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Response of *Microcystis aeruginosa* BCCUSP 232 to barley (*Hordeum vulgare* L.) straw degradation extract and fractions

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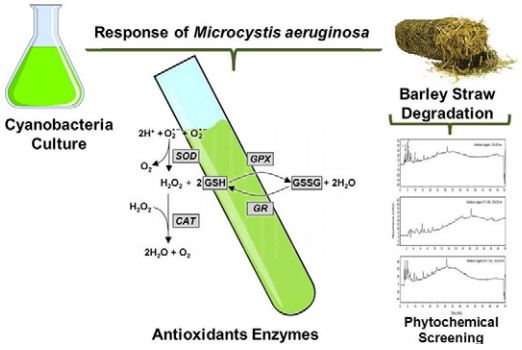
HIGHLIGHTS

- *H. vulgare* extract significantly reduced the cell density in a concentration dependent manner.
- The extract had an immediate inhibitory effect on the biomass of the cyanobacterium.
- Internal H_2O_2 concentration increased after exposure to the crude extract.
- Changes in POD, SOD and GST activities of *M. aeruginosa* exposed to *H. vulgare* crude extract.
- Reactive oxygen species, lipid peroxidation and antioxidant enzyme activities were altered.

GRAPHICAL ABSTRACT



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ABSTRACT

The eutrophication of aquatic ecosystems is a serious environmental problem that leads to increased frequency of cyanobacterial blooms and concentrations of cyanotoxins. These changes in aquatic chemistry can negatively affect animal and human health. Environment-friendly methods are needed to control bloom forming cyanobacteria. We investigated the effect of *Hordeum vulgare* L. (barley) straw degradation extract and its fractions on the growth, oxidative stress, antioxidant enzyme activities, and microcystins content of *Microcystis aeruginosa* (Kützing) Kützing BCCUSP232. Exposure to the extract significantly ($p < 0.05$) inhibited the growth of *M. aeruginosa* throughout the study, whereas only the highest concentration of fractions 1 and 2 significantly ($p < 0.05$) reduced the growth of the cyanobacterium on day 10 of the experiment. The production of reactive oxygen species (ROS), lipid peroxidation and antioxidant enzyme activities were significantly ($p < 0.05$) altered by the extract and fractions 1 and 2. Phytochemical profiling of the extract and its fractions revealed that the barley straw degradation process yielded predominantly phenolic acids. These results demonstrate that barley straw

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extract and its fractions can efficiently interfere with the growth and development of *M. aeruginosa* under laboratory conditions.

1. Introduction

The eutrophication of water resources is a serious environmental problem that is driven by nutrient loading from industrial and urban wastewater, and leaching of agricultural inputs (Heisler et al., 2008). Consequent upon the eutrophication of aquatic ecosystems, the frequency of cyanobacterial blooms and the concentration of cyanotoxins are increased. This interferes with the management of water resources, and deteriorates water quality (Barrett et al., 1996; Ferrier et al., 2005). Cyanotoxins include potent neurotoxins, hepatotoxins, cytotoxins and endotoxins that have been implicated in animal and human deaths (Sivonen and Jones, 1999; Gugger et al., 2005; Rodriguez et al., 2007; Pearson et al., 2010), and their accumulation in plant and animal tissues has led to their detection in human diet (Gangstad, 1986; Vymazal, 1995; El-Shehawy et al., 2012). As a result of the risk associated with cyanotoxins exposure, the World Health Organization (WHO) recommended 1 μg L^{-1} as the maximum permissible level of microcystin-LR in drinking water (WHO, 2004).

Biological, chemical and physical methods have been employed for the control of the harmful algae and their associated problems. Physical methods comprise filtration, centrifugal separation and ultrasound treatments (Chorus and Bartram, 1999; Kotopoulis et al., 2009; Liang and Nan, 2009); chemical methods employ compounds such as copper sulfate and hydrogen peroxide (Drabkova et al., 2007); and biological methods use selected fish and zooplankton species to control the population of harmful algae and cyanobacteria (Chorus and Bartram, 1999; Chen et al., 2007). Most of these methods have not been very successful, are very expensive and are primarily used as emergency solutions. There is the need to develop efficient, sustainable and cost effective techniques with little or no environmental footprints (Ferrier et al., 2005). As an alternative, compounds of plant origin can be employed in controlling microorganisms (e.g. microalgae and cyanobacteria)

