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Effect of *Spirulina platensis* crude extract on catalase activity and stability

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Abstract

Spirulina platensis has recently received considerable attention because of its potential application in the prevention and/or management of cancer and many other diseases. However, little is known about the effects of *S. platensis* on the antioxidant defense system. This study aimed to investigate the effects of this microalga on the function and structure of the antioxidant enzyme catalase. The catalase activity was measured under steady-state kinetic conditions. Spectrophotometric methods were executed to understand *S. platensis* -induced alterations in catalase. Results showed that low concentrations of *S. platensis* extracts stimulated catalase activity, whereas higher concentrations inhibited its activity in a non-competitive manner. Catalase activity was inhibited by 27% and 80% using the aqueous and methanol extract, respectively. The Stern-Volmer plots indicated that *S. platensis* methanolic extract quenched catalase fluorescence emission with both static and dynamic mechanisms. The values of thermodynamic parameters indicated that the binding reaction is a spontaneous and exothermic process, and hydrogen bonds and van der Waals forces play a major role in binding the compounds in *S. platensis* extract to catalase. The number of obtained binding sites was ~2. Circular dichroism spectroscopy data showed that high concentrations of *S. platensis* extract led to a significant decrease in α-helix and ß-sheet content, accompanied by an increase in the enzyme's random coil content. It can be concluded that the application of *S. platensis* extract has a dual effect on the structure and function of catalase, stimulatory or inhibitory, depending on its concentration. Therefore, it may have potential benefits as an antioxidant or tumor suppressor.

1. Introduction

Microalgae are an excellent natural source of interesting secondary metabolites with different biological activities, such as antimicrobial, antioxidant, anti-inflammatory, and anticancer activity. Recently, the demand for natural antioxidants as an alternative to synthetic antioxidants has increased. Microalgae are rich in

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antioxidants and are considered a potential source of natural antioxidants by the food, cosmetic, and nutraceutical industries (Roy et al., 2021; Bravo-Tello et al., 2017; Gogineni & Hamann, 2018). The antioxidant properties of blue-green microalgae are mainly attributed to their natural pigments, including β-carotenes, phycoerythrins, xanthophylls, and phycocyanins, as well as their phenolic compounds (Park et al., 2018; Wu et al., 2011). Among the various microalgae, *S. platensis* has received much attention due to its therapeutic properties as well as the FDA approval of its safety and non-toxicity (Sharoba et al., 2014).

S. platensis is a microscopic and filamentous blue-green microalga that has been advocated for use in the prevention of cancer, hypercholesterolemia, hyperglycerolemia, diabetes, hypertension, and certain inflammatory diseases (Jerez-Martel et al., 2017; Joventino et al., 2012). In addition, *S. platensis* is a good nutrient for human and animal nutrition due to its being rich in protein, beta-carotene, and essential fatty acids such as γ-linolenic acid, other omega-3 and omega-6 fatty acids, and various minerals (Farag et al., 2016). Therefore, *S. platensis* is rapidly being added to the food basket of people worldwide (Sharoba et al., 2014). However, despite the reported antioxidant properties of some compounds of *S. platensis* (Park et al., 2018; Wu et al., 2011), as far as we know, no studies have examined its effect on antioxidant enzymes.

Numerous studies have shown that increased expression and activity of antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase, inhibit the cell division of cancer cells (Zalewska-Ziob et al., 2019). In addition to cancer, antioxidant enzymes play an important and effective role in the prevention of various inflammatory diseases, such as lupus, arthritis, as well as cardiovascular disease, gastric ulcer, hypertension, diabetes, age-related eye diseases, and nervous system diseases such as Alzheimer's and Parkinson's diseases (Ahmad et al., 2017; Forrester et al., 2018).

Catalase, in particular, is considered one of the most important antioxidant enzymes. It is found in nearly all living organisms. In mammalian cells, the highest catalase activity is found in the liver and kidney mitochondria as well as in the cytoplasm of red blood cells. Having one of the highest turnover numbers known, catalase protects cells from ROS-induced oxidative damage. One catalase molecule can decompose millions of hydrogen peroxide (H_2O_2) molecules into harmless products, oxygen, and water (Panahi et al., 2019). H_2O_2 is considered one of the non-radical reactive oxygen species that is continuously produced in aerobic organisms. This molecule is readily dissolved in lipids and, therefore, diffuses rapidly between cell membranes. The accumulation of H_2O_2 in cells leads to the oxidation of important cellular macromolecules, such as proteins, lipids, and DNA that ultimately lead to mutagenesis and cell death (Vieceli et al., 2014; Halliwell & Gutteridge, 2015). In addition to neutralizing the deleterious effects of H_2O_2, catalase is involved in regulating the aging process in eukaryotes so that mutation of this enzyme can decrease life span, but conversely, increase the lifespan and delayed heart disease and cataract among rats as its expression increases (Selvaratnam and Robaire, 2016; Schriner et al., 2005). In addition, there are some reports of the role of reduced catalase activity in the development of diseases such as vitiligo (Mansuri et al., 2017), diabetes (Lenzen, 2017), and Alzheimer's (Youssef et al., 2018).

Due to the importance of catalase in maintaining human health as well as the various therapeutic properties of *S. platensis*, the present study aims to investigate the effect of different *S. platensis* extracts on the catalytic activity. Additionally, we studied the effects of the extracts on the secondary and tertiary structure of catalase to determine whether the antioxidant effects of *S. platensis* can be exerted through its impact on catalase, an enzyme in the body's antioxidant system.

2. Materials and methods

2.1. Reagents

Crystalline bovine liver catalase was purchased from Sigma-Aldrich. The stock solution was prepared by the dissolution of 2 mg of pure catalase in one ml phosphate buffer. H_2O_2 30% was also obtained from Sigma-Aldrich. All the materials needed to prepare Zarrouk's medium were obtained from Merck.

2.2. Source organism and cultivation conditions

The *S. platensis* strain (PCC 7345) used in this work was donated from the Algae Culture Collection of the Food Biotechnology Research Institute, ABRII, Tabriz, Iran. S. platensis was grown in Zarrouk's medium under controlled conditions (pH 9, 30 ± 2 °C, 12 /12 hour light-dark cycles provided by fluorescent lamps at a light intensity of 4000 lux). At the end of the 16th day, the biomass was harvested and then dried at 40 °C (Fedekar Fade Madkour et al., 2012).

