producer of bioactive secondary metabolites and therapeutic enzymes. The total extracts and their fractions demonstrated significant antioxidant activity and good cytotoxic properties on various human cancer cell lines.
Objective: To study effect of an extract of the Basrah soil isolate strain *B. cereus* IND 10 which is newly isolated from agricultural fertilizer, as a potential anticancer agent.
Methodology: Extract of *B. cereus* IND 10 and application through MTT assay MCF-7 and HBL-100 cell lines for growth inhibition Evaluation of antioxidant activity by DPPH free radical scavenging assay.
Results: The extract enzymatically obtained showed significant cytotoxic properties on MCF-7 cells with an IC_{50} of 9.684 µg/mL, which was significantly less than that of doxorubicin (42.08 µg/mL), showing higher antiproliferative potential in vitro. The extract showed an IC_{50} of 19.8 µg/mL in HBL100 cells while doxorubicin had an IC_{50} of 93.58 µg/mL. The extract also exhibited antioxidant activity with renewed DPPH radical scavenging was 37.87%. Moreover, extract treatment markedly increased GPX1 levels compared to the untreated control, suggesting that it may improve cellular antioxidant capacity. Following treatment, SOD2 expression was shown to increase 1.64 times.
Conclusion: Bacterial extract of new investigated strain IND 10 showed the best performance as compared to doxorubicin (improved cytotoxic effect on MCF-7 cell line and less cytotoxic activity towards HBL100 - nonmalignant breast adenocarcinoma).

Keywords: Bacterial extract, *Bacillus cereus*, anticancer activity, MCF-7, DPPH

Introduction

Bacillus cereus (*B. cereus*), a Gram-positive, rod-shaped, motile aerobic or facultative anaerobic bacterium [1]. Though it has primarily been considered a foodborne pathogen [2], it is an underutilized source of bioactive secondary metabolites and therapeutic enzymes. New studies on *B. cereus* strain taken from marine soil sediment, (*B. cereus* PSMS6) showed that the methanolic extracts contained mutual edible molecules and bioactive compounds [3]. These extracts as well as their isolated fractions exhibited both significant antioxidant capacity and high cytotoxicity against human cancer cell lines. These treated cancer cells showed membrane blebbing and DNA fragmentation both indicating an apoptosis-inducing mechanism [4]. Owing to the deep secondary metabolism of *B. cereus*, these findings lead this bacterium becoming a promising source of antitumor agents [5].

Several bioactive compounds from cell-free supernatant of *B. cereus* including proteases, lipopeptides, L-asparaginase and secondary metabolites have been characterized to impart anticancer activity [6]. Its anticancer effects pass through several pathways that communicate with each other; apoptosis Inducing: Major Antitumor mechanism — Chromatin condensation, DNA fragmentation and mitochondria damage [7], selective oxidative stress: Although the extract with strong antioxidant activity [8], some of them serve to promote intracellular ROS in cancer cells and stimulate oxidative-stress-mediated apoptosis selectively [9], these include various bioactive secondary metabolites that have been suggested as potential correlates of the anticancer effects in vivo [9], enzymatic activities: L-asparaginase depletes asparagine in cancer-cell, but both proteases and lipopeptides stimulate apoptosis, inhibit metastasis and promote loss of integrity for the cancer cell [10], selective cytotoxicity: as more display in section doi 10.1038/s41598-018-20418-w, the *B. cereus* extract showed preferentially cytotoxic effects against cancer cells towards normal cells, suggesting a potential therapeutic application [11], and inhibition of Angiogenesis and Metastasis: Microbial enzymes and microbial metabolites can inhibit tumor vascularization as well as invasion/metastasis from cancer cells [12].

Various factors influence the anticancer activity of *B. cereus* extract, including bacterial culture and extraction conditions. Bioactive enzymes and secondary metabolites production are strongly influenced by the composition of culture medium as well as temperature, incubation time, pH and aeration. The extraction methods/solvents used control the types and yield of active compounds obtained from the bacterial extract [13]. Metabolite and enzyme structure and composition is also a significant element that correlates with anticancer activity. The concentration dependency of cytotoxic effects, compound stability that determines biological effectiveness, efficacy and bioavailability [14]. The anticancer efficacy might differ according to the cancer cell types, since those possess diverse components of signal pathways and different sensitivity to apoptosis. Mechanisms mediating extract activity include inducing apoptosis, inhibiting the proliferation of cells and workplace disruption [15]. Anticancer activity is dependent on the specific strain

and different *B. cereus* strains produce active metabolites and enzymes. Moreover, the bioactive enzymes and their respective gene expression levels play an important role in differentiating the bacterial extract with therapeutic potential [16].

Methodology

Anticancer Activity of Bacterial Enzymatic Extract on the Growth of Cell Lines

The research was carried out at the Iraqi Company for Biotechnology/ Basrah city. The cell lines that were used for this study obtained from Center for Cancer Research and Molecular Genetics/ Baghdad/ Iraq (Table 1). However, cell lines were grown and sub cultured in the Histology Laboratory/ Department Biology/ College of Education for Pure Sciences/University of Basrah under appropriate sterile condition until use in experimental procedures.

Table 1: Types of cell lines

Type	Location	Origin	References
MCF-7	Brest cancer	Human	[17]
HBL100	Breast epithelial cells	Human	[18]

Determination of IC_{50}

Following treatment in four independent biological replicates, the half-maximal inhibitory concentration (IC_{50}) of each tested material against each cell line was determined. The proportion of growth inhibition was calculated for all concentrations and each biological replicate. To calculate the IC_{50}, the inhibition data were then inputted into Graph Pad Prism software (version 8.0.1) and nonlinear regression analysis was performed [19].