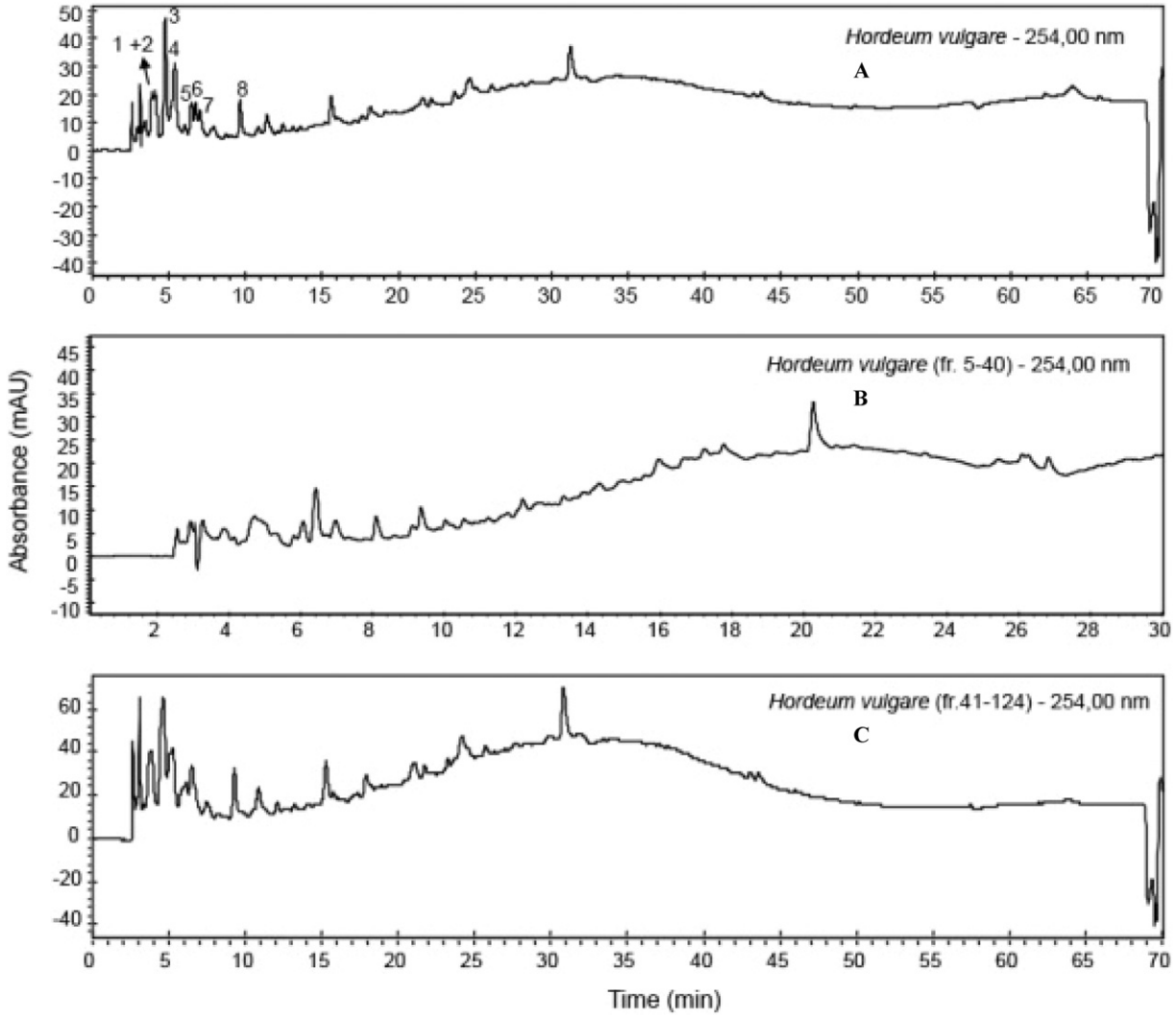

Fig. 1. HPLC-PAD chromatographic profile of *H. vulgare* extract (A), fraction 1 of *H. vulgare* extract (B) and fraction 2 of *H. vulgare* extract (C). Elution system: A (Methanol + 0.1% Form. ac.) and B (Water + 0.1% Form. ac.). Gradient: 10–60% A in B over 60 min. Phenomenex® Luna C18 column (250 × 4.6 mm i.d., 5 μm), HPLC (Jasco®), flow 1.0 mL min^{-1}, λ = 254 nm, Injection volume: 20 μL. Column oven: 40 °C.

that threaten public and environmental health, and agricultural production (Smith and Doan, 1999; Gibson et al., 1990; Pillinger et al., 1992; Park et al., 2006; Leflaive and Ten-Hage, 2007; Zak et al., 2011; Bártová et al., 2011; Hajimahmoodi et al., 2010). This encourages the search for bioactive compounds from different plants. Barley (*Hordeum vulgare* L.) is a promising source of bioactive compounds, and has been previously shown to inhibit the growth of freshwater algae and cyanobacteria (Ferrier et al., 2005; Ó Huallacháin and Fenton, 2008, 2010; Rajabi et al., 2010). The degradation products of barley straw contain polyphenols and quinones that have been implicated in the inhibition of algal and cyanobacterial species (Pillinger et al., 1992; Everall and Lees, 1997; Martin and Ridge, 1999; Waybright et al., 2009).

