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Original Article

Characterisation and Cytotoxic Activity Evaluation of Superoxide Dismutase (SOD) Enzyme Purified from *Escherichia coli.*

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Abstract

Background: Superoxide dismutases (SODs) are antioxidant metalloenzymes produced by all living cells, including *E. coli*. SODs enzymes have several roles in treatment of different diseases, including tumors. Its catalysing the dismutation of superoxide free radicals ($O_2^{\bullet-}$) to H_2O_2 and H_2O to protect cells against their harmful effects. This might prevent damage to tissues. **Objective:** Production and purification of antitumor enzyme (superoxide dismutase) from local isolate *Escherichia coli*. **Methods**: SOD was partially purified from *E. coli* using ammonium sulphate precipitation, DEAE-cellulose ion exchange and sephadex G-150 Gel filtration chromatography. Subsequently, the molecular weight of the purified SOD was determined using SDS-PAGE. Moreover, the activity and stability of SOD were examined under different pH and temperature values. Finally, cytotoxic potential assessed against colon cancer and normal cell lines. **Results:** Crude extract of SOD was partially purified using ammonium sulphate precipitation, DEAE-cellulose ion and sephadex G-150 Gel filtration chromatography, whereas specific activity of SOD was increased from 314.2 into 1357 U/mg protein with 3.5-fold and yield (52.5%). The activity and stability of SOD were examined under different pH and temperature values. The maximum SOD activity was 95 U/ml at pH 6, while the stability of enzyme (90 – 100%) at pH range (6-8). The maximum activity at 37°C. At 32-37°C, SOD enzyme was stable with remaining activity of 100%. Various agents (NaCl, $CaCl_2$, H_2O_2, MnSO4 and urea) were used to estimate their effects on enzyme stability. The enzyme was maintaining its activity (100%) in presence of NaCl, $CaCl_2$ and H_2O_2, while completely lose its activity in presence of urea (0.1M), and this activity was increased into 140% in presence of MnSO4 (0.1 M). Cytotoxicity of purified SOD was exanimated on primary dermal fibroblast normal (Hdfn) and a human colorectal adenocarcinoma cell-line (Caco-2) cell lines. The viability of (Caco-2) was decreased into 45% after treatment with SOD enzyme at 400 µg/ml after 24 hrs. On the other hand, the viability of HdFn was decreased into 75% after treatment with SOD enzyme at concentration of 400 µg/ml after 24 hrs. **Conclusion***:* Lower IC_{50} values indicate a stronger inhibition of cell viability at higher doses of SOD. Which suggesting its potential therapeutic for future applications.

Keywords: *Characterisation; Cytotoxicity; E. Coli; Purification, Superoxide Dismutase*

Introduction

Superoxide dismutases (SODs) (EC 1.15.1.1) are metalloenzymes found in eukaryotes and some prokaryotes. These enzymes are located in various cellular compartments: Cu, Zn-SOD (or SOD1) is found in the cytosol and mitochondrial intermembrane, Mn-SOD (or SOD2) is located in the mitochondrial matrix and inner membrane, and Cu, Zn-SOD (or SOD3) is present in the extracellular compartment (Rosa *et al*., 2021). SODs catalyse the conversion of the superoxide anion free radical (•O2−) into hydrogen peroxide (H2O2) and molecular oxygen (O2). Subsequently, the hydrogen peroxide produced is reduced to water by enzymes such as catalase (CAT), glutathione peroxidase (GPx), and thioredoxin (Trx)-dependent peroxiredoxin (Prx) (Islam *et al*., 2022). For SODs to eliminate free radicals and metabolise harmful intermediates to their full catalytic potential, they require

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cofactors such as iron, manganese, copper, and zinc. Aspartate, histamine, and histidine make up the side chains, and the metal-binding site is situated between the two domains of SOD. Cofactors like these are known to replenish themselves throughout the catalytic process by donating electrons to (O2). Scientists have identified several types of SOD, which are oligomeric proteins that include metals such as iron, manganese, copper, and zinc as cofactors (Stephenie *et al*., 2020). Many Gram-negative bacteria, such as *Escherichia coli*, contain three types of superoxide dismutase (SOD), categorised based on their cellular location and metal cofactor. These enzymes protect cellular macromolecules from the superoxide produced externally by host phagocytes during infection (Doukyu & Taguchi, 2021).

Cancer remains one of the most challenging diseases globally, leading to an increased demand for accurate treatment methods (World Health Organization, 2018). Moreover, these enzymes have been the subject of extensive medical research due to their potential to treat a wide range of illnesses, including diabetes, cancer, brain damage, and other ailments (Alateyah *et al*., 2022). Further investigation into the medicinal benefits of SODs is expected throughout the current research phase, resulting in more advancements in this area (Zheng *et al*., 2023). Therefore, this study aimed to characterise partially purified superoxide dismutase from a local isolate of *E. coli* (data not shown) and examine its cytotoxic effect on cancer cell lines in comparison with normal cell lines.