Cytotoxicity of Bacterial Enzyme Extract on Normal Breast Cells (HBL100): MTT assay on HBL-100 cell lines

The cytotoxicity of the enzymatic extract from bacteria and doxorubicin on HBL 100 non-tumorigenic cell line by MTT assay. This assay is based on the reduction of the yellow MTT dye by metabolically active cells through mitochondrial enzymes, principally succinate dehydrogenase, to insoluble purple formazan crystals; therefore, the final absorbance intensity is directly proportional to viable cell quantity. Cells were plated on 96-well plates at appropriate density (approx. 1 × 10^4 cells/well) in complete growth medium, and left to adhere for an additional 24 h in a humidified atmosphere at 37°C/5% CO_2 generating a semi-confluent monolayer. The growth medium was removed and cells were incubated with five serial concentrations (percentage units of prepared bacterial enzymatic extract, 5%, 10%, 20%, 40%, 60%) for the bacterial enzyme treatment. We treated another well with doxorubicin diluted down to five concentrations (16%, 31%, 63%, 125% and 250%), because doxorubicin is a reference compound which possess proven cytotoxic effect. The experiment has a negative control (not-treated cells), solvent control if required, and blank wells containing medium and reagents without cells. In the experimental design, each treatment was done in triplicate, and plates were incubated for (48) hours. After the treatment periods, an appropriate volume of MTT solution (5 mg/mL) was added in each well and plates were incubated for 2-4 hours at 37 °C in the dark till formazan crystals appeared. Medium was then removed and 100 µL DMSO added to each well for the dissolution of the formazan crystals and plates shook gently over a period of 5-10 minutes, to ensure complete dissolution. OD620 using a micro plate reader, with blank wells to subtract for blank absorbance. The percentage inhibition of cells was calculated according to:

$$\text{Inhibition of cell survival (\%)} = (\text{OD of treated cells} / \text{OD of control cells}) \times 100$$

Dose-response curves were generated to calculate the half-maximal inhibitory concentration (IC_{50}) from percent inhibition data for both the enzymatic extract and doxorubicin in HBL 100 cell lines as described previously [20].

DPPH Scavenging antioxidant activity assay

The DPPH free radical scavenging assay is a commonly established spectrophotometric method for evaluation of the antioxidant capacity of bioactive compounds and natural extracts. The assay is founded on the reduction of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) into a non-radical form where antioxidants donate electrons or hydrogen atoms to DPPH°; and this characteristic is widely used as one way of determining antioxidant activity. This reaction displays a specific color on transition from deep violet to pale yellow, which can be represented in quantitative manner using spectrophotometer (commonly performed at 517 nm). The absorbance decrease as a function of radical scavenging activity of the test sample wherein lower absorbance indicates higher antioxidant activity. In this case, antioxidant activity is usually expressed in terms of percentage (%) inhibition of DPPH radical and half-maximal inhibitory concentration (IC_{50}). lower value of IC_{50} indicates higher antioxidant activity. The free radical scavenging potential of the bacterial enzymatic extract was monitored by DPPH method using ascorbic acid as standard comparator [3]. This method is based on the reduction of stable radical DPPH by electron- or hydrogen-donating compounds, and decreasing color intensity causes a lower absorbance at 517 nm. An appropriate concentration of DPPH solution was prepared in methanol and stored in dark until used. The enzymatic extract was diluted in three serial concentrations (10%, 40% and 60%). Ascorbic acid of various concentrations (10%,40%,60%) prepared in same or other appropriate standard conc for comparing. Each concentration of the extracts was mixed with a fixed volume of DPPH solution in either 96-well plates or test tubes. A control reaction with DPPH only (no extract) and solvent were prepared. One blank sample of each concentration of the extract (extract plus solvent free DPPH) was also included in order to eliminate any color due to the intrinsic characteristics of the extract. Samples were incubated for 30minutes at room temperature in the dark and absorbance was taken at 517nm.

$$\text{DPPH Scavenging Activity (\%)} = (\text{A control} - \text{A sample}) / \text{A control} \times 100$$

With an extract blank, we first need to correct the sample reading:

$$\text{Corrected sample} = \text{Sample} - \text{Sample blank}$$

For this the absorbance is corrected and percent inhibition is calculated. To compare the antioxidant activity of enzymatic

extract with that of ascorbic acid, which was used to determine the relative antioxidant power [21].

Assessment of Superoxide Dismutase (SOD2) and Glutathione Peroxidase (GPX1) Activities in MCF-7 Cells Following IC_{50} Treatment

The levels of the antioxidant enzymes superoxide dismutase (SOD2) and glutathione peroxidase (GPX1) were then determined to assess changes in the cellular antioxidant defense system in MCF-7 cells after exposure for 24 h to an enzymatic extract from day-6 bioengineered bacteria at its half-maximal inhibitory concentration (IC_{50}). Commercially available ELISA-based assay kits (Elabscience, UK) were used to determine the activities of each enzyme according to the manufacturer's instructions. These cells were then analyzed after establishing the IC_{50} value using the mtt cell viability assay. The MCF-7 cells were plated in suitable dispersion societies, split into 2 examples groups set up in organic at least triplicate; (i)e negative control non treated bunch and (ii) a treatment group with the bacterial gut enzymatic chemical at its characterized IC_{50}. Cells were incubated in standard culture conditions (37°C, 5% CO_2 in a humidified atmosphere) for 48 h.

After 24 h, the culture medium was thoroughly aspirated and the cells were washed twice with ice-cold phosphate-buffered saline (PBS) to remove any residual medium and treatment compounds. Then, cells were harvested and lysed using protein lysis buffer provided or recommended by the manufacturer. All procedures were performed on ice when appropriate to minimize protein degradation and maintain enzyme activity. Cell lysates were centrifuged at 4ø C to pellet cell debris, and the resulting supernatants were collected for biochemical analysis (clarified CFPs). Total protein concentration was assessed via a bicinchoninic acid (BCA) protein assay to confirm equal total protein loading between samples. The activities of SOD_2 and GPX_1 were subsequently measured based on the protocols provided with respective kits and presented after normalizing to the total protein amount in each sample. Results are expressed as enzyme activity per mg total protein, which allowed the reliable comparison of the antioxidant status between treated and untreated cells [22].

Superoxide dismutase (SOD_2) activity assay

Due to its association with pitting corrosion initiation, superoxide dismutase (SOD_2) activity is often monitored by the standard biochemical assay that measures the capacity of a test solution to carry out the dismutation of superoxide radicals (O_2^-) into molecular oxygen and hydrogen peroxide. The assay is normally carried out via a colorimetric approach on stimulation of superoxide-mediated reduction of a mixed color indicator. In this method, higher SOD_2 activity leads to more inhibition of color development and thus enzyme activity can be determined spectrophotometrically. Results are often expressed in enzymatic units of SOD_2 activity per milligram total protein (U/mg protein) supplying a definition for comparison the antioxidant capacity between samples [23]. Procedures for the Elab Science SOD_2 kit was performed following the manufacturer's specifications. In brief, samples and reagents that produce superoxide anions and a chromogenic detector were firstly added to wells or tubes according the directions in the kit protocol. This assay is based on the ability of SOD_2 in the sample to inhibit formation of a colored formazan product; therefore, color intensity is inversely proportional to SOD_2 activity. The samples were incubated for the kit designating time after mixing reagents and absorbance was read at manufacturers choosing womb (450nm). SOD_2 activity was determined employing the equations present in the manual of kit and expressed as U (units) as proposed by the manufacturer [24].