Although it has been previously shown that barley straw extract is capable of inhibiting the growth of cyanobacteria, the mechanism of action was not defined. Changes in growth, oxidative stress and antioxidant response provide new insights into the physiological changes toxin producing cyanobacteria undergo during exposure to barley straw extracts. Furthermore, previous studies did not considered changes in toxin production of cyanobacteria as a function of the presence and concentrations of barley extract and fractions.

So the objective of the present study was to investigate changes in growth, toxin content, oxidative stress and antioxidant response of *M. aeruginosa* during exposure to barley straw degradation extract and fractions. Beyond the conventional growth inhibition tests, the results obtained from the present study will contribute to the understanding of the mechanism of action of the degradation products of barley straw on the cyanobacterium *Microcystis*.

2. Material and methods

2.1. Plant material and extract preparation

Hordeum vulgare L. straw was obtained from an Agricultural plantation of the Instituto Agronômico de Campinas, São Paulo, Brazil. The straw was cut into 2.0 cm pieces, weighed and extracted by degradation in distilled water (2 g L^{-1}). The decomposition process was carried out in polyethylene screw capped containers, which were aerated with an air pump for 50 days at room temperature. Afterwards, the extract was filtered, frozen and lyophilized following the procedures outlined by Ferrier et al. (2005).

In order to fractionate barley straw extract, 2.0 g of lyophilized extract was dissolved in 12 mL MeOH:H_2O (60:40, v/v), sonicated for 10 min, and the solution centrifuged for 15 min (1200 $\times g$). The supernatant was filtered and chromatographed using gel permeation chromatography (GPC) on Sephadex® LH-20 column. The samples were eluted with MeOH:H_2O(60:40, v/v). Every 15 min, fractions of approximately 15 mL were collected.

The GPC separation of *H. vulgare* gave 60 fractions that were screened by TLC and grouped according to their similarities (Rf and color) into sub-fractions. Thus it was possible to verify that the