2.3. Preparation of *S. platensis* extracts

First, 0.5 g of dry biomass was soaked in 50 ml double distilled water and left at room temperature for 24 hours to prepare an aqueous extract of *S. platensis*. The resulting mixture was then centrifuged at 3,000 g for 15 min. The supernatant was filtered (Whatman No. 1) and then transferred to a glass plate and dried in the incubator (45 °C). The methanol extract of *S. platensis* was prepared by the same method, except 80% methanol was used as a solvent instead of water. The dried extracts were stored at 4 °C until used in the experiments.

2.4. Assessing catalase activity in the presence of *S. platensis* extract

Catalase activity was measured using a spectrophotometer (Varian, CARY 100 Bio, USA). The reaction mixture contained 980 µl of phosphate buffer (50 mM, pH 7.0), 30 mM hydrogen peroxide as substrate, and a constant catalase concentration (10 µl, 2.3 nM). The change in absorbance was recorded every 20 seconds for 3 minutes at 240 nm, and the initial enzyme velocity was determined from the slope of the curve of the change in H_2O_2 absorbance at 240 nm plotted against time (Aebi, 1984). In addition, the changes in catalase activity (2.3 nM) were measured in the presence of different concentrations of each extract (0.01-800 µg / ml) and a fixed concentration of H_2O_2 prepared in the same method described above in order to evaluate the impact of aqueous and methanol extracts of *S. platensis* on catalase activity. The results illustrated in the graphs are the mean of at least three repetitions for each experiment.

2.5. Determination of inhibition mechanism

In order to determine the inhibition mechanism of catalase activated by the aqueous and methanol extracts of *S. platensis*, enzyme (2.3 nM) activity was measured in the presence of different concentrations of *S. platensis* extracts (600 and 800 µg / mL) and substrate after a pre-incubation period between the catalase and the extracts. Three pre-incubation times were tested (0, 10, and 30 min); for simplicity, only 10 min results have been shown as they covered more than 50% of the inhibition rates.

2.6. Estimation of kinetic parameters

The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) values were determined by drawing Lineweaver-Burk plots using initial velocities measured over a substrate concentration ranging from 5.88 to 22.25 mM. The apparent K_i value (the equilibrium dissociation constant for the enzyme-inhibitor complex) was obtained by plotting the slope of each line in the Lineweaver–Burk plots against different concentrations of *S. platensis* extracts. The apparent K_i value was

confirmed by a Dixon plot, plotting 1/v against various concentrations of *S. platensis* extracts. Measurements were performed in triplicate.

2.7. Structural studies of catalase

2.7.1. Electron absorption spectrum

The electron absorption spectra of catalase were recorded in the presence of different concentrations of methanol *S. platensis* extracts (0-800 µg/mL) with a Hitachi U-3900 spectrophotometer in the range of 250 - 700 nm at 25 °C. The initial reaction mixture contained 50 mM phosphate buffer (pH 7) and 0.7 µM catalase in both the sample and reference cuvettes. The absorption spectrum of the enzyme was recorded after adding 20 µl of the extract and 20 µl of distilled water to the sample and reference cuvettes, respectively. The concentration of catalase was determined spectophotometrially at 405 nm using a molar extinction coefficient of $3.24 \times l0^5\ M^{-1}\ cm^{-1}$.

2.7.2. Fluorescence spectroscopy

Fluorescence spectra were recorded using a Cary Eclipse fluorescence spectrophotometer in the presence of different concentrations of *S. platensis* extract (0–800 µg/ml) to evaluate changes in the tertiary structure of the catalase. The excitation wavelength was set at 280 nm, and emission spectra were recorded from 300 to 450 nm at two temperatures of 25 and 37 °C. All the measurements were performed in a cuvette with a 1.0 cm path length.

2.7.2. Circular dichroism (CD) measurements

The CD spectra of catalase (0.8 µM) were studied in the far –UV region (190 - 250 nm) with an Avive 215 CD spectrometer at 25 °C to investigate the changes in the secondary structure of catalase in the presence of different concentrations of *S. platensis* extract (0.01, 0.05, 200, and 800 µg / ml). A 1 mm cuvette with a path length of 0.1 cm and a concentration of 0.8 µM catalase was used. In addition, CDNN software was used to deconvolute the CD spectra to quantify changes in the secondary catalase structure.

2.8. Statistical analysis

Statistical analysis was performed using Minitab version 19 software. Data were presented as mean ± SD. Differences between means were statistically analyzed using one-way ANOVA followed by Tukey's multiple comparison tests. P values less than 0.05 were considered significant.

3. Results and Discussion

3.1. Catalase activity in the presence of different concentrations of *S. platensis* extract

Catalase activity was measured in the presence of very low concentrations (10 ng/ml equivalent to 0.01 µg / ml) to high concentrations of aqueous and methanol extracts of S. platensis (800 µg/ml) for three different enzyme incubation times with extracts (0, 10, and 30 minutes). As shown in Figure 1a and b, very low concentrations of both extracts (0.01 and 0.05 µg/ ml) increased catalase activity from 1 to 13.8%, with the highest increase of 13.8% obtained in the presence of 0.01 µg/ml methanol extract ($p < 0.05$). The rate of increase in enzyme activity decreased as the pretreatment time of the enzyme increased from zero to 30 minutes, with the smallest increase in enzyme activity of 1% obtained after 30-min pretreatment of the enzyme with 0.05 µg/ml of the aqueous extract ($p < 0.05$). The effect of methanol extract on increasing the enzyme activity was about 5-7% higher than that of the aqueous extract, which was observed up to 5 µg / ml. However, the aqueous extract indicated a stimulating effect, up to 0.5 µg / ml. The concentration of 0.5 µg / ml of both aqueous and methanol extracts of S. platensis

slightly increased (2% to 4%; $p < 0.05$) catalase activity only immediately after their addition to the enzyme. However, this concentration of extracts failed to change catalase activity after 10 or 30 minutes of exposure to the enzyme. The stimulatory effect of the enzyme at low concentrations of *S. platensis* extracts and short times of enzyme pretreatment with the extract (up to ten minutes) may be related to the stability of the enzyme in its optimal configuration. Approximately one-third of all proteins are completely or partially intact, and it was found that the tendency of some elements to bind to a fully folded state is far greater than that of their tendency to bind to an unfolded or partially folded state of that protein (van Anken and Braakman, 2012). Therefore, it is possible that the *S. platensis* extract bounded to the fully folded enzyme at low and stimulating concentrations. However, binding in other states of the enzyme may also be possible at higher concentrations or as the incubation time increases. Thus, filling several binding sites on an enzyme can alter enzyme conformation and inhibit its activity. This is in agreement with our results, indicating there is more than one binding site for *S. platensis* extract compounds on catalase.