Glutathione peroxidase (GPX_1) activity assay

A coupled enzymatic assay utilizes the enzyme-catalyzed oxidation of reduced glutathione (GSH) into glutathione disulfide (GSSG) to measure Glutathione Peroxidase (GPX_1) activity. This assay is based on the continuous reduction of GSSG back to GSH by glutathione reductase in the presence of nicotinamide adenine dinucleotide phosphate (NADPH), which oxidizes NADPH to $NADP^+$. The gradual fall in NADPH concentration is spectrophotometrically measured at 340 nm and used as an indirect marker of GPX_1 activity [25]. GPX activity detected in crude enzyme extracts from *Bacillus cereus* IND10 using the Elab Science Cell Glutathione Peroxidase (GPX1) Activity Assay Kit. According to the manufacturer's instructions. The assay is based on the ability of GPX to catalyze reduction of peroxides using reduced glutathione (GSH) as a substrate, with oxidation of GSH coupled via consumption of reducing agent NADPH that can be monitored by the measurement of absorbance at 340 nm over time. *B. cereus* culture is grown under optimized conditions and the centrifuge at 6000 rpm, 20 min to remove bacterial cells and filtered by a sterile syringe filter 0.22 mm in order to ensure residual removal of bacterial cells to get bacteria enzyme extract. The relevant volume of this extract was then added to the reaction mixture in accordance with the order and volumes outlined by the kit, and absorbance at 340 nm (A340) was measured at both initial and final time points as specified within the kit manual. The absorbance change was determined according to the kit manufacturer's equation for GPX1 activity. Results were represented in mg or pg protein suggested by the kit manual [26].

Results and Discussion

Cytotoxicity of Bacterial Enzyme Extract on Normal Breast Cells (HBL100)

The MTT assay was used to evaluate the cytotoxic effect of enzymatic extract on a normal human breast cell line (HBL-100) at concentrations from 5% to 60%. However, there was a relatively weak association within the concentration range tested (IC_{50} = 19.8%; R^2 = 0.4487). The results showed that the enzymatic extract caused a moderate decrease in viability of HBL-100 cells. The highest growth inhibition values were obtained with the lowest tested concentration (5%), about 58–60% followed by a decrease to around 50–52% at both 10% and 20%. At higher concentrations, however, the inhibitory effect decreased progressively to 44–45% inhibition (at 40%) and to 39–40% inhibition (at 60%). This surprise inverse concentration-response trend suggests that increasing the enzymatic extract concentration, cytotoxicity toward normal breast cells did not proportionally increase. A relatively moderate concentration of the extract is needed to obtain a 50% reduction in viability on HBL-100 cells, making its calculated IC_{50} value 19.8%. Yet, the low R^2 (0.4487) means that there is much variation in experimental data and indicates that this

cytotoxic response does not follow a classic sigmoidal dose-dependent curve as (Fig. 1) shown.

This variability may be due to the fact that, since the biochemical composition of the enzymatic extract is complex, different bioactive components could play opposing biological activities. However, at higher concentrations aggregation of active molecules and low bioavailability, enzymatic instability, and activation of adaptive cellular defense mechanisms may mitigate the overall cytotoxic effect [27]. Biologically, the observation that cytotoxicity is not a smooth function of enzyme concentration (Fig. 1) reinforces the conclusion that within the range of concentrations tested in our study, there is minimal toxicity of buds on normal HBL-100 cells.

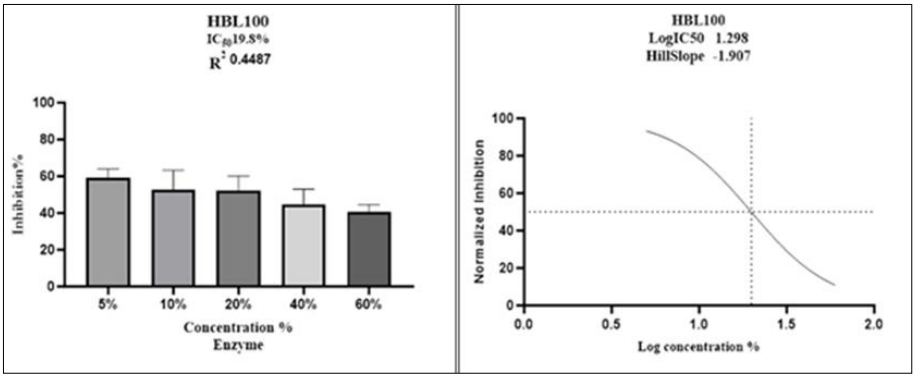

Fig 1: Cytotoxic effect of enzyme extract on HBL100 cells

Cytotoxicity of Doxorubicin on Normal Breast Cells (HBL100)

MTT assay shows the cytotoxic effect of doxorubicin depending on the dosages in normal human breast epithelial cell line HBL-100 (Fig. 2). Doxorubicin effected a concentration-dependent decrease in cell viability, as the percentage inhibition of growth progressively increased with increasing doxorubicin concentrations (15.6–250 µg/mL). The lowest concentration (15.6 µg/mL) resulted in only a slight inhibition of HBL-100 cell growth, indicating a low toxicity of the microscopic dose on normal cells. An increase in the section of growth inhibition at 31.2 and62.5 µg/mL was apparent, showing a corresponding increase in cellular sensitivity to doxorubicin with increasing concentration of drug. The inhibitory effect became apparent at 125 µg/mL, and the maximum concentration (250 µg/mL) had the strongest inhibition of cell viability (>50% inhibition). This dose-dependent cytotoxic activity of doxorubicin was confirmed by this progressive response. IC_{50} value calculates around 93.58 µg/mL indicates that approximately the concentration required to inhibit 50% of HBL-100 cell viability. These results show that doxorubicin is not only an important anticancer drug but also a compound that shows significant toxicity to normal breast epithelial cells. Thus, doxorubicin is a powerful chemotherapeutic drug, but its cytotoxicity to normal cells is still one of the key factors limiting clinical application [28].