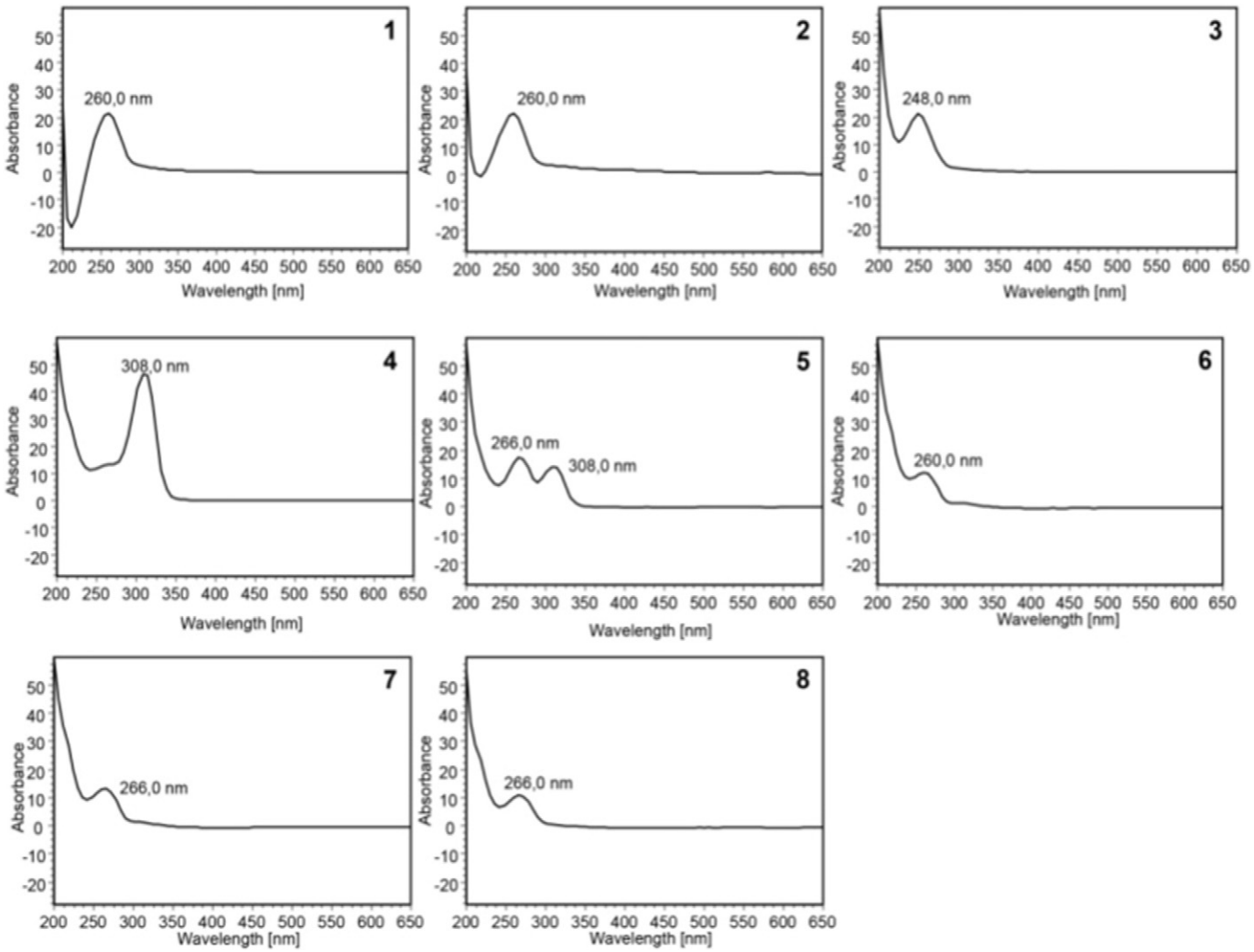

Fig. 2. (A) Maximum absorption bands in the UV region for phenolic acids found in the *Hordeum vulgare* straw extract.

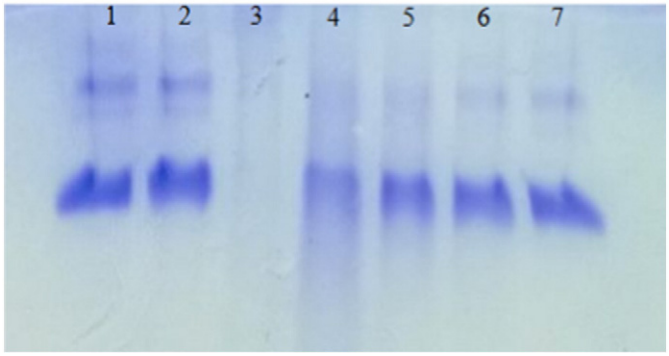

Fig. 3. Coomassie Brilliant Blue stained polyacrylamide gel showing oxidative degradation of albumin. 1) BSA (negative control); 2) BSA + Cu; 3) BSA + Cu + H_2O_2 (Positive control); 4) BSA + Cu + 500 μg mL^{-1} extract; 5) BSA + Cu + 250 μg mL^{-1} extract; 6) BSA + Cu + 100 μg mL^{-1} extract; 7) BSA + Cu + 10 μg mL^{-1} extract.

preliminary separation by GPC concentrated more polar compounds in fractions collected at the beginning of the separation process, while later fractions comprised phenolic acid derivatives and flavonoid compounds, which were reassembled into two fractions (fraction 1 and fraction 2). Once reassembled, the sub fractions were dried until complete removal of solvents.

2.2. Phytochemical screening of H. vulgare extract

Using gallic acid as a standard, the Folin–Ciocalteu method was used to measure total phenol content of the extracts (Stagos et al., 2012). To each 0.1 mL (1000 μg mL^{-1}) aliquot of the extract, 5 mL distilled water and 0.5 mL Folin–Ciocalteu reagent were added. After 3 min, 1.4 mL 25% Na_2CO_3 and 3 mL distilled water were added, and the mixture incubated for1 h. Subsequently, the absorbance of the mixture was measured at 725 nm. All measurements were performed in triplicate and the results expressed in μg gallic acid/mg extract.

Total flavonoid concentration was measured using the complexation of flavonoids with $AlCl_3$, a process that changes absorption bands to higher wavelengths (Yao et al., 2013). One milliliter extract aliquot (1000 μg mL^{-1}) was mixed with 4 mL 70% ethanol and 0.5 mL $NaNO_2$ to give a 5% solution. After 6 min of incubation, 0.5 mL 10% $AlCl_3$ solution and 3 mL 1 M NaOH solution were added to the mixture, and then the total volume brought to 10 mL with distilled water. The mixture was vortexed, and the absorbance read at 510 nm. All assays were carried out in triplicate, and the flavonoid concentrations given in μg rutin/mg extract.

Prior to high performance liquid chromatography (HPLC) analysis, 10 mg aliquot of barley straw extract was dissolved in 1 mL methanol/water (1:1) and filtered through a 0.45 μm pore size SPE syringe filter. Chromatographic analysis was performed with a Jasco® Model PU-2089S Plus HPLC system coupled to a MD-2015 photodiode array detector (scan range 200–900 nm), an AS-2055 auto sampler with 50 mL loop, and a CO-2060 column oven (Jasco®, Hachioji, Tokyo, Japan). Reverse phase immobilized 250 × 4.6 mm idoctadecylsilane column (Phenomenex Inc., Torrance, CA, USA) and 4 × 3 mm id guard column were used (Phenomenex Inc., Torrance, CA, USA). Mobile phase comprised methanol + 0.1% Formic acid (A), and Water + 0.1% Formic acid (B). Gradient was 05–100% of A in B for 60 min. Chromatograms were obtained at 254, 330 and 360 nm. The Jasco *ChromPass* v.1.8.1.6 software was used for acquisition and processing of chromatographic data.