On the other hand, both aqueous and methanol extracts of *S. platensis* had an inhibitory effect on catalase activity at higher concentrations (10 to 800 µg / ml), which gradually increased as the concentration of extracts and incubation time increased. The inhibitory effect of the methanol extract was more than that of the aqueous extract, with only 50% of the enzyme activity remaining in the presence of 600 µg / ml methanol extract. The enzyme lost 80% of its activity at a concentration of 800 µg / ml of this extract. Whereas the highest concentration and time tested (800 µg / ml and 30 min) for the aqueous extract of *S. platensis* inhibited only 27% enzyme activity (Figures 1a and b).

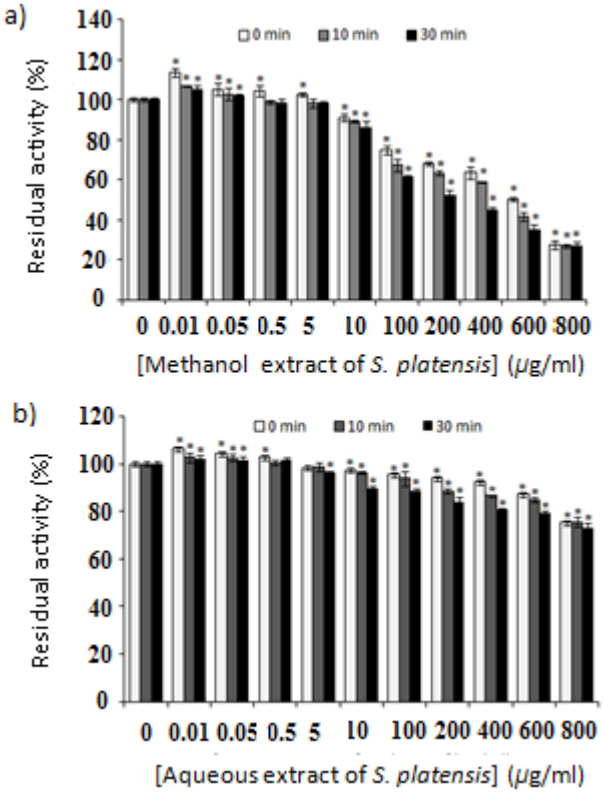

Figure 1. The residual activity of catalase in the presence of different concentrations of a) methanol and b) aqueous extracts of *S. platensis* (0–800 µg/ml). Data were expressed as mean ± SD from three different experiments. The asterisk (*) indicates a significant difference from the control group (*, $P < 0.05$).

3.2. Determining the inhibition pattern and kinetics parameters

The inhibition kinetics of catalase at different concentrations of the aqueous and methanol extracts of *S. platensis* was analyzed using Lineweaver-Burk plots (Figures 2 and 3).

Both extracts lowered the V_{max} but not the K_m, indicating *S. platensis* extracts were non-competitive inhibitors of catalase. In the absence of an inhibitor, the K_m and V_{max} values were 27 mM and 0.93 mM/s, respectively. When the concentrations of aqueous and methanol extracts of *S. platensis* increased, the V_{max} values decreased by 33% and 79%, respectively, while the k_m values remained unchanged. Dixon plot experiments were performed in the presence and absence of the

extracts with several substrate concentrations (H_2O_2) to determine the inhibition constant (K_i).

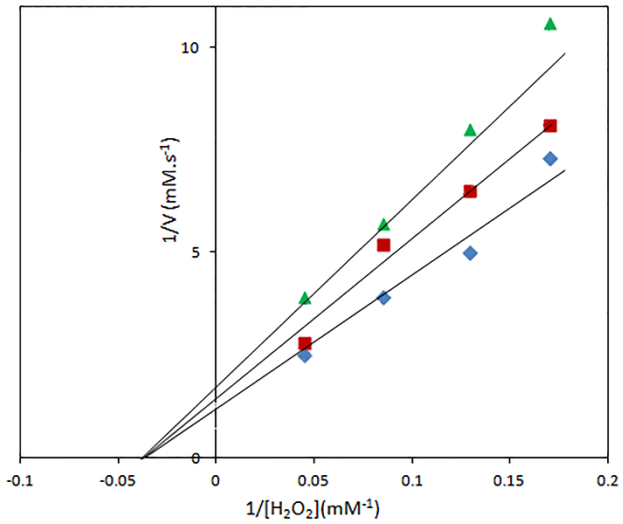

Figure 2. Lineweaver–Burk plot of catalase activity in the absence (♦) and presence of aqueous extract of *S. platensis* at different concentrations: 600 µg / ml (■) and 800 µg / ml (▲).

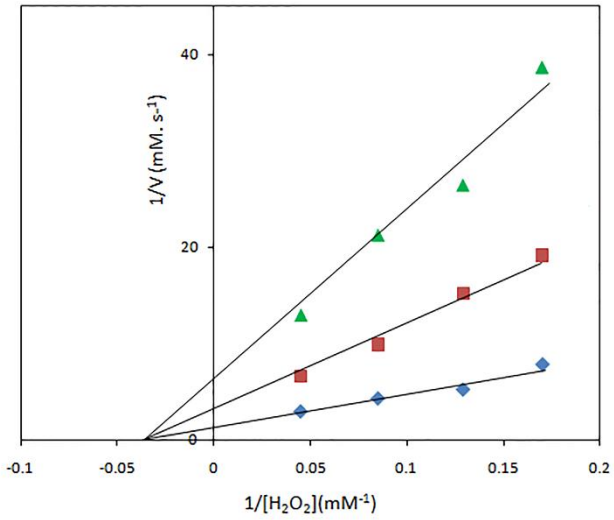

Figure 3. Lineweaver–Burk plot of catalase activity in the absence (♦) and presence of methanol extract of *S. platensis* at different concentrations: 600 µg / ml (■) and 800 µg / ml (▲).