In addition, the dose-response curve had a coefficient of determination (R^2 = 0.8819), showing that nearly 88.2% of growth inhibition variation could be accounted for by how much doxorubicin concentration was altered. This strong correlation reflects a good fit of the regression model and supports the reliability of the calculated IC_{50} value. Overall, these findings demonstrate a clear concentration-dependent cytotoxic effect of doxorubicin on HBL-100 cells, with increasing drug concentrations resulting in progressively greater inhibition of cell viability.

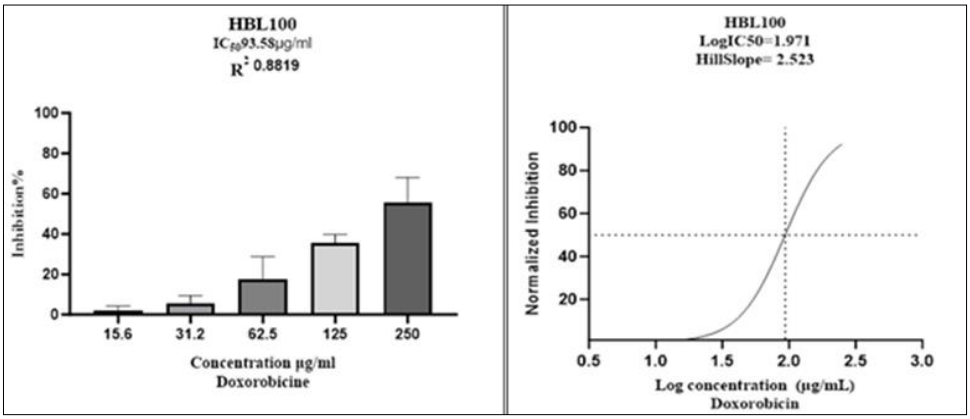

Fig 2: Cytotoxic effect of Doxorubicin on HBL100 cells

Cytotoxic Effect of Doxorubicin on MCF-7 Cells

The cytotoxicity of Doxorubicin to MCF-7 human breast cancer cells was calculated using the MTT assay. (Table 2 & Fig. 3) showed that the cytotoxicity of Doxorubicin was concentration-dependent, the higher the drug concentration, the stronger inhibition effect on cell growth. The half-maximal inhibitory concentration (IC_{50}) of Doxorubicin was determined to be 42.08 µg/mL according to the dose-response curve, which is the concentration of compound that results in a 50% reduction cell viability under experimental conditions.

Table 2: Cytotoxic effect of Doxorubicin on MCF-7 cells

Concentration (µg/mL)	Mean Inhibition (%)	Standard Deviation (SD±)
15.6	1.03	3.94
31.2	19.60	6.29
62.5	44.76	4.64
125	54.85	4.19
250	59.02	2.27

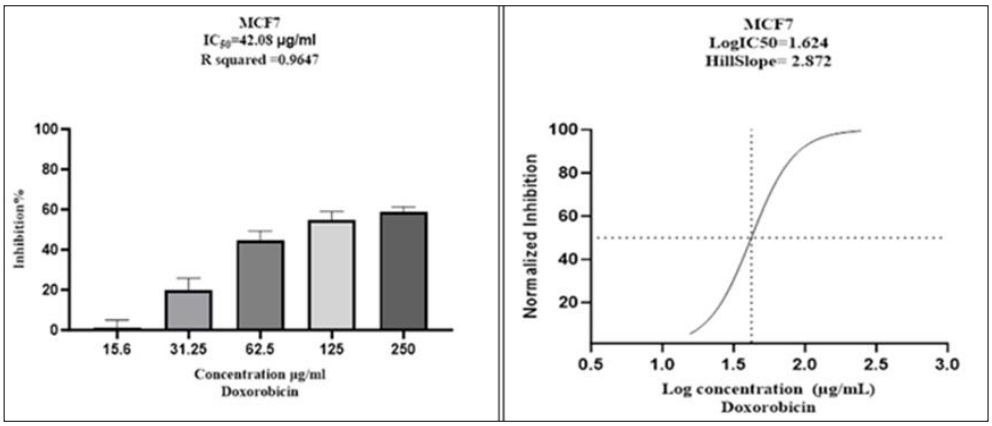

Fig 3: Cytotoxic effect of Doxorubicin on MCF-7 cells

In this study, the cytotoxic activity of doxorubicin against MCF-7 human breast cancer cells was assessed and it was found that the IC_{50}=42.08 µg/mL during a treatment period (48) hr. It also confirms that doxorubicin is a potent antiproliferative agent of MCF-7 cells and reinforces its status as a first-line chemotherapeutic drug used to treat breast cancer. We have explored the cytotoxic effects of Doxorubicin on MCF-7 human breast cancer cell line where an IC_{50} was found to be 42·08 µg/mL. Doxorubicin, known as an anthracycline antibiotic, is a well-studied chemotherapeutic agent of choice for treating different types of cancers (including breast cancer) [29]. It has three main ways of working, intercalation with DNA, inhibition of topoisomerase II activity, and induction of reactive oxygen species resulting in DNA damage and apoptosis. These effects include intercalation into DNA, inhibition of topoisomerase II activity and induction of reactive oxygen species (ROS) leading to oxidative stress, DNA damage, cell cycle arrest and activation of apoptotic pathways. Taken together, these molecular events play a role in the inhibition of tumor cell proliferation and finally lead to apoptosis [30].

The Enzyme's Cytotoxic Effect against MCF-7 Cells

The MTT assay was performed to quantify the cytotoxic effects of the tested enzyme against MCF-7 human breast cancer cells. Demonstrated in (Table 3 & Fig. 4), the enzyme has a concentration-dependent inhibitory effect on lower active concentrations, as the percentage of growth inhibition increased progressively and reached a plateau at 0.2% and 0.4%. Nonetheless, at 0.6%, the finding of an inhibitory effect was reduced, indicating that as cytotoxic response mediated by bacterial enzymes increases with concentration the hostility decreases in response to higher concentrations. The enzyme IC_{50} was calculated based on the dose–response curve, which was found to be 9.684% (about 10%).