2.3. Evaluation of pro-oxidative activity of H. vulgare extract

2.3.1. Sample preparation

Relative mobility of electrophoresis was measured according to the method of Hsieh et al. (2005). Bovine serum albumin (BSA, 2 mg mL^{-1}) was diluted in 10 mM phosphate buffer (pH 7.4). The $H_2O_2/CuSO_4$ oxidation system consisted of 50 mM H_2O_2 and 1 mg mL^{-1} CuSO4 solution (Hunt et al., 1988). The negative control of the tests contained 1 mL of BSA solution and 1 mL phosphate buffer; positive control had 1 mL of BSA, 0.5 mL $H_2O_2/CuSO_4$ solution and 0.5 mL phosphate buffer; and the treatment contained 1 mL BSA, 0.5 mL $H_2O_2/CuSO_4$ solution and 0.5 mL of *H. vulgare* extract at 10, 100, 250 and 500 μg mL^{-1}. The negative control, positive control, and treatments were incubated at 37 °C for 48 h. BSA electrophoresis was performed using polyacrylamide gel electrophoresis (SDS-PAGE).

2.3.2. One-dimensional gel electrophoresis (SDS-PAGE) preparation

The pro-oxidative activity of different concentrations of *H. vulgare* extract was analyzed using one-dimensional electrophoresis in polyacrylamide gel (Laemmli, 1970; Kocha et al., 1997). The 12% polyacrylamide gels (22.2% acrylamide/0.6-bis acrylamide; 1 M Tris-HCl, pH 8.8; distilled water; 10% SDS; 10% ammonium persulfate, TEMED) were mounted on K33-10 V electrophoresis system 10 × 10 cm plates (Kasvi, Brazil). The negative control, positive control and treatment samples were incubated in a sample buffer (1 M Tris-HCl, pH 6.8, 4% SDS, 20% Glycerol, 5% B-mercaptoethanol, 0.2% bromophenol blue) at 95 °C for 5 min, and then applied to sodium dodecyl sulfate polyacrylamide gel. The BSA protein was electrophoretically separated at 200 mA and 100 V. After the run, the gels were stained with Coomassie solution (Coomassie Bright Blue R-250 in 50% methanol and 10% acetic acid) and stored in 7% acetic acid until image scanning. In addition, for better visualization of the electrophoretic profile of some samples, staining was done with silver nitrate.

2.4. Bioassays with Microcystis aeruginosa BCCUSP232

For the assays, *Microcystis aeruginosa* BCCUSP232 was obtained from the Brazilian Cyanobacteria Collection of the University of Sao Paulo. The cyanobacterium produces microcistin-LR and RR (Bittencourt-Oliveira et al., 2011). The cultures were maintained in ASM-1 culture medium at pH 7.4 (Gorham et al., 1964) under controlled conditions (30 μmol m^{-2} s^{-1} light intensity, 14:10 h light:dark cycle photoperiod, and 23 ± 0.5 °C temperature).

All bioassays were carried out in 1000 mL Erlenmeyer flasks containing 600 mL of culture medium. For each treatment, 2.3 × 10^5 cells mL^{-1}

Table 1
Growth of *M. aeruginosa* BCCUSP232 exposed to different *Hordeum vulgare* L. straw extract concentrations (50, 100, 250 and 500 mg L^{-1}).

Cell density (× 10^3 cells mL^{-1})					
	Concentrations	4th day	6th day	8th day	10th day
H. vulgare extract	50 mg L^{-1}	595 ± 84a	1.284 ± 171a	1.662 ± 201a	3.073 ± 491a
	100 mg L^{-1}	340 ± 58a	651 ± 183b	762 ± 283b	1.684 ± 517b
	250 mg L^{-1}	418 ± 102a	962 ± 217b	273 ± 000c	817 ± 483c
	500 mg L^{-1}	307 ± 115a	918 ± 359b	000 ± 000d	000 ± 000d
Control		495 ± 102a	1.129 ± 157a	1.718 ± 164a	2.595 ± 189a

Results are means ± standard deviation for $n = 3$. Means with different alphabets per column are significantly different ($p < 0.05$).

Table 2
Growth of *M. aeruginosa* BCCUSP232 as a function of different concentrations (25, 50 and 100 mg L^{-1}) of fraction 1 obtained from *Hordeum vulgare* L. straw extract.

Cell density ($\times 10^3$ cells mL^{-1})					
	Concentrations	4th day	6th day	8th day	10th day
Fraction 1	25 mg L^{-1}	623 ± 24a	957 ± 71a	1.590 ± 165a	2.473 ± 141a
	50 mg L^{-1}	657 ± 24a	907 ± 0b	1.473 ± 47a	2.373 ± 0b
	100 mg L^{-1}	607 ± 47a	873 ± 94b	1.307 ± 188c	2.057 ± 306c
Control		573 ± 24a	951 ± 0a	1.557 ± 47a	2.462 ± 23a

were used as the initial cell density (day 0). After 96 h at exponential growth phase, *M. aeruginosa* was exposed to different concentrations (50, 100, 250 and 500 mg L^{-1}) of *Hordeum vulgare* extract. The negative control contained only water. In a second series of experiments, exponential growth phase *M. aeruginosa* was exposed to the different fractions obtained in section 2.2 above. For each fraction, the cyanobacterium was exposed to 25, 50 and 100 mg L^{-1} concentration. During exposure to each extract and fraction treatment, the cyanobacterium was incubated for 10 days under the same conditions as the stock cultures described above. All experimental treatments were carried in triplicates.