Figures 4 and 5 show the results of the Dixon plot analyses for the aqueous and methanol extracts of *S. platensis*, respectively.

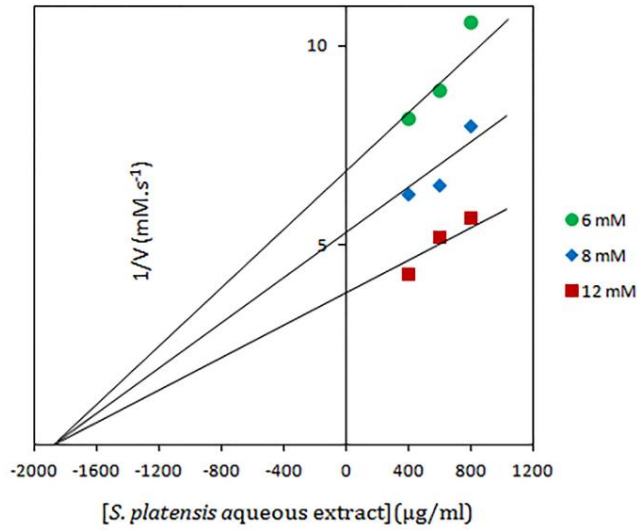

Figure 4. Dixon plot for the determination of the dissociation constant (K_i) value of the aqueous extract of *S. platensis* at three different concentrations of H_2O_2.

Values of K_i for aqueous and methanol *S. platensis* extracts obtained were 1.8 mM and 0.29 mM, respectively (Table 1).

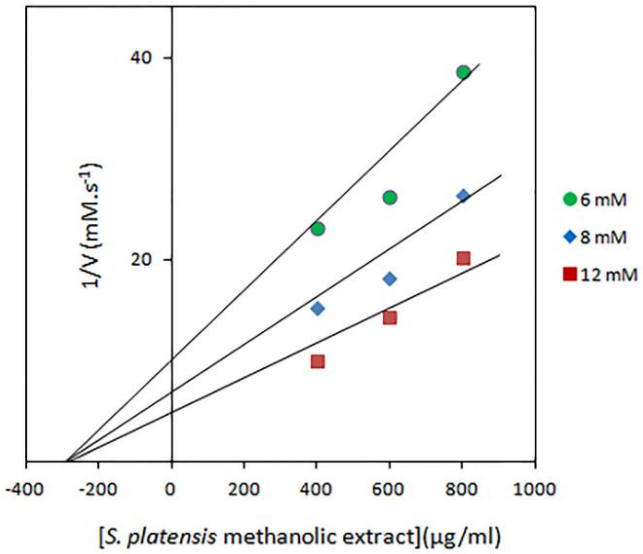

Figure 5. Dixon plot for the determination of the dissociation constant (K_i) value of the methanolic extract of *S. platensis* at three different concentrations of H_2O_2.

Table 1. Kinetic parameters of aqueous and methanol *S. platensis* extracts on catalase activity.

***S. platensis* extracts (mg /mL)**	**K_m (mM)**	**V_m (mM.s^{-1})**	**V_m / K_m (s^{-1})**	**K_i (mM)**
Without extract (0)	27.03	0.93	0.034	0
Aqueous extract (0.6)	27.03	0.71	0.026	1.8
Aqueous extract (0.8)	27.03	0.62	0.023	1.8
Methanolic extract (0.6)	27.03	0.37	0.014	0.29
Methanolic extract (0.8)	27.03	0.20	0.007	0.29

The non-competitive inhibitor can be bound either to a free enzyme or an enzyme-substrate complex, forming an inactive enzyme-substrate-inhibitor complex. Therefore, the aqueous and methanol extracts of *S. platensis* bind to a different position than the substrate; they can reduce or eliminate the catalytic activity of the enzyme by altering its conformation and accordingly influencing its catalytic site. The observed changes in the electron absorption spectrum, as well as the intrinsic fluorescence emission spectrum of catalase in the presence of the different concentrations of the extracts, confirmed this issue.

In the following part of the experiments, only the methanol extract of *S. platensis* was evaluated because this extract had a considerably stronger inhibitory and stimulatory effect on the catalase enzyme activity compared to the aqueous extract of *S. platensis* at different concentrations and times of incubation. The effect of this extract was studied using the changes in the electron absorption, fluorescence, and CD spectra.

3.3. Structural studies of *S. platensis* on catalase

3.3.1. UV–Vis absorption studies

The absorption spectra of catalase after 10 min of incubation with 0–800 µg / ml concentrations of methanol extract of *S. platensis* are shown in Figure 6. The UV-Vis spectrum of natural catalase (0.7 µM) shows two maxima at 278 and 405 nm (Soret-band), which is due to the aromatic amino acids and the $\pi \rightarrow \pi^*$ transition of electrons in the heme group (Bartoszek & Suákowski, 2006).

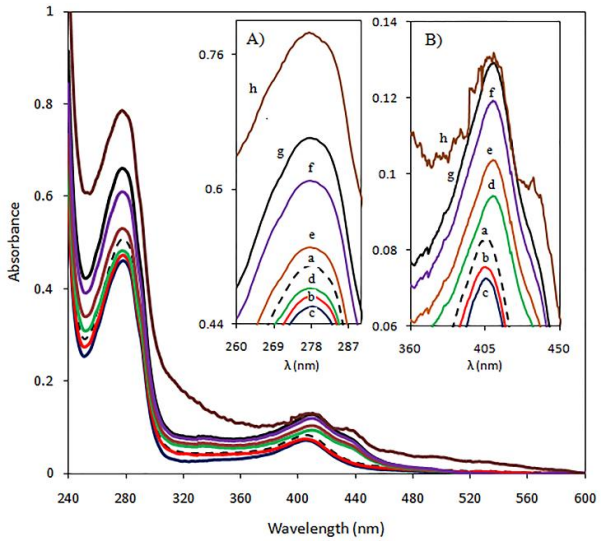

Figure 6. Electronic absorption spectra of catalase incubated with *S. platensis* methanol extract. For any given spectrum, catalase (0.7 µM), phosphate buffer (50 mM, pH 7), and *S. platensis* methanol extract (0.01 to 800 µg / ml) were added to the sample cuvette, whereas phosphate buffer (50 mM, pH 7), and *S. platensis* methanol extract (at the same concentration as in the sample cuvette) were added to the reference cuvette. Insets: absorbance changes at 287 nm (A), and 405 nm (B) in the presence of (a) 0.01, (b) 0.05 µg / ml, (c) 0. 5 µg / ml, (d) 10 µg / ml, (e) 200 µg / ml, (f) 400 µg / ml, (g) 600 µg / ml, and (h) 800 µg / ml *S. platensis* methanol extract.