Table 3: Cytotoxic effect of the enzyme on MCF-7 cells

Concentration (%)	Mean Inhibition (%)	Standard Deviation (SD±)
0.05	45.45	12.04
0.1	62.42	3.42
0.2	67.79	3.63
0.4	67.71	6.14
0.6	46.80	4.26

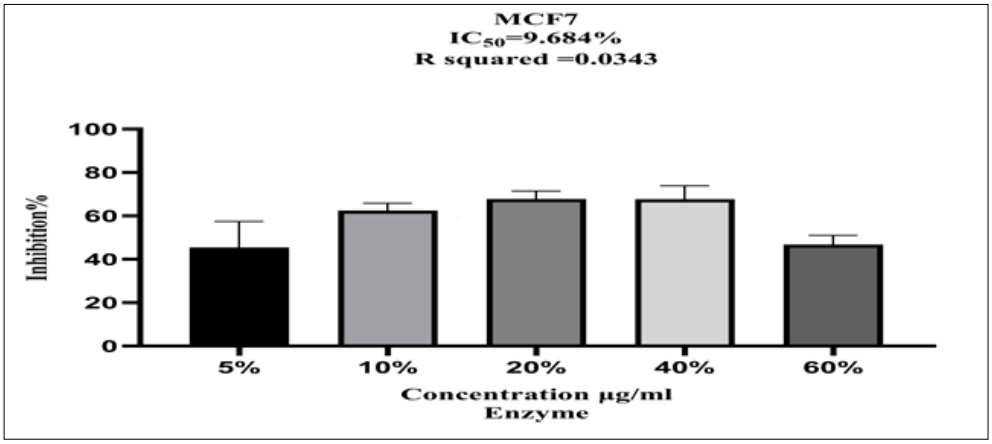

Fig 4: Cytotoxic effect of the enzyme on MCF-7 cells

IC_{50} of the enzyme showed a complex cytotoxic effect against MCF-7 breast cancer cells as we evaluated in this study, with an IC_{50} value equal to 9.684%. With the increase of enzyme concentration, cell growth inhibition was stronger, and with 0.2% and 0.4%, they reached a peak value of (67.79 ± 1.83) %/(67.71 ± 5) %. Interestingly, a decrease in cytotoxic effect was observed at the highest concentration (0.6%), with inhibition reaching only 46.80%. This biphasic non-linear dose–response pattern is different from the subjected concentration-dependent increase in cytotoxicity generally observed for most anticancer agents. There are many things that could explain this. High concentrations of the enzyme can also result in protein aggregation or conformational changes which reduces their activity and prevents them from sufficiently interacting with target cells. Furthermore, saturation of the cellular target, the instability of enzymes in culture conditions or the detection interferences caused by high concentrations can also be deriving reasons for reduced cytotoxicity. Others (little known bioactive substances derived from biologically active compounds or enzymes) have similar reports of a non-monotonic dose-response relationship where the biological activity being assessed is not increasing with dose but rather occur optimal in certain concentration range.

Different enzymes have been studied for their anticancer effect mediated by numerous mechanisms, including the induction of apoptosis, alteration of tumor metabolic pathways and degradation of molecules required for cancer cell survival and proliferation [31]. For instance, lactase was reported to have antitumor activity by degrading 17β-estradiol, thus inhibiting the growth of estrogen receptor-positive MCF-7 breast cancer cells [32]. Similarly, enzymes related to glycolytic metabolism including lactate dehydrogenase are potential therapeutic targets as their inhibition interrupts energy-producing pathway and induces apoptotic cellular death in cancer cells [33]. The decrease in inhibitory activity at 0.6% enzyme may indicate that the enzyme does not show an entirely dose-dependent cytotoxic function on MCF-7 cells. This non-linear behavior might be indicative of a range concentration with an ideal ratio between the enzyme and the substrate that shows maximum bioactivity. High concentrations of this optimum might lead to reduced enzyme stability, structural changes, aggregation or substrate saturation result in diminishing anticancer activity. An alternative, and plausible explanation for this observation is a biphasic dose-response, where the enzyme produces different biological effects at varying concentrations. Alternatively, MCF-7 cells may respond to greater enzyme concentrations by activating adaptive or cytoprotective mechanisms that reduce the overall inhibitory response. The relatively high standard deviation observed here, at the lowest concentration (0.05%), again suggests increased variability during analysis at this dose that may reflect biological heterogeneity or decreased sensitivity of the assay to low treatment levels. This variability emphasizes the need to validate these results in further independent experiments. As shown in (Fig. 5), doxorubicin cytotoxicity shows a linear dose-dependent pattern on MCF-7 cells while the enzyme shows a non-linear dose-dependent pattern on MCF-7 cells.

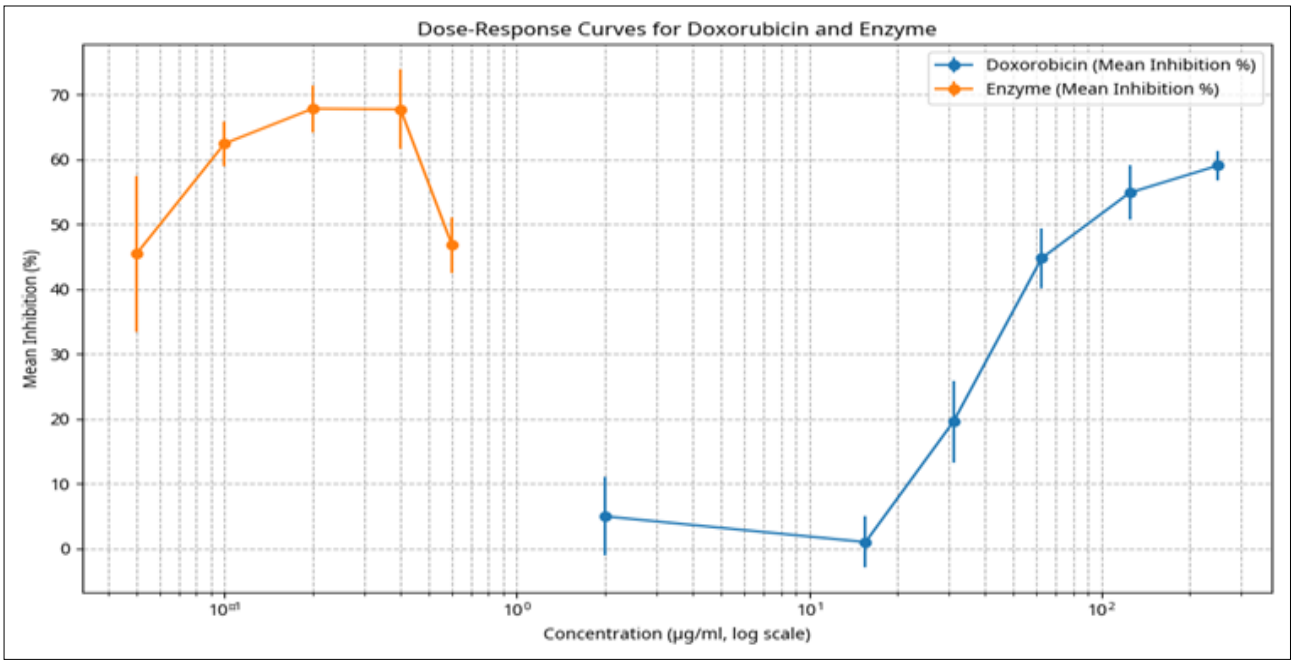

Fig 5: Dose-response curve for Doxorubicin and enzyme extract

Antioxidant Activity of Bacterial Enzyme Extract (DPPH Radical Scavenging Assay)

The Antioxidant activity of the enzyme was conducted by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, with ascorbic acid as the standard reference antioxidant. The antioxidant capacity was expressed as the percentage of DPPH radical inhibition (Inh%). (Table 4 & Fig. 6) shows the experimental results.