Sample aliquots were collected every two days for growth and biomass determination. Cell density was determined using a combination of optical density at 750 nm ($y = 3E\text{-}08\times + 0.0058$, $R^2 = 0.9992$), and microscopic counts with the aid of a Fuchs Rosenthal counting chamber.

2.5. Biochemical analyses

For biochemical analyses, *M. aeruginosa* biomass was obtained from 40 mL of culture by centrifugation at 1200 ×*g* for 5 min, and the resulting pellet (biomass) stored at −80 °C. The pellet was resuspended in 2 mL 0.1 M phosphate buffer (pH 6.5) containing 1% (w/v) polyvinylpyrrolidone (PVP). The mixture was homogenized by vortexing for 10 s, centrifuged (10,000 ×*g*, 0 °C, 10 min) and the supernatant stored at −20 °C for biochemical assays.

Internal H_2O_2 levels in *M. aeruginosa* were determined by monitoring the formation of titanium peroxide (Jana and Choudhuri, 1982). A 750 μL aliquot of the extract was mixed with 250 μL of 0.1% titanium chloride (in 20% H_2SO_4). Absorbance of the red-orange solution was measured at 410 nm. H_2O_2 concentration was calculated using its extinction coefficient (0.28 L $mmol^{-1}$ cm^{-1}) and expressed as μmol/cell.

The formation of Malondialdehyde (MDA) was measured using the thiobarbituric acid (TBA) method (Madhava Rao and Sresty, 2000) with some modifications. To 1 mL aliquot of the extracts was added 4 mL 0.5% (w/v) TBA in 20% TCA (w/v), and the reaction mixture incubated at 95 °C for 30 min in a water bath. The reaction was immediately stopped by cooling in an ice bath, and the mixture centrifuged at 10000 g for 15 min and vortexed. The absorbance of the mixture was read at 532 nm and corrected at 600 nm. MDA concentration (mM)

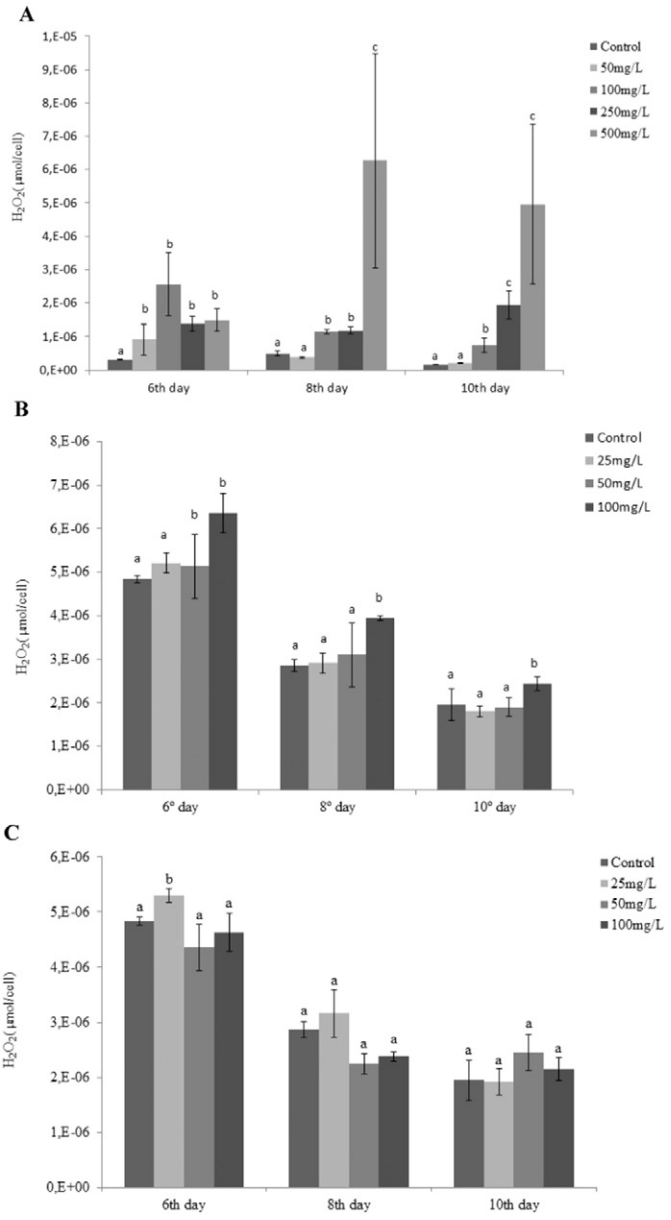

Fig. 4. Intracellular H_2O_2 content of *M. aeruginosa* BCCUSP232 exposed to different concentrations of *H. vulgare* extract (A), fraction 1 of *H. vulgare* extract (B), and fraction 2 of *H. vulgare* extract (C). Error bars represent standard deviation for $n = 3$. Means with different alphabets are significantly different ($p < 0.05$).