There was a slight decrease in the intensity of both absorption peaks in the presence of three *S. platensis* methanol extract concentrations that stimulated the catalase activity (0.01, 0.05, and 0.5 µg / ml). However, in the presence of inhibitory concentrations of the extract (10-800 µg / ml), the peak intensity of catalase at 278 nm increased with a blue shift (from 278 t0 275). At 405 nm, it increased with a red shift (from 405 t0 414), indicating some compounds of *S. platensis* extract

can form complex with catalase and alter its structure.

The increase in the intensity of the peak at 278 nm in the presence of high *S. platensis* extracts represents the unfolding of catalase. However, the absorbance change around 405 nm indicates that changes have occurred in the position corresponding to tryptophan residues located in the vicinity of the heme pocket. It has previously been reported that one tryptophan residue is located at the bottom of the channel end, the active site of catalase (Zamocky et al. 2008). Therefore, the resulting changes indicate that in addition to the overall three-dimensional structure of catalase, the conformation of the active site of the enzyme (heme group) is also affected by the compounds present in the *S. platensis* extract. Catalase intrinsic fluorescence spectroscopy was used to confirm the induction of the three-dimensional shape change of the enzyme under the influence of *S. platensis* extract and the changes in the absorption spectrum.

3.3.2. Fluorescence studies

Fluorescence spectra of the catalase (0.85 µM) in the presence of different concentrations of *S. platensis* methanol extract at 25 and 37 °C are represented in Figures 7 and 8, respectively. As is evident in these two figures, there is a single emission peak at 350 nm due to the fluorescence emission of the tryptophan residues in catalase.

The results of intrinsic fluorescence of catalase at both temperatures indicated that concentrations of 0.01 - 0.5 µg / ml of extract (catalase activity stimulating concentrations) increased the fluorescence emission of the enzyme with a blue shift emission maxima from 350 to 356 nm. Although high concentrations of extract (10-800 µg / ml) decreased the intensity of intrinsic fluorescence emission with a red shift from 6 nm in the presence of 10 µg / ml to 1 nM in 800 µg / ml concentration. In addition, the decrease in emission increased by increasing the extract concentration.

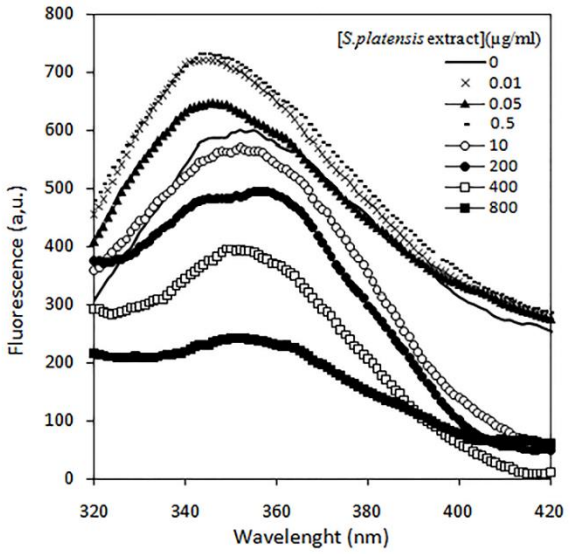

Figure 7. Fluorescence emission spectra of catalase and catalase pre-incubated for 10 min with various *S. platensis* methanol extract from 0.01 to 800 µg / ml in 50 mM phosphate buffer (pH 7) at 25 °C (λ_{ex} = 280 nm).

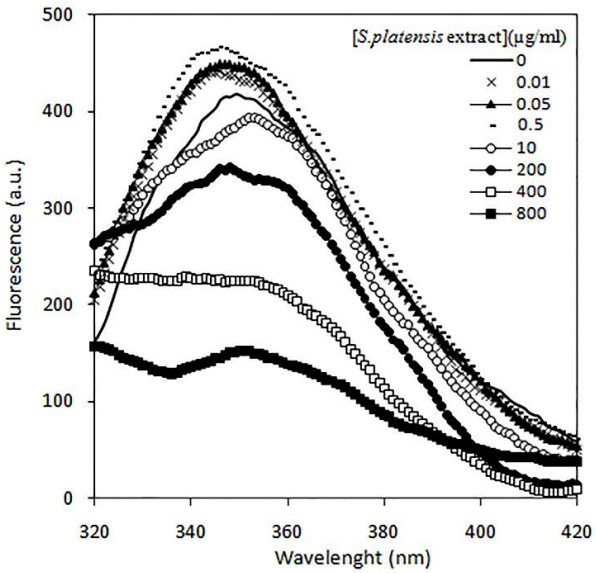

Figure 8. Fluorescence emission spectra of catalase and catalase pre-incubated for 10 min with various *S. platensis* methanol extract from 0.01 to 800 µg / ml in 50 mM phosphate buffer (pH 7) at 37 °C (λ_{ex} = 280 nm).

Figure 9 illustrates the impact of temperature on the fluorescence emission of catalase in the absence and presence of different concentrations of *S. platensis* methanol extract. As shown, the rate

of the decrease of catalase fluorescence emission intensity at 37 °C with any given concentration of the extract is slightly lower than that of the quenching rate corresponding to the same extract concentration at 25 °C. In addition, the increase in the catalase fluorescence peak at 25 °C was higher than the one at 37 °C at stimulatory concentrations. In fact, the rate of induced changes in the three-dimensional structure of the enzyme at different concentrations of methanol *S. platensis* extract was greater at 25 °C than at 37 °C.