Material and Method

E. Coli Isolate

A total of 160 urine samples from patients with UTIs who visited Al-Yarmouk Teaching Hospital and Al-Karkh General Hospital in Baghdad were collected between September 2023 and November 2023. The samples were subjected to cultural characteristic examinations, microscopic examination (Gram staining), biochemical tests (catalase, oxidase, and methyl red), and the VITEK2 system. SOD was extracted from the most efficient isolate, and the crude enzyme extract was subjected to several treatments, as follows:

Determination of SOD Activity

Nutrient agar media was streaked with *E. coli* local bacterial isolates, and the cells were cultured at 37°C for 24 hours. After that, one colony was selected and left to incubate in a shaker incubator for one day at 37°C on top of trypticase soy broth. After 24 hours of incubation, *E. coli* grown in trypticase soy broth was placed in a centrifuge for 15 minutes at 4°C at 10,000 rpm. The particle was carefully collected after the supernatant was removed. Then, one ml of NaOH and 6 ml of distilled water were added to the pellet obtained from the previous step. Glass beads were then placed into the tube, and the mixture was shaken for 1 hour. Afterward, the mixture was placed in a centrifuge at 4°C at 10,000 rpm for 15 minutes. The supernatants were examined for superoxide dismutase activity according to Beyer and Fridovich (1987). Each test tube contained a mixture of 75 µl SOD solution, 2.25 ml of working solution, and 750 µl of distilled water. The blank control tube contained 2.25 ml of working solution and 750 µl of distilled water without SOD, with 75 µl of distilled water used instead of the SOD solution. Then, 75 µl of riboflavin solution was added to each tube, and each tube was thoroughly mixed using a vortex mixer. The optical density of each tube was measured at 560 nm. Subsequently, the tubes were exposed to UV light at room temperature for 30 minutes, and the optical density was measured again. The SOD activity was calculated according to the method described by Fulghum and Worthington (1984).

$$\text{Inhibition percentage} = \frac{(OD\ of\ C_A - OD\ of\ C_B) - (OD\ of\ S_A - OD\ of\ S_B)}{(OD\ of\ C_A - OD\ of\ C_B)} \text{x } 100\%$$

OD: Optical density at 650 nm; C: Control; S: Sample; A: After UV light exposure; B: Before UV light exposure.

The enzyme unit (SOD) is defined as the amount of enzyme that causes a decrease in NBT reduction by 50%:

$$\text{Activity of SOD} = \frac{\text{Inhibition percentage (\%)}}{50\%} \times \frac{1000 \times 3}{Vs\ (\mu l)}$$

Vs = Volume of enzyme solution added

Determination of specific activity

The specific activity of an enzyme was determined using the following equation [15]:

$$\text{Specific activity}\left(\frac{\text{U}}{\text{mg}}\text{protein}\right) = \frac{\text{Enzyme activity}\left(\frac{\text{U}}{\text{ml}}\right)}{\text{Protein concentration}\left(\frac{\text{mg}}{\text{ml}}\right)}$$

Determination of Protein Concentration

Bradford method was used to measure concentration of protein (Bradford, 1976), following the procedure described by (AL-Sa'ady & Hilal, 2020).

Purification of SOD

Ammonium Sulphate Precipitation

Cultures of E. coli weqa `434re placed in centrifuge (4oC; 15 min; 1000 rpm). Ammonium sulfate (80% saturation) was added into the crude enzyme (supernatant) with gentle mixing using magnetic stirrer (20 min; 4°C). The extract was placed in centrifuge (4oC; 15 min; 1000 rpm). A suitable volume of phosphate buffer (pH 7; 0.05M) was added into the precipitate of enzyme. Enzyme activity and concentration of protein were determinate before and after treatment.

DEAE-Cellulose Chromatography

According to Whitaker and Bernhard (1972), A DEAE-Cellulose column was prepared, potassium phosphate buffer (0.05 M, pH 7) was used for washing the concentrated enzyme, that poured in the resin. Raising NaCl concentrations (0.1, to 1 M) was used to elute fractions. The rate of flow was kept at 30ml/h. At 280 nm, UV-VIS spectrophotometer was used to estimate the absorbance of each fraction. For each fraction, the activity of SOD was estimated, fractions with desired SOD activity were kept at 4°C for further experiment.

Gel Filtration Chromatography

Based on the recommendations of the Company of Pharmacia Fine Chemicals, sephadex G-150 was made. Potassium phosphate buffer (0.05 M, pH 7) was used to equilibrate the sephadex G-150 (1.5×40cm) column. Active fractions of SOD were applied into column. Flow rate of 30ml/h. was carried out for elution. Elution profile was monitored to measure the absorbance of each fraction at 280 nm and target enzyme was assessed.

Characterisation of SOD

Evaluation of SOD Molecular Weight

SDS-PAGE was used to evaluate the molecular weight and purity of SOD, as described by (Garfin, 1990).

Effect of different pH values on SOD activity and stability

Different buffers were prepared with ionic power of 0.05 M, including acetate buffer (pH 5, 6), phosphate buffer (pH 7 and 8), and tris base buffer (pH 9). The effect of pH on activity of partial purified SOD, enzyme activity was determined at each pH value. The effect of pH on stability of partial purified SOD, enzyme was incubated at 37 ºC for 30 min with different pH buffers, Ice bath was used to cool the tubes. Then, enzyme activity was estimated.