Table 4: DPPH Radical Scavenging Activity (Inh%) of Enzyme and Ascorbic Acid at Different Concentrations

Ascorbic Acid (Inh% ± SD)	Enzyme (Inh% ± SD)	Concentration (µg/mL)
88.84 ± 1.54	37.87 ± 3.94	10
95.51 ± 2.06	24.88 ± 1.01	40
96.59 ± 4.03	10.04 ± 7.22	60

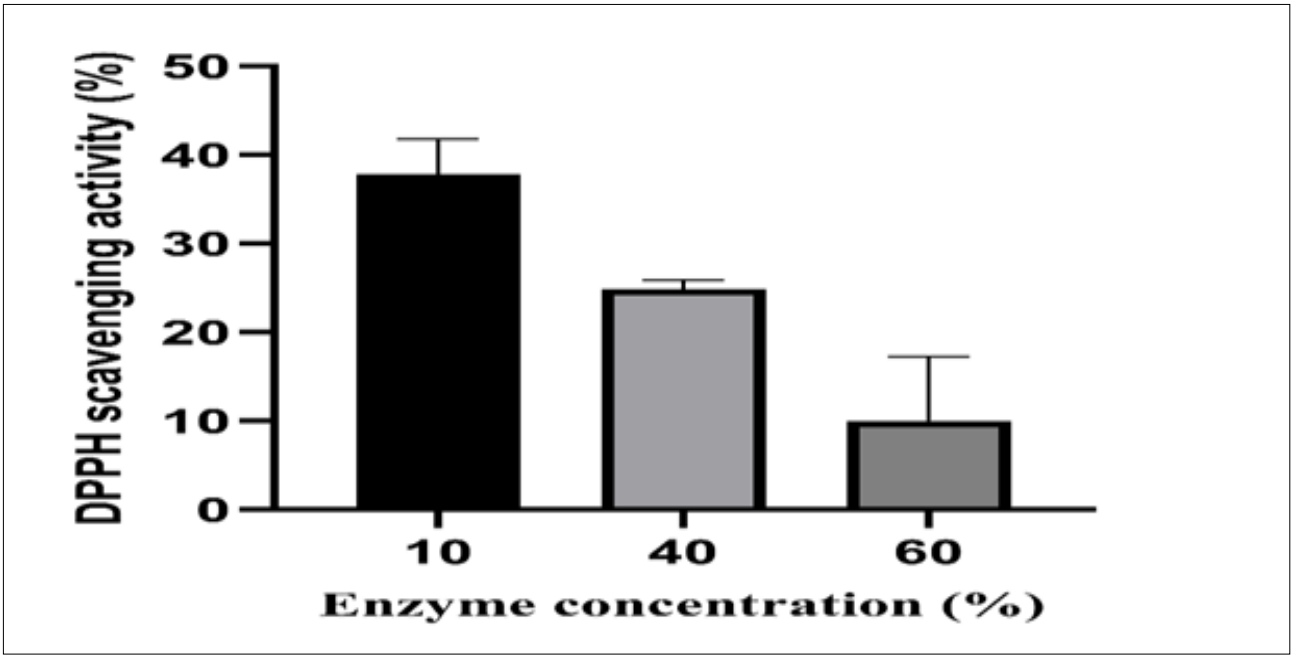

Fig 6: DPPH radical scavenging activity (Inh%) of enzyme at different concentrations

Radical scavenging capacity decreased with increasing enzyme concentration, and the resulting trend of antioxidant activity exhibited an opposite dependence on the tested enzyme. Maximum DPPH radical scavenging activity (37.87%) was recorded in lowest concentration (10 µg/mL) tested for each extract. In contrast, the scavenging activity gradually decreased to 10.04% at the highest concentration (60 µg/mL). The drop-in antioxidant activity at high concentrations is an unexpected finding that has been explained in previous studies and is likely due to the concentration-dependent shift of some antioxidants from protective function to a pro-oxidant one. At higher concentrations antioxidant compounds, such as enzymes or polyphenols (a natural form of antioxidants), could do auto-oxidation or interact with trace transition metal ions, causing the formation of ROS instead of neutralizing them. Thus, this pro-oxidant effect may reduce the general free radical scavenging capability detected in the DPPH assay leading to lower inhibition percentages in higher enzyme concentrations [34]. Similar results have been stated in previous reports that specific bioactive compounds display a concentration-dependent dual action being responsible for antioxidant activity at lower concentrations and pro-oxidant at higher ones [35]. In a similar context, another study emphasized that the transition from antioxidant to pro-oxidant activity is highly dependent on concentration and is an important variable to be considered when interpreting results from biological antioxidant tests [36]. These observations sufficiently justify the studied reduction in radical scavenging activity at higher enzyme concentrations as shown in our study.

Most of these reports on enzymatic antioxidants identify concentration-dependent increases in the radical scavenging activity [37], which contrasts with our results. Such a trend has generally been attributed to more active catalytic sites being available at higher enzyme concentrations, thus enabling better free radical neutralization. One such example is that there exists a threshold beyond which increasing concentration will have minimal effect, compared with the linear increase seen until this threshold in enzymatic systems [38]. The reduced antioxidant activity noted at higher concentrations of the enzyme in this investigation could be attributed to a few reasons. Molecular crowding or specific enzyme aggregation at high concentrations, which decrease the availability of catalytically active sites is one of the possible explanations. These structural changes could limit the effective interaction of the enzyme molecules with DPPH radicals, resulting in a decrease of radical scavenging activity measured through spectrophotometry. Similar concentration-dependent limitations have also been suggested in studies of enzymatic antioxidant systems [39].