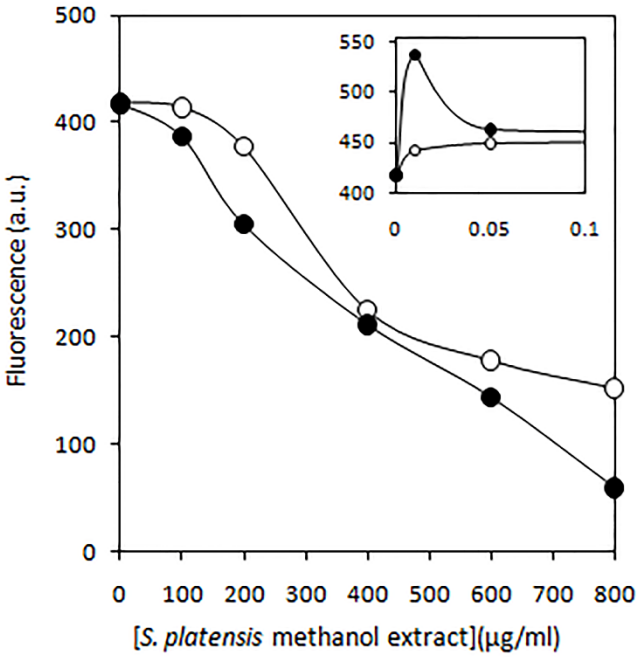

Figure 9. Changes in the maximum intensity of catalase at various concentrations of *S. platensis* methanol extract at 25 °C (●) and 37 °C (○).

The intrinsic fluorescence of proteins provides considerable information about their structure and dynamics in a solution. Conformational changes in protein, substrate binding, or denaturation lead to changes in the tryptophan emission spectra (Lakowicz 2006). Catalase is a homotetramer containing six tryptophan residues in each subunit that contribute to its fluorescence properties (Putnam et al., 2000). In this study, the intrinsic fluorescence analysis of catalase indicated that adding inhibitory concentrations of *S. platensis* extract to catalase at both 25 and 37 °C reduced the intrinsic fluorescence emission associated with a red shift, indicating tryptophan localization to a more polar environment. Conversely, increased emission and a blue shift were observed in the presence of low and stimulating concentrations of *S. platensis* extract, indicating an increase in the hydrophobicity of the environment around tryptophan residues. In other words, these results confirm the conformational changes of catalase after its interaction with *S. platensis* extract and are in accordance with the UV–Vis and CD spectroscopy findings.

3.3.3. Quenching Mechanism

The experimental data were analyzed according to the well-known Stern-Volmer equation (Equation 1) to predict the fluorescence quenching mechanism.

$$F_0/F = 1 + K_{sv}\ [Q] \qquad \text{(Equation 1)}$$

Where F_0 and F are the fluorescence intensities of the enzyme in the absence and presence of *S. platensis* extract, respectively, and K_{sv} and [Q] are the Stern-Volmer quenching constant and concentration of *S. platensis* methanol extract as a quencher, respectively.

As shown in Figure 10, the Stern-Volmer plot is linear up to the concentration of 400 µg / ml of *S. platensis* extract. It exhibits an upward curvature at higher extract concentrations, indicating the contribution of both static and dynamic mechanisms in quenching of fluorescence emission intensity of catalase.

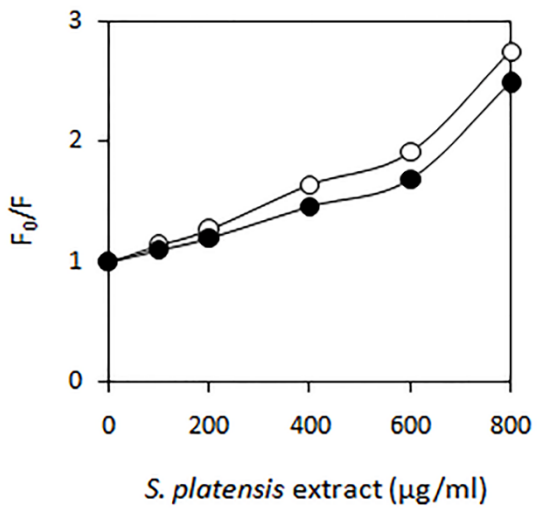

Fig.10. Stern-Volmer plots for fluorescence quenching of catalase by different concentrations of *S. platensis* methanol extract at 25 °C (●) and 37 °C (○).

The modified Stern-Volmer equation (2) was used to determine the fraction of total fluorophores accessible for a quencher.

$$F_0/(F_0-F)=(1/K_{sv}f_a)(1/[Q])+1/f_a \quad \text{(Equation 2)}$$

Where f_a is the fraction of fluorophores accessible to the quencher F and F_0, and K_{sv} and [Q] are as defined above. K_{sv} and f_a were determined from the values of the slope and intercept of the plot of $F_0 / (F_0-F)$ vs. 1 / [Q], respectively (Figure 11).

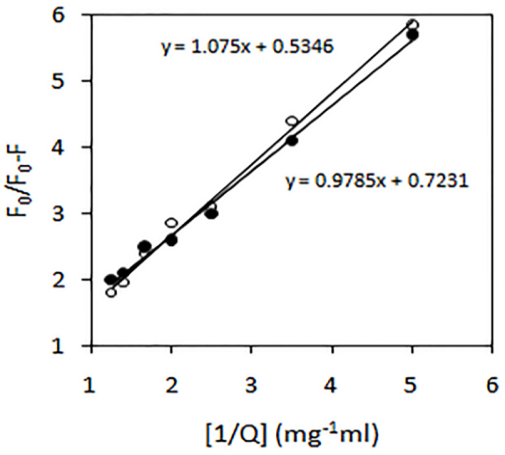

Figure 11. Modified Stern-Volmer plots of catalase in the presence of various concentrations of *S. platensis* methanol extract at 25 °C (●) and 37 °C (○).

The f_a values at temperatures of 25 and 37 °C were found to be 1.9 and 1.4, respectively, indicating approximately 79% and 58% of the enzyme fluorophores were accessible to and affected by compounds of *S. platensis* extract. The K_{sv} values were found to be 0.73 mg^{-1}.ml at 25 °C and 0.49 mg^{-1}.ml at 37 °C.

3.3.4. Binding constants and the number of binding sites

The binding constants (K_a) and the number of quencher binding sites (n) on a catalase molecule at different temperatures were respectively obtained from the intercept on the y-axis and the slope of plots of log [(F_0 - F) / F] versus log [Q] based on equation (3).

$$\text{Log } (F_0 - F)/F = n\log[Q]+\log K_a \quad \text{(Equation 3)}$$

As shown in Figure 12, there are two binding sites for the compounds present in the *S. platensis* methanol extract on the enzyme, and increasing the temperature caused no change in the number of binding sites.