Effect of different temperatures on SOD activity and stability

A graph of enzyme activity versus temperature was made to determine the ideal temperature of enzymatic activity. Various temperature ranges (27–52°C) were utilised to analyse SOD activity. On

other hand, SOD that had been partially purified was pre-incubated for 30 min at various temperatures (32, 37, 42, 47, and 52 ºC) in a water bath. It was then quickly cooled on an ice bath. The remining activity (%) was then estimated.

Determination the effect of various ions and inhibitors on SOD activity

A variety of ions and inhibitors (NaCl, $CaCl_2$, H_2O_2, $MnSO_4$ and urea) were made at concentrations of 0.1M each. For 30 min, these agents were pre-incubated with SOD at room temperature. Then, the enzyme's activity and remaining activity were measured.

Cytotoxic effect of SOD on cancer and normal cell lines

The cytotoxic effect of SOD was determined using Intron Biotech Kit (Korea). Hdfn normal and CaCo-2 cancer cells (1x104 - 1x106 cells/ml) were grown in microtiter plate with 96 wells. The culture media were used to bring the total volume to 200µl for each well. Sterilised parafilm was used to seal the microplate, which was then gently mixed. The microplate was placed in an incubator (24 hrs.; CO_2; 37ºC). After incubation, the media were removed and 2-fold serial dilutions (0.5, 2.5, 10, 50, 100 and 200 µM/ml) prepared and placed into the wells. For each concentration and controls (serum free media-treated cells), triplicates were prepared. The microplate was sealed with sterilised parafilm, gently mixed and it was placed in incubator (4 hrs.; CO_2; 37ºC). After incubation, cuprizone (50 µM/ml) was added to each well for 24 hours. Subsequently, 10 µl of MTT solution was added to each well. The microplate was sealed again with sterilised parafilm and gently mixed. Microplate was placed in incubator (4 hrs.; CO_2; 37ºC). Carefully, media were removed and solution of solubilisation (100 µl) was added to every well for 5 min. ELISA reader was used to measure the absorbance (at 575 nm). Half-maximum inhibitory concentration (IC50) was calculated using following equation:

$$Y= D+ A-D / 1+10 (x-logC)B$$

Whereas A: Minimum asymptote; B = Hill's slope; C = Inflection point; C is the concentration of analyte where y=(D-A)/2; D = Maximum asymptote.

Results

Purification of SOD from E. coli

Crude extract of SOD was partially purified using ammonium sulphate precipitation at 80% saturation ratio. Based on results in Table 1, yield of SOD was reached 57.8%, specific activity of 314.2 U/mg protein with purification fold of 1.2. Then, DEAE-cellulose ion exchange chromatography was used to increase purity of SOD. According to findings in Figure 1, one peak of protein was appeared during the washing step, and another protein peak appeared after elution by the gradient concentrations of sodium chloride. All these peaks were assayed to detect SOD activity. Results showed that eluted proteins (fractions 59-67) contained most of SOD activity, and enzyme specific activity was measured to be 750 U/mg with yield of 56.8% and 2.9 purification fold (Table 1).

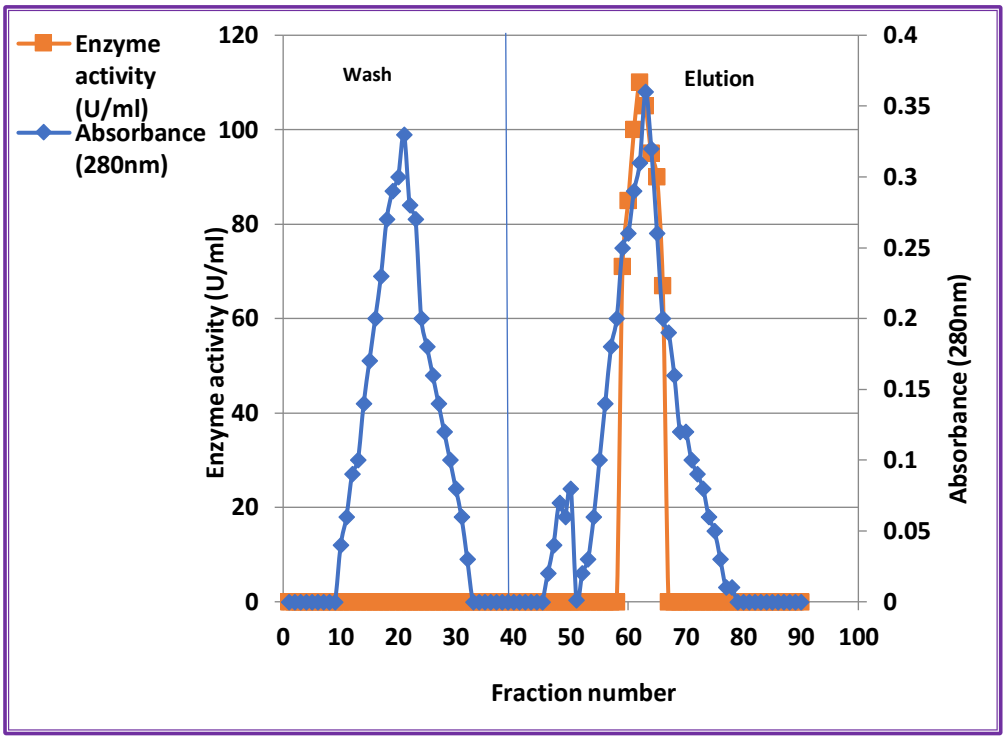

Figure 1: *Ion Exchange Chromatography for SOD Purification from E. Coli Using DEAE-Cellulose Column (1.5 X 25 Cm) Equilibrated with Potassium Phosphate Buffer (0.05 M; Ph 7), Eluted with Nacl Gradient (0.1-1 M) In Flow Rate 30 Ml/H for Each Fraction*

Using Sephadex G-150 Gel filtration chromatography, single peak was observed in Figure 2, indicated that specific activity of SOD was increased into 1357 U/mg proteinwith 3.5-fold and yield (52.5%).