was calculated using the extinction coefficient of 155 mM^{-1} cm^{-1} (Demiral and Türkan, 2005):

$$[MDA](mM) = \frac{\text{Abs } 532 - \text{Abs } 600}{155,10}$$

In order to determine specific activity of antioxidant enzymes, total protein content was quantified. Using BSA as protein standard, total

Table 3
Growth of *M. aeruginosa* BCCUSP232 as a function of different concentrations (25, 50 and 100 mg L^{-1}) of fraction 2 obtained from *Hordeum vulgare* L. straw extract.

Cell density ($\times 10^3$ cells mL^{-1})					
	Concentrations	4th day	6th day	8th day	10th day
Fraction 2	25 mg L^{-1}	590 ± 23a	973 ± 0ab	1.507 ± 141a	2.307 ± 47b
	50 mg L^{-1}	607 ± 47a	1.040 ± 94ab	1.590 ± 23a	2.273 ± 94b
	100 mg L^{-1}	573 ± 38a	1107 ± 47b	1.623 ± 23a	2.207 ± 94b
Control		573 ± 24a	951 ± 0a	1.557 ± 47a	2.462 ± 23a

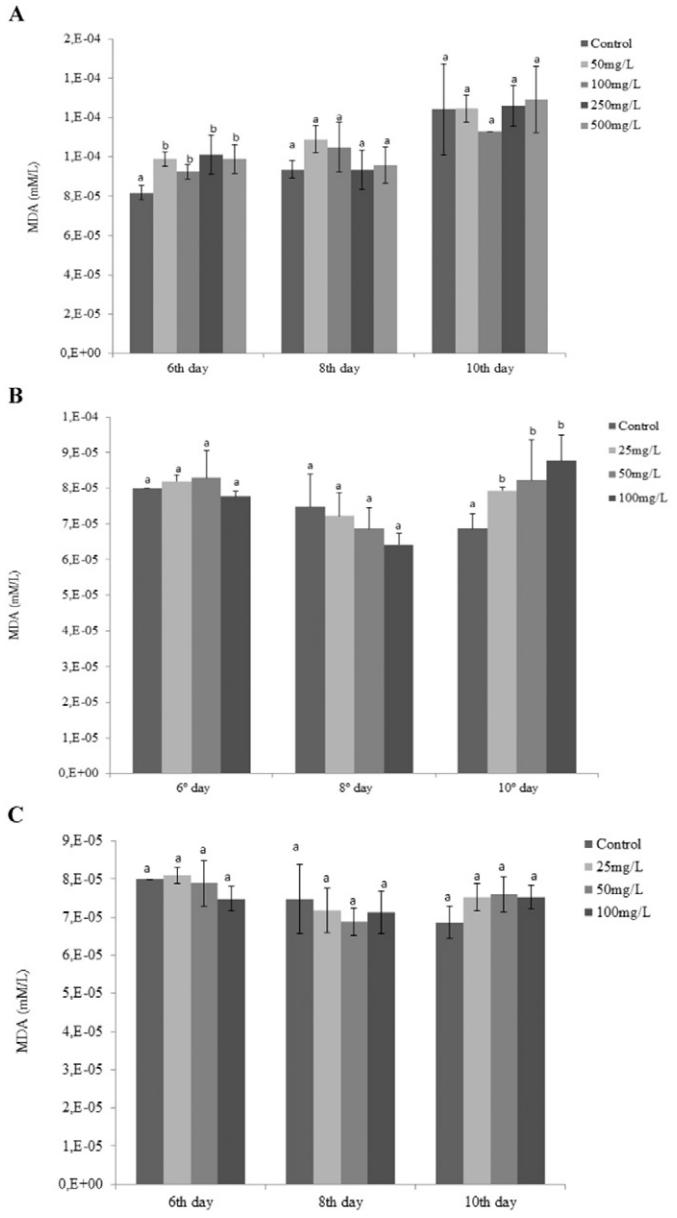

Fig. 5. Lipid peroxidation in *M. aeruginosa* BCCUSP232 exposed to different concentrations of *H. vulgare* extract (A), fraction 1 of *H. vulgare* extract (B), and fraction 2 of *H. vulgare* extract (C). Error bars represent standard deviation for $n = 3$. Means with different alphabets are significantly different ($p < 0.05$).

protein concentration was measured following the procedure described by Bradford (1976). Superoxide dismutase activity was determined spectrophotometrically according to Misra and Fridovich (1972). Keeping SOD concentration below 1 unit (U), the 3 mL reaction mixture used to measure the activity of the enzyme contained 6.7 mM potassium phosphate buffer (pH 7.8), 45 μM methionine, 5.3 mM riboflavin and 84 μM nitro blue tetrazolium chloride (NBT). One enzymatic unit was defined as the amount of enzyme required to inhibit 50% NBT reduction. Peroxidase activity was assayed following the method of Reddy et al. (1995). To 3 mL of 0.05 M pyrogallol solution in 0.1 M phosphate buffer (pH 6.5), 0.5 mL 1% H_2O_2 was added and gently mixed, and the reaction initiated by the addition of 0.1 mL enzyme extract. The activity (nkat mg^{-1} protein) of the enzyme was proportional to the change in absorbance per minute.