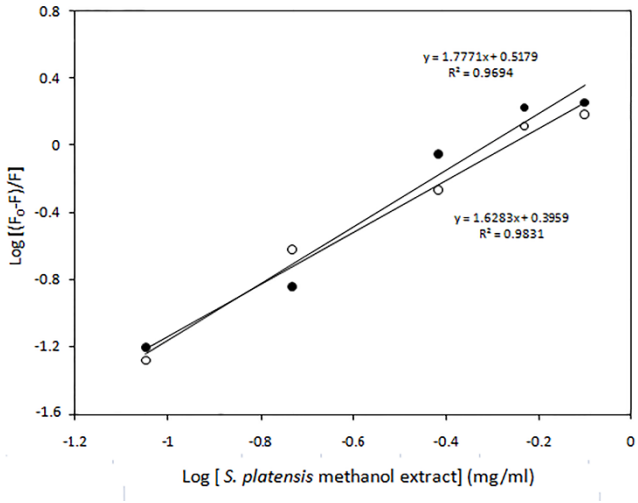

Figure 12. The plot of log [($F-F_0$)/F] vs. log [*S. platensis* methanol extract], according to equation 3, for fluorescence quenching of catalase in the presence of *S. platensis* methanol extract at 25 °C (●) and 37 °C (○).

The binding constants of compounds of *S. platensis* extract with catalase were calculated to

be 2.5 × 10^{-3} and 3.3 × 10^{-3} mg^{-1}.ml at 25 and 37 °C, respectively. Based on these results, the binding affinity increased as the reaction temperature increased. In other words, the reaction was exothermic.

The number of binding sites indicated that compounds in *S. platensis* extract could bind to the catalase through approximately two positions, and no change is made in the number of binding sites as the temperature increases from 25 to 37 °C. However, the binding constant value increased as the temperature increased, demonstrating that the stability of the *S. platensis* extract - catalase increased with the rising temperature.

3.3.5. CD studies

Circular dichroism spectroscopy gives us some interesting information about the changes in the secondary structure of proteins due to the changes in experimental conditions, such as the effect of inhibitory and activating compounds, temperature, ligand binding, etc. (Jasim et al., 2018).

The alterations in the secondary structure of catalase after the addition of different concentrations of *S. platensis* extract to the enzyme at 25 and 37 °C were studied by CD in the far-UV–CD region (190-250 nm). As shown in Figure 13, the CD spectra of catalase exhibit two distinct extremes at 208 and 222 nm, indicating a predominately α-helical content for catalase. The changes in the shape and intensity of the CD peaks at 208 and 222 nm occurred in the presence of different concentrations of *S. platensis* methanol extract, indicating significant secondary structural changes. The calculated contents of the secondary structure elements were summarized in Table 2. Upon addition of *S. platensis* extract, the *α*-helical content increased by up to 14% for concentrations up to 0.05 µg / ml and then began to decrease; reaching 55.8% of controls for the 800 µg / ml extract concentration. In addition, increases in the *β*-sheet fraction were observed for extract concentrations 0.01 and 0.05 µg / ml; but at higher extract concentrations, the *β*-sheet content decreased and went from a 43.3% decrease with the 200 µg / ml extract to a 56.7% decrease with the 800 µg / ml extract. Concomitantly, the random coil fraction decreased by up to 34.8% for extract concentrations up to 0.05 µg / ml and then began to increase, reaching a 2-fold control value for the 800 µg / ml extract concentration. Since the same data were obtained at 37 °C, the data at this temperature are not shown.

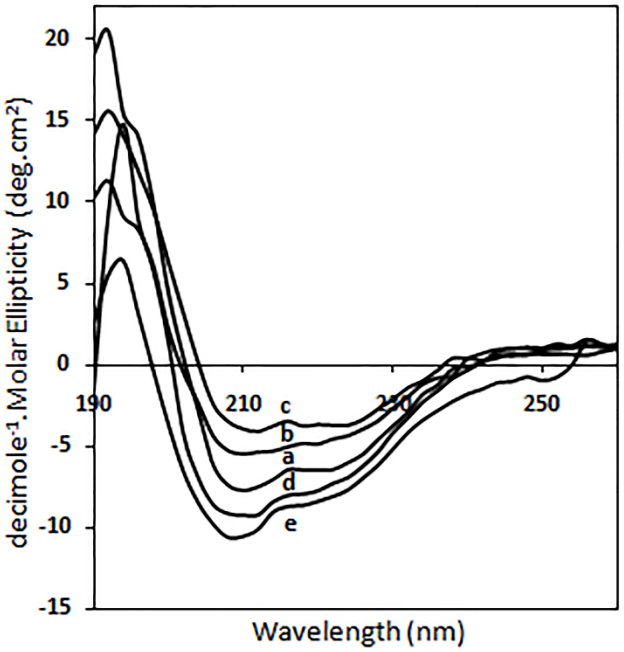

Figure 13. Far-UV CD spectra of catalase in the absence (a) and presence of various concentrations of *S. platensis* methanol extract, (b) 0.01, (c) 0.05, (d) 200, and (e) 800 µg / ml, at 25 °C temperature.

Table 2. Content of the secondary structure of catalase – *S. platensis* extract complex at 25 °C.

Sample (µg/ml)	*α*- Helix%	*β*- Sheet%	Random coil%
0	28.40±0.3	37.40±0.8	34.20±1.2
0.01	29.00±0.5	46.40±2.0	24.60±3.0
0.05	30.30±2.1	46.20±1.2	24.30±2.4
200	19.30±3.2	31.60±2.5	50.10±3.6
800	15.40±0.5	16.20±1.1	68.40±2.5

The results of CD catalase spectroscopy in the presence of different concentrations of the *S.*

platensis extract indicated that the low concentrations of the extract (0.01 - 0.05 µg /ml) drive catalase towards becoming more stable as the percentage of stable structures of α-helix and β-sheet increases. Conversely, the concentrations of the *S. platensis* extract that inhibits the enzyme activity (200 and 800 µg / ml) drive the enzyme towards unfolding and instability as the percentage of α-helix and β-sheet structures decreases and the random coil structures increase. In addition, the rate of changes in the β-sheet structures was higher than in the alpha-helices in the presence of both stimulatory and inhibitory concentrations of *S. platensis* extract (Table 2). Since the β-sheet structure is considered one of the most stable secondary protein structures, the results of CD studies indicated a higher tendency of low concentrations of *S. platensis* extract to bind to the enzyme's folded state and stabilize it. However, higher concentrations affected its relative opening and instability. In other words, the results are consistent with those obtained from fluorescence and UV–vis spectroscopy studies.