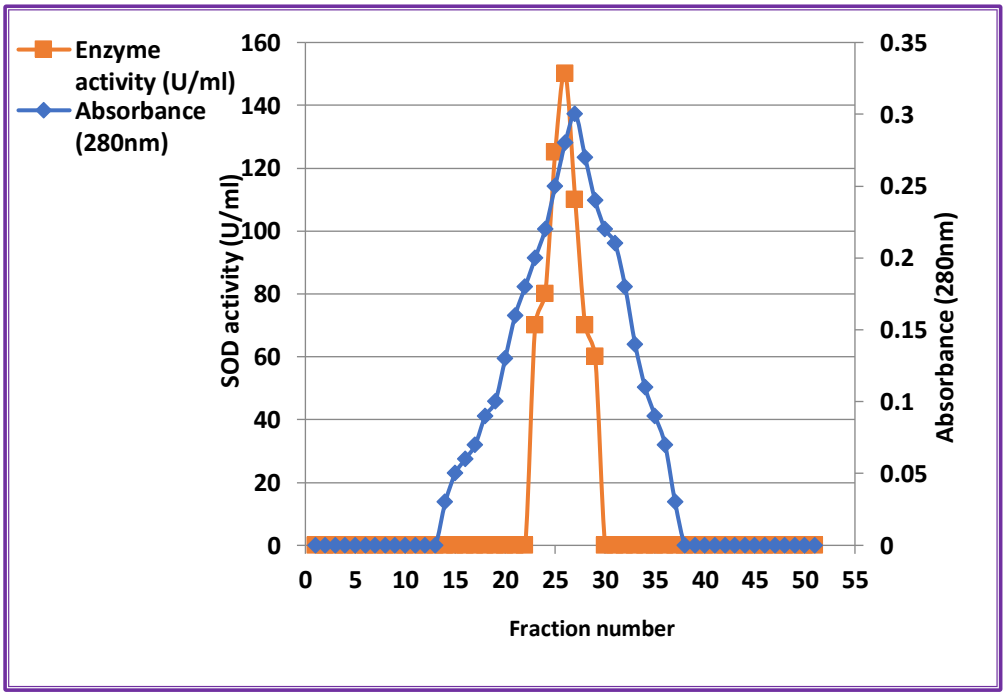

Figure 2: *Gel Filtration Chromatography for SOD Purification from E. Coli Using Sephadex G-150 Column (1.5 x 40 cm) Equilibrated with Potassium Phosphate Buffer (0.05 M; Ph 7), Eluted with the Same Buffer in Flow Rate 30 Ml/H for Each Fraction*

Table 1: *Purification steps for SOD from E. coli.*

Purification step	Volume (ml)	Enzyme activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg protein)	Total activity (U)	Purification (folds)	Yield (%)
Crude enzyme	50	76	0.3	253.3	3800	1	100
Ammonium sulphate precipitation 80%	20	110	0.35	314.2	2200	1.2	57.8
DEAE-cellulose	24	90	0.12	750	2160	2.9	56.8
Sephadex- G150	21	95	0.07	1357	1995	5.3	52.5

Characterisation of SOD

Determination of Molecular Weight of SOD

SDS-PAGE was used to estimate the molecular weight of SOD. The results, as shown in figure (3), were showed that the molecular weight of SOD was 54 kDa. The migration distance of a protein complexed with the strong cationic detergent sodium dodecyl Sulfate (SDS) separated on Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) can be used to calculate its apparent Molecular Weight (MW).