Conversely, ascorbic acid displayed the anticipated concentration-dependent antioxidant activity with DPPH radical scavenging rising steadily from 88.84% at 10 µg/mL to 96.59% at 60 µg/mL (Fig. 7). Sparing action dose-dependently followed this pattern and is well in agreement with the antioxidant nature of ascorbic acid that acts as a potent secondary antioxidant donating a hydrogen atom or electron to neutralize the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) through the mechanism of hydrogen atom transfer (HAT) [40,41]. The presence of highly metabolic scavenging activity across all selected concentrations further reaffirmed its role as a reference antioxidant and affirmed the reliability of the DPPH assay performed in this study.

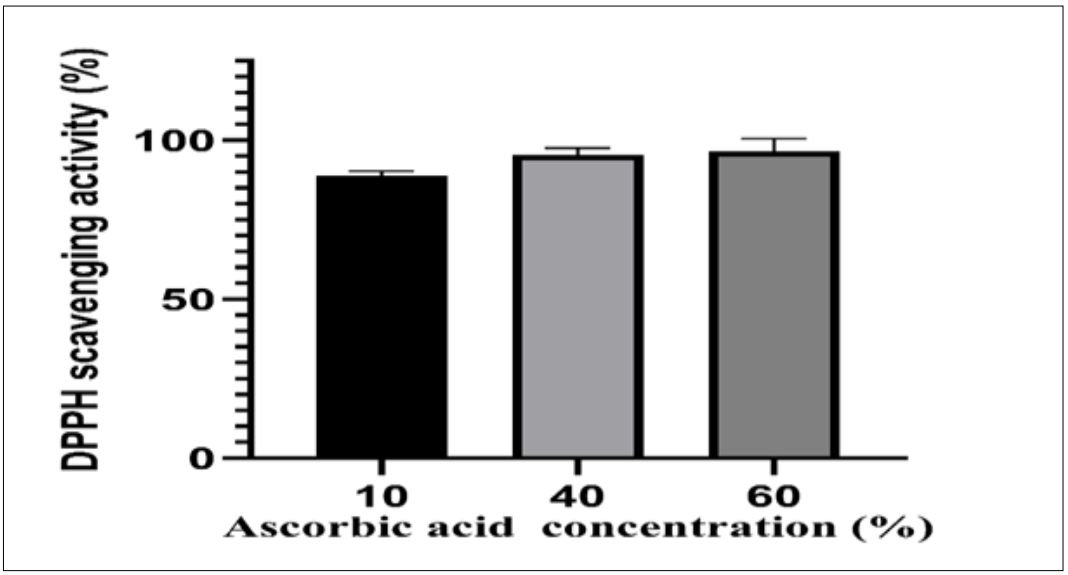

Fig 7: DPPH radical scavenging activity (Inh%) of ascorbic acid at different concentrations

Modulation of Endogenous Antioxidant Enzymes (GPX and SOD2)

Glutathione Peroxidase 1 (GPX1)

A typical calibration curve, obtained by plotting the mean optical density (OD) values vs GPX1 concentrations (pg/mL), gave a linear relationship for the multiple analyte range of the assay. The linearity of the assay as assessed by a calibration curve had a coefficient of determination (R^2) of 0.9855 and confirmed the reliability and accuracy of the method within the range 0–2000 pg/mL for GPX1 quantification.

Results Total protein concentrations, as measured in all samples from 1.013 to 1.590 mg/mL. These-medications served as the key normalization factor for GPX1 quantification, acting to equilibrate any differences in protein content across samples that may confound comparison of enzyme levels. Normalizing GPX1 concentrations to total protein (pg/mg protein), that provides a more valid and relevant biological comparison between the control and treatment groups. Normalized GPX1 concentrations (pg/mg protein) between the control and treatment (25262 ± 1293 pg/mg vs. The average GPX1 level in the control group was 19746 goodness pg/mg protein, and the treatment group had a statistically greater mean GPX1 of 24177 goodness pg/mg protein. The increase was statistically confirmed by means of the t-test ($p<0.05$). These results show that the treatment leads to increased levels of GPX1, which is likely because the dose and time points used were sufficient to up-regulate the expression and/or activity of this important antioxidant enzyme, thereby enhancing cellular defenses against oxidative stress. as exhibited in the below-mentioned (Fig. 8 & Fig. 9).

Glutathione peroxidase 1 (GPX1) is a major intracellular antioxidant enzyme that protects cells against oxidative damage. It promotes the catalytic reduction of H_2O_2 to water, restricting ROS formation and buffering oxidative damage [42]. GPX1 exerts its antioxidant function to maintain the cellular redox homeostasis and protects life-essential biomolecules/organelles such as cellular membranes, mitochondria, proteins and DNA from ROS-induced damages. Due to its pivotal role in maintaining cellular integrity [43].

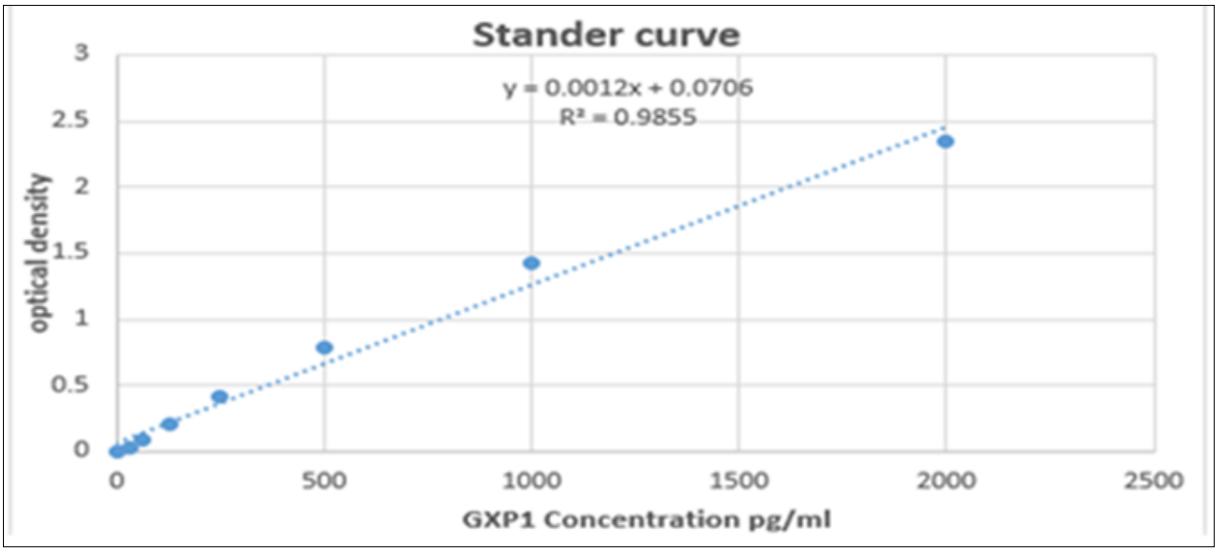

Fig 8: Standard calibration curve for quantitative determination of GPX1 concentrations by ELISA