3.4. Determination of interaction forces between catalase and *S. platensis* extract

The non-covalent protein–ligand interactions are divided into four groups based on the size and signs of the thermodynamic parameters: $\Delta S° > 0$ and $\Delta H° > 0$ (hydrophobic interactions); $\Delta S° > 0$ and $\Delta H° < 0$ (electrostatic interactions), and $\Delta S° < 0$ and $\Delta H° < 0$ (hydrogen bonds and van der Waals forces) (Mu et al., 2011). The interaction forces between catalase and methanol extract of *S. platensis* were determined using equations 4 (van Hoff equation) and 5.

$$\ln K_1/K_2 = (-\Delta H°/R)(1/T_2 - 1/T_1) \quad \text{(Equation 4)}$$

$$\Delta G° = \Delta H° - T\Delta S° = -RT \ln K \quad \text{(Equation 5)}$$

Where K_1 and K_2 are the binding constants obtained from equation 3 at the corresponding temperatures (T_2 and T_1). R stands for the gas constant (Table 3).

Table 3. Thermodynamic parameters of catalase–S. platensis methanol extract interaction at two temperatures of 25 and 37 °C.

Temperature (°C)	ΔG° (KJmol^{-1})	ΔH° (KJmol^{-1})	ΔS° (JK^{-1}mol^{-1})
25	14.85-	-22.45	-0.0251
37	-14.73	-22.45	-0.0248

As can be seen, all three parameters of ΔG°, ΔH°, and ΔS° are negative. The negative value of ΔG° is indicative of spontaneous interactions of compounds of *S. platensis* methanol extract to the enzyme. Also, negative values of ΔH° and ΔS° are usually taken as evidence for hydrogen bonding and/or van der Waals forces between *S. platensis* extract and catalase.

The findings are consistent with the low binding constant values obtained for the extract. In addition, the negative values obtained for ΔG° at 25 and 37 °C suggest that the interaction of *S. platensis* methanol extract with catalase is performed through spontaneous intermolecular interactions. Additionally, the negative sign of ΔS° and ΔH° shows that the interactions of the *S. platensis* methanol extract and catalase are mainly enthalpy driven.

It is not possible to compare and discuss the results of this study with other researchers because, to the best of our knowledge, no study has been conducted on the interaction of *S. platensis* extract with catalase. However, the antioxidant properties of *S. platensis* and its beneficial impacts in preventing or ameliorating some diseases have previously been reported, and there are several studies on the impact of certain algae on the activity of antioxidant enzymes under *in vivo* conditions. For example, Salem and Ibrahim (2011) indicated that different extracts of some marine algae, such as *Ulva rigida*, *Enteromorpha clathrata*, *Jania adherens*, and *Corallina elongate*, exert anticancer activity against the Ehrlich Ascites Carcinoma (EAC) cell line and increase the activity of some antioxidant enzymes. Furthermore, they showed the methanol and

chloroform extract of *Ulva rigida* showed the highest cytotoxicity, stimulatory effect on catalase, and superoxide dismutase activity among the various extracts tested. Another study investigating the antioxidant properties of *Sargassum muticum* seaweed extract on the growth of male Sprague – Dawley rats pointed out that the addition of the algae extracts in the diet of rats significantly reduced lipid peroxidation and glutathione peroxidase activity. However, it failed to significantly alter the activity of catalase and superoxide dismutase (Balboa et al., 2019).

Overall, the present work indicates that *S. platensis* extract has different effects on the structure and activity of catalase depending on its concentration and, consequently, the amount of cellular H_2O_2. These non-radical reactive oxygen species are produced during and in many physiological and pathological conditions and play a dual role in disease and health. The spatiotemporal accumulation pattern of H_2O_2 determines whether this molecule will act as a signaling messenger or cause oxidative damage (Dickinson et al., 2011). Moreover, hydrogen peroxide plays a role in regulating the synthesis and activity of many transcription factors. Therefore, catalase can modulate the expression of numerous genes by altering the amount of H_2O_2 in mammalian cells (Marinho et al., 2014). Thus, by inhibiting or enhancing the activity of catalase, the most important H_2O_2-degrading enzyme, *S. platensis*, can increase and/or decrease the amount of H_2O_2 and its associated cellular processes, respectively. In other words, low concentrations of *S. platensis* could act as antioxidants, while higher concentrations could significantly reduce catalase activity, increase the concentration of H_2O_2 and, accordingly, produce beneficial effects in tumor tissue alone. Further *in vitro* and *in vivo* investigation of the antitumor activity of *S. platensis* could help explain this hypothesis.

Conclusion

Based on our results, *S. platensis* aqueous and methanol extracts have a dual effect on the catalase enzyme because the *S. platensis* extract has an active and protective role for the catalase enzyme at low concentrations (less than 0.5 µg / ml), while it inhibits the catalase non-competitively at higher concentrations. Further, thermodynamic studies indicated that the compounds in the extract of *S. platensis* spontaneously bind to the two binding sites on the enzyme through hydrogen and van der Waals interactions. Based on the CD results, the proposed mechanism is that the extract compounds at low concentrations initially bind to fully folded catalase, leading to an increase in its stability and activity. However, binding to the other site occurs in the unfolded or partially folded states of the enzyme at higher concentrations, inducing a conformational change in the active site of the enzyme that inhibits its activity. Thus, the *S. platensis* extract has the potential to strengthen the body's antioxidant system at low concentrations by activating the catalase enzyme. On the other hand, the application of *S. platensis* methanol extract at high concentrations may induce apoptosis in cancer cells by inhibiting catalase activity by thus increase H_2O_2. However, further research is needed to prove these assumptions.

Conflict of Interest

The authors declare that there is no conflict of interest.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors. This article does not contain any studies with human participants or animals performed by any of the authors.

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