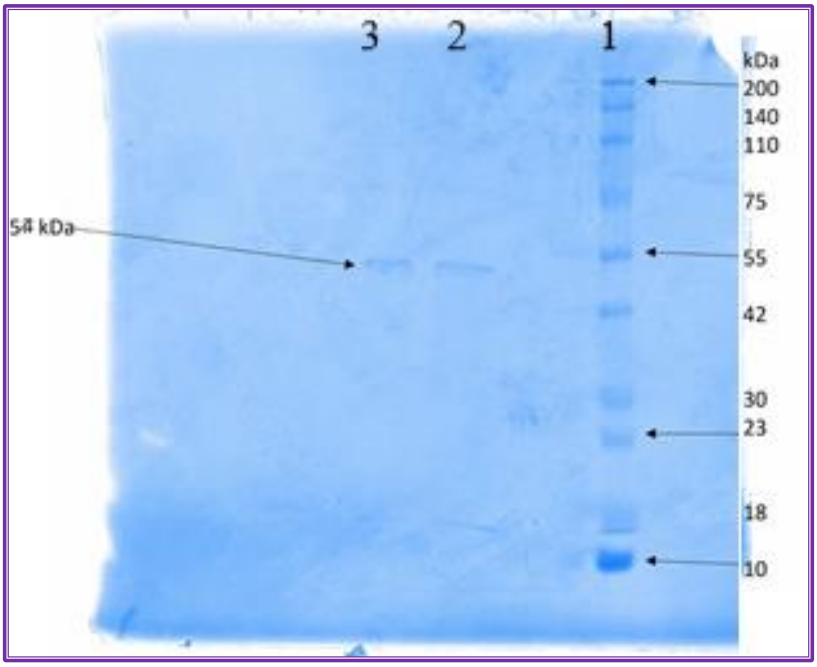

Figure 3: *SOD Molecular Weight Using SDS-PAGE, where (1): Molecular Weight Ladder (2): Gel Filtration Protein Band. (3): Ion Exchange Protein Band*

Optimal pH for SOD activity and Stability

Five values of pH (5, 6, 7, 8 and 9) were examined to estimate the optimal pH for SOD activity and stability. The results in Figure (4A) showed that the maximum SOD activity was 95 U/ml, achieved at pH 6, then this activity was decreased at neutral and alkaline pH values (pH 7-9), whereas the activity was reduced into 80 U/ml at pH 9. According to the findings, as displayed in Figure (4B), the stability of enzyme (90 – 100%) was achieved at pH range (6-8), whereas the stability of SOD was reduced out of this range. Half of SOD activity was remained at pH 5, while 70% of remaining activity was achieved at pH 9.

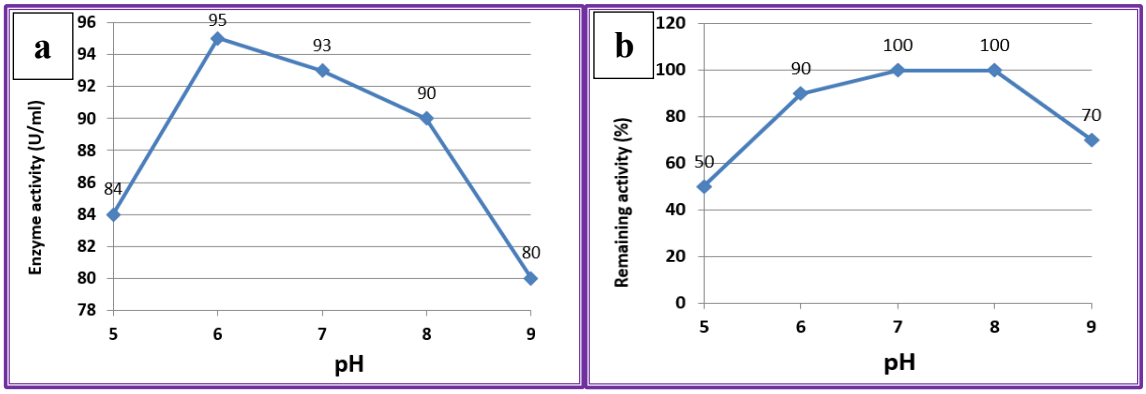

Figure 4: *Effect of Different Ph Values on (A) Activity and (B) Stability of Partial Purified SOD Produced by E. Coli.*

Optimum Temperature for SOD Activity and Stability

The effect of different temperatures was used to estimate activity and stability of purified SOD. The results, as shown in Figure (5A), indicated that the activity of SOD was increased with rise of temperatures and reached the maximum activity at 37°C; then, this activity was decreased into 63 U/ml at 52°C. At 32°C, SOD enzyme was stable with remaining activity of 100%, whereas this activity stable until 37°C. Then, SOD began to lose its stability, whereas SOD retained 90, 80 and 65% of activity at 42, 47 and 52°C, respectively, as shown in Figure (5B).

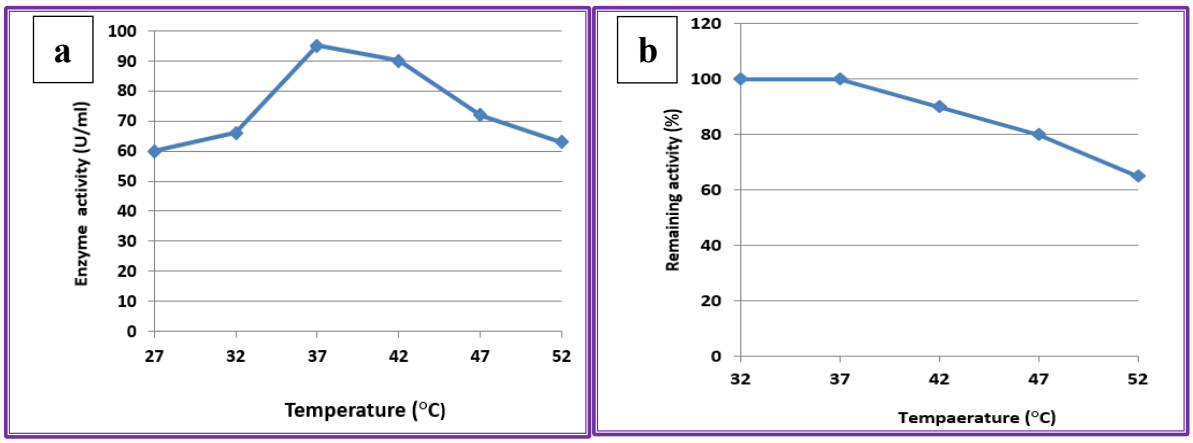

Figure 5: *Effect of Different Temperature Values on (A) Activity and (B) Stability of Partial Purified SOD Produced by E. Coli.*