Fig 9: Effect of treatment on GPX1 concentrationGPX1 concentration (pg/mg protein)

Glutathione Peroxidase 1 (GPX1) is a widely expressed selenium-dependent antioxidant enzyme in mammalian cells. Research-GC2B47 2200293014042 Peroxidase (POX) acts as an antioxidant enzyme: its catalysis the reduction of hydrogen peroxide and organic hydroperoxides, generating less harmful products which prevent oxidative damage to cells and are very important in maintaining cellular redox homeostasis [44]. Compared to the controls, our study showed a significantly higher concentration of GPX1 in the treatment group ($P < 0.05$). This observation suggests that the intervention improved the cellular antioxidant defense system, and might also increase capacity for detoxifying tumorigenic ROS produced within the cell and lowering oxidative stress. The expression of GPX1 is, therefore, upregulated and may be an adaptive protective response that helps sustain cellular integrity in the face of oxidative stressors. These data are supported by extensive evidence that first highlighted the crucial protective function of GPX1 in maintaining redox homeostasis and shielding tissues from oxidative injury. An earlier study characterized that both upregulation of GPX1 expression or activity, along with treatment with GPX1 mimetics such as epsilon, could significantly bolster cellular protection from oxidative damage by augmenting antioxidant defense mechanisms [45]. Likewise, GPX1 overexpression has been correlated with decreased cellular sensitivity to oxidative stress, which also reinforces the point of the importance of maintaining intracellular redox balance [42]. These results present a compelling case that increased GPX1 expression or activity appears to boost the antioxidant capacity of cells by reducing oxidative stress and protecting against oxidative injury in our cell line models.

3.2 Superoxide dismutase2 (SOD2)

Human superoxide dismutase 2 (SOD2) levels were measured by ELISA against a standard calibration curve [46]. Standard curve based on serial dilution of purified SOD2 standard (SOD2−) The optical density (OD) at 450 nm was measured in the presence or absence of various dilutions of a purified SOD2 standard (0–20 ng/mL). The standard curve showed a good agreement with the regression model, which gave the R^2=0.999 as shown in (Fig. 10). The excellent linearity demonstrated by this assay established its correctness and reproducibility, allowing for accurate quantification of SOD2 levels present in experimental samples.

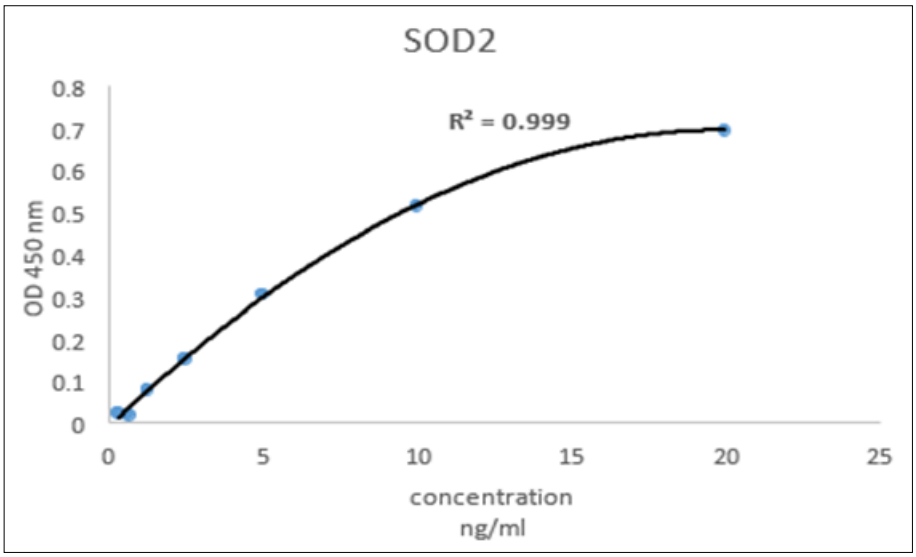

Fig 10: Standard calibration curve of SOD2.The standard curve was generated using serial concentrations (0-20) ng/mL after blank subtraction

This SOD2 concentration was normalized to total protein content (ng/mg) and the results are shown in (Fig. 11), where it can be seen that there is no statistically significant difference between SOD2 concentrations within the same treatment groups as well as across different time points. The mean SOD2 concentration of the control group (n = 18) was 0.544 ± 0.395 ng/mg and that of the treatment group (n = 14) was a higher level, with an average SOD2 concentration at variance from the control by two-sample t-test analysis approaching statistical significance (P 0.05, ns). The treatment group was determined to have a higher average SOD2 levels, nevertheless the differences among groups were not statistically significant, as there was too much variability between mice in both groups. These results suggest that when analyzed under the experimental conditions of this investigation, SOD2 expression was not significantly affected by treatment even with an upward trend. These results evidenced an increase in SOD2 levels in the treatment group, the SOD2 concentration being 1.64 times higher than that of the control group (control vs treatment). This increase may represent a biological response to treatment via the induction of cellular defense mechanisms, implying increased protection against oxidative stress. This lack of statistical significance may be due to a number of factors such as sample size, intra-group variability or the actual effect size of the treatment [47]. The reliability of the quantification method is confirmed by the high R^2 value (0.999) of the standard curve, which indicates that these SOD2 concentrations can be considered accurate within this experimental setup.

Fig 11: Effect of treatment on SOD2 levels normalized to total protein content

Conclusion

A report on a newly isolated strain of *B. cereus* IND 10 also showed that its bacterial extract (BE) exhibited higher anticancer activity against the MCF-7 cell line compared to doxorubicin and lower HBL100 cell line cytotoxicity, good antioxidant activity on MCF-7 cells, and up regulation of GPX1 and SOD2.

Consent for publication

Not applicable

Availability of data and material

Data are available upon request.

Competing Interests

The authors declare no competing interests related to the research, authorship, or publication of this article. This manuscript has not been previously published and is not under consideration by any other journal.

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