Effects of ions and inhibitors on SOD stability

Various ions and inhibitors (NaCl, $CaCl_2$, H_2O_2, $MnSO_4$ and urea) were used to estimate their effects on enzyme stability. The results, as displayed in table (2), showed that the enzyme was maintaining its activity (100%) in presence of NaCl, $CaCl_2$ and H_2O_2, while it completely lose its activity in presence of urea (0.1M). In addition, this activity was increased into 140% in presence of $MnSO_4$ (0.1 M).

Table 2: *Ions and Inhibitors on SOD Stability*

Reagent	Concentration (M)	Remaining activity (%)
Control (Enzyme)		100
NaCl	0.1	100
$CaCl_2$	0.1	100
H_2O_2	0.1	100
$MnSO_4$	0.1	140
Urea	0.1	0.0

Cytotoxic Effect of SOD on Tumor Cells

The cytotoxicity of SOD was examined on Colon Cancer (Caco-2) and normal (Hdfn) cell lines. The results, as displayed in Table 6 and Figure 6, indicated that the viability of both cell lines was dose-dependent on SOD, with their viability gradually decreasing as the concentrations of SOD increased. The viability of Caco-2 was reduced to 41.35% after treatment with SOD enzyme at 400 µg/ml, while the viability of Hdfn was reduced to 72.56% after treatment with SOD enzyme at a concentration of 400 µg/ml after 24 hours. The SOD enzyme concentration at which 50% inhibition of a certain biological activity (cytotoxic impact on cancer cell lines) occurs is known as the half-maximal inhibitory concentration (IC_{50}) (Park *et al*., 2022). When comparing the relative potency or effectiveness of SOD on Hdfn and Caco-2, the IC_{50} value is frequently used. A higher potency of SOD is indicated by lower IC_{50} values, which were 138.2 for Hdfn and 29.76 for Caco-2 cell lines.

Table 3: *Cytotoxic Effect of Superoxide Dismutase on Normal Cell Line (Hdfn) and Colon Cancer Cell Line (Caco-2) After 24 Hrs.*

	HdFn		CaCo-2	
Conc.	**Mean**	**SD**	**Mean**	**SD**
400	72.56967	0.834408	41.358	1.237295
200	77.54633	1.449964	52.81667	1.654562
100	88.503	1.400201	60.68667	1.280027
50	93.78867	0.240513	71.798	0.942473
25	94.599	0.240913	84.60667	2.935035
12.5	95.79467	0.438024	94.02	1.188031

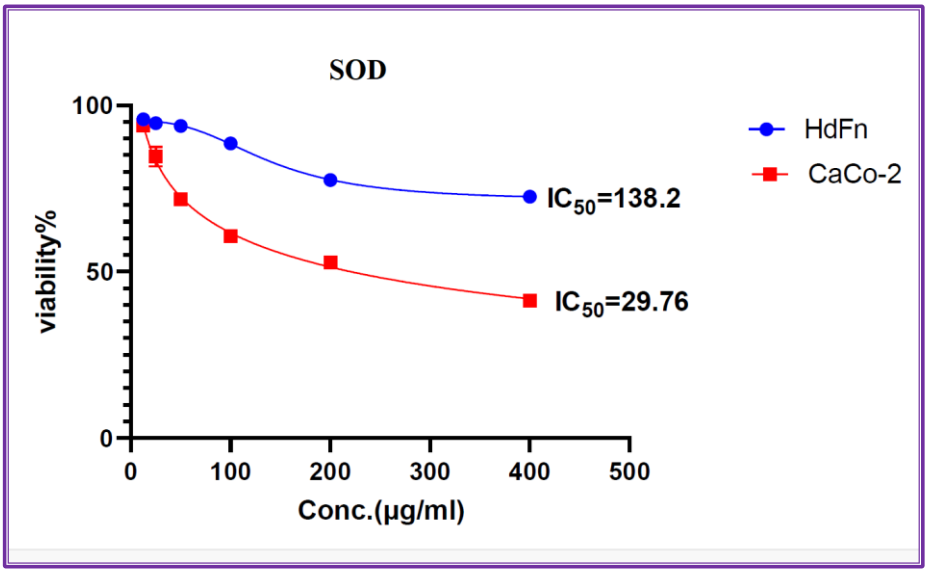

Figure 6: *Cytotoxic Effect of Superoxide Dismutase on Normal Cell Line (Hdfn) and Colon Cancer Cell Line (Caco-2) After 24 Hours*

Discussion

Some of the many advantages provided by DEAE-Cellulose resin include its charge-difference-dependent separation principle, simplicity, high capacity, ease of handling, high resolution power, and excellent separation (Abdalah *et al*., 2018). In agreement with these findings, several studies have used DEAE-Cellulose ion exchange chromatography and gel filtration chromatography to purify SOD from various organisms. Abdel-Monsef *et al*. (2023) used DEAE-cellulose anion exchange column

chromatography and Sephacryl S-300 gel filtration column chromatography to purify SOD obtained from Egyptian honeybee venom (*Apis mellifera lamarckii*). The yield reached 53.9% with a specific activity of SOD (588 U/mg protein) using the DEAE-cellulose exchanger, whereas gel filtration reduced the yield to 21.0% with a specific activity of SOD (1250 U/mg protein). Additionally, Ozdemir *et al*. (2021) precipitated SOD from *Punica granatum* L using ammonium sulfate and purified it using DEAE chromatography, achieving a specific activity of SOD of 166 U/mg protein with a 16.60-fold purification. Habib *et al*. (2023) reported that the specific activity of SOD obtained from *Cellana rota* snail increased to 520.7 units/mg protein, displaying a 26.5% yield and a 6.6-fold purification using DEAE-cellulose column and Sephacryl S-300 chromatography. Moreover, various purification folds and yield percentages for SOD have been reported, including 103.7-fold and 70% yield for *Leishmania peruviana* and 101.6-fold and 86% yield for *Leishmania amazonensis* (Longoni, Marín & Sánchez-Moreno, 2014); and 29.5, 17.7, and 13.3 folds with yields of 34.9%, 20.1%, and 9.4% from *Hyalomma dromedarii* (Ibrahim *et al*., 2013).

SDS-PAGE approach was developed in 1969 and is still widely used due to its simplicity Matsumoto, Haniu and Komori, 2019. In study reported by Zeinali, Homaei& Kamrani (2017), the MW of SOD from Avicennia marina was 31 kDa using SDS-PAGE.

The findings indicated that the activity of SOD gradually increased with an increase in pH until it reached pH 6, after which the activity began to decrease. Changes in the geometry of the enzyme or substrate, ionisation of groups in the substrate, and ionisation of groups in the enzyme's active site are some potential mechanisms through which pH influences enzyme activity (Chimbekujwo, Ja'afaru & Adeyemo, 2020). In a study reported by Guan *et al*. (2017), the optimal pH for SOD activity, obtained from *Alvinella pompejana*, was 7.4. Additionally, Tuteja *et al*. (2015) reported that the SOD activity in *Pisum sativum* showed a broad range of pH sustainability. The optimal activity of the enzyme was found to be in the pH range of 6–8.0. About 70% of its optimal activity was retained at the acidic pH of 5.0 and the alkaline pH of 10.0. However, 90% of the maximal activity was lost at a lower pH (pH 4.0). It is suggested that SOD is not pH-dependent, although results from recombinant proteins have shown contradicting outcomes (Holdom *et al*., 2000).

As the pH of the buffer solution approaches its ideal value, the activity of the enzyme diminishes due to ionic changes in the enzyme's active site and substrate, as well as alterations in its secondary and tertiary structures (Wang *et al*., 2024). Moreover, most enzymes can undergo irreversible denaturation in very acidic or basic conditions (Acharya & Chaudhuri, 2021). It has been discovered that the SOD from the thermoacidophilic *Alicyclobacillus* strain remains highly stable across a broad pH range of 2.0 to 10.0 (Dong *et al*., 2021).

Temperature variations have an impact on a number of characteristics of an enzymatic process, such as pH, enzyme substrate affinity, and prosthetic group ionisation (Amaal, Sahar & M-Ridha, 2023) High stability is attributed to a number of characteristics, including a narrow solvent-exposed surface area, β-barrel fold, a tight hydrophobic dimer interface, large ion-pair networks, and improved salt bridge formation (Tuteja *et al*., 2015). The highest enzyme activity was achieved at 37ºC with an observed decrease in activity at less or a higher temperature of incubation. The results showed an increase in enzyme reaction activity until it reached 37ºC then it began to decline Figure (4-a). In other hand, SOD enzyme was stable with remaining activity of 100%, this activity stable until 37ºC. Then, SOD began to lose its stability, However, SOD retained 90, 80 and 65% of activity at 42, 47 and 52ºC, respectively figure (4-b). this might be because the enzyme molecules' movement energy increased as they shared the reaction with the substrate, increasing the likelihood that they would clash with it (Pokharel, Ma & Chang, 2020). For rEsmtMnSOD protein obtained from Eriocheir sinensis, when the temperature ranged from 10 to 30 ºC, its enzymatic activity exhibited no significant change and kept at the level higher than 2000 U/mg (Wang *et al*., 2015). The SOD activity, from Pisum sativum, was found to be temperature resistant till 55°C, beyond which the activity declined sharply (Tuteja *et al*., 2015). It has been reported that the relative activity of SOD, obtained from Alvinellapompejana, at 15℃ was approximately 2 times the activity at 37℃ and was 23% at 50℃ (Guan *et al*., 2017).

However, the optimal temperature for SOD reaction, extracted from thermoacidophilic Alicyclobacillus strain, was 35◦C, and was highly stable at any certain temperature up to 80◦C (Dong *et al*., 2021).

Since SOD is metalloenzyme (Barwinska-Sendra *et al*., 2020), it is clearly that the presence of metal ions result is improving of its activity. The effect of metal ions on the superoxide disutase activity depends on the origin of the enzyme. It has been reported that SOD of *Pseudoalteromonas* sp. (rPsSOD), expressed in E. coli, and exhibited well capability to 2.5 M NaCl (62.4%) (Wang *et al*., 2016). The activity of many enzymes, notably SOD, can be significantly impacted by urea (Gou *et al*., 2017). In disagreement with the findings of this study, the activity of CuZn-SOD, obtained from Pisum sativum, was not significantly affected by 4 M of urea and imidazole (Amaal, Sahar & M-Ridha, 2023). SOD that extracted from Caragana jubata and expressed in E. coli, showed specific features, including resistance of urea (Kumar *et al*., 2016). In research on drought-stressed maize, foliar-applied urea was found to significantly boost SOD, peroxidase (POD), and hydrogen peroxidase (CAT) activities under conditions of water stress. The effect of hydrogen peroxide on enzyme activity was also examined to determine the active site metal ion. Table (2) indicated that the enzyme isolated from the local strain E. coli is a MnSOD since it preserved its full activity when heated with H_2O_2. These findings supported MValderas& Hart (2001) that MnSOD remains active when heated with H2O2. According to Lefebre & Valvano (2001), H_2O_2 inhibits FeSOD and CuZnSOD but not MnSOD. Also, manganese sulphate increases enzyme activity in this study. Valderas & Hart (2001) found Mn ion near the active site of S. aureus SOD enzymes, where metal ions affect metalloenzymes differently. Each enzyme prefers its parent metal (Krezel & Maret, 2021).Manganese sulphate stimulated superoxide O_2-elimination with the enzyme (Rady *et al*., 2022).

The increasing demand for new antitumor medications with reduced non-specific cytotoxicity and fewer adverse effects has driven the search for novel pharmacologically active molecules from natural products. This has led to a greater emphasis on using polypharmacological approaches to treat complex disorders with a variety of anticancer mechanisms (Bishayee & Sethi, 2016). In this line, using a new approach to treating complex disorders, including cancer, is necessary. Since cancerous cells already exhibit elevated levels of ROS under normal conditions, which reduces their buffering capacity, they are more vulnerable to ROS, including SOD, attack than normal cells (Saqib *et al*., 2018). Elevated levels of Reactive Oxygen Species (ROS) cause damage to mitochondria and nuclear DNA, which in turn triggers apoptosis (Wang *et al*., 2017).

In a study reported by Law *et al*., (2017), the use of SOD demonstrated that the extract from the strain Streptomyces colonosanans MUSC 93JT exhibited strong antioxidant activity. this extract also showed anticancer efficacy against human colon cancer cell lines without significantly harming normal human colon cells. This extract exhibited 83.32 ± 2.62% SOD-like activity at the maximum tested dose of 2 mg/mL. In the investigation of its effects on cancer cells, it demonstrated significant anticancer efficacy without causing notable harm to normal human colon cells (He *et al*., 2020), It was found that human hepatocellular carcinoma ($HepG_2$) cells treated with 10 µg/mL of PS (polystyrene) and PS-COOH (carboxyl-modified polystyrene) showed 1.68- and 1.80-fold increases in SOD activity (13.18 U/mg protein), respectively, compared to the control. The novel SOD mimetic GC4419 enhanced thioredoxin reductase (TrxR) activity in healthy cells but lowered it in cancerous cells, making cancer cells more vulnerable to oxidative stress (El-Mahdy *et al*., 2020). Additionally, Epigallocatechin Gallate (EGCG) suppresses breast cancer cells (Her2/neu) by activating Forkhead box O (FoxO) proteins, which protect cells from oxidative damage-induced apoptosis by inducing the antioxidant enzymes MnSOD and catalase (CAT) (Evacuasiany *et al*., 2014).

In conclusion, this study contributes to the understanding of thyroid diseases in our population, shedding light on their distribution, demographic patterns, and histopathological characteristics. The findings underscore the importance of tailored approaches in the diagnosis, management, and prevention of thyroid disorders, considering the observed variations in sex and age distributions. Future research endeavors could explore additional factors influencing thyroid diseases, including genetic predispositions and environmental exposures, to further enhance our knowledge and inform public health strategies.

While the study provides useful data on the cytotoxic effects of SOD on HdFn and Caco-2 cells, there are several limitations, particularly related to the short-term nature of the treatment, the lack of in vivo validation, and the absence of mechanistic insights. Expanding on these areas in future studies would strengthen the conclusions and provide a more comprehensive understanding of SOD's potential as a therapeutic agent.

Conclusion

As the SOD enzyme concentration increases, both Hdfn and Caco-2 cell lines exhibit a dose-dependent cytotoxic response. Lower IC_{50} values indicate a stronger suppression of cell viability caused by SOD. Based on these data, it is possible that SOD has cytotoxic effects on the cancer cell line. This effect may prove useful in the future for developing therapeutic applications or for understanding the mechanisms behind SOD-induced cytotoxicity.

Conflict of Interest

The authors declare that they have no competing interests.

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