Glutathione S-transferase activity was measured at 340 nm using 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) as substrates (Mauch and Dudler, 1993). Enzymatic reaction was started by the addition of 50 μL enzyme extract to 1 mL of reaction mixture (3.6 mM GSH and 1 mM CDNB in 0.1 M potassium phosphate buffer, pH 6.5). The change in absorbance at 340 nm was proportional to GST activity (nkat mg^{-1} protein). Catalase (CAT) activity of the cyanobacterium was assayed using its perioxidatic function (Johansson and Borg, 1988). Methanol was used as the hydrogen donor for CAT, which results in the formation of formaldehyde. Subsequently, the formaldehyde was measured colorimetrically with Purpald, and a unit (U) of CAT activity was equivalent to the amount of enzyme that caused the formation of 200 μM formaldehyde. CAT activity was expressed in nkat mg^{-1} protein.

2.6. Microcystins analysis

Microcystins extraction was performed on frozen culture samples in 1.5 mL microcentrifuge tubes. The samples were thawed at room temperature, and rapidly subjected to 3 freeze (in liquid nitrogen for 30 s) – thaw (in a water bath at 37 °C for 5 min) cycles. Total microcystins concentration was measured using BEACON ELISA plate kits (Beacon Analytical Systems Inc., USA), following the manufacturer's instructions. The absorbance of the color reaction was measured at 450 nm with an

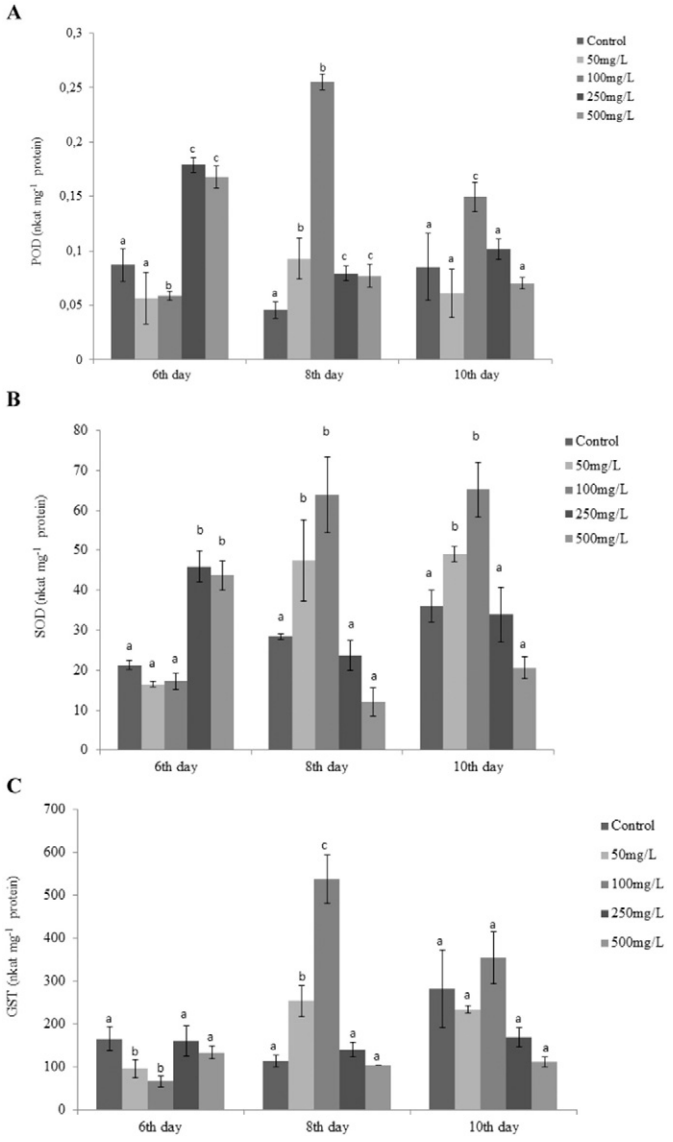

Fig. 6. Effect of different concentrations of *H. vulgare* extract on POD (A), SOD (B) and GST (C) activities of *M. aeruginosa* BCCUSP232. Error bars represent standard deviation for $n = 3$. Means with different alphabets are significantly different ($p < 0.05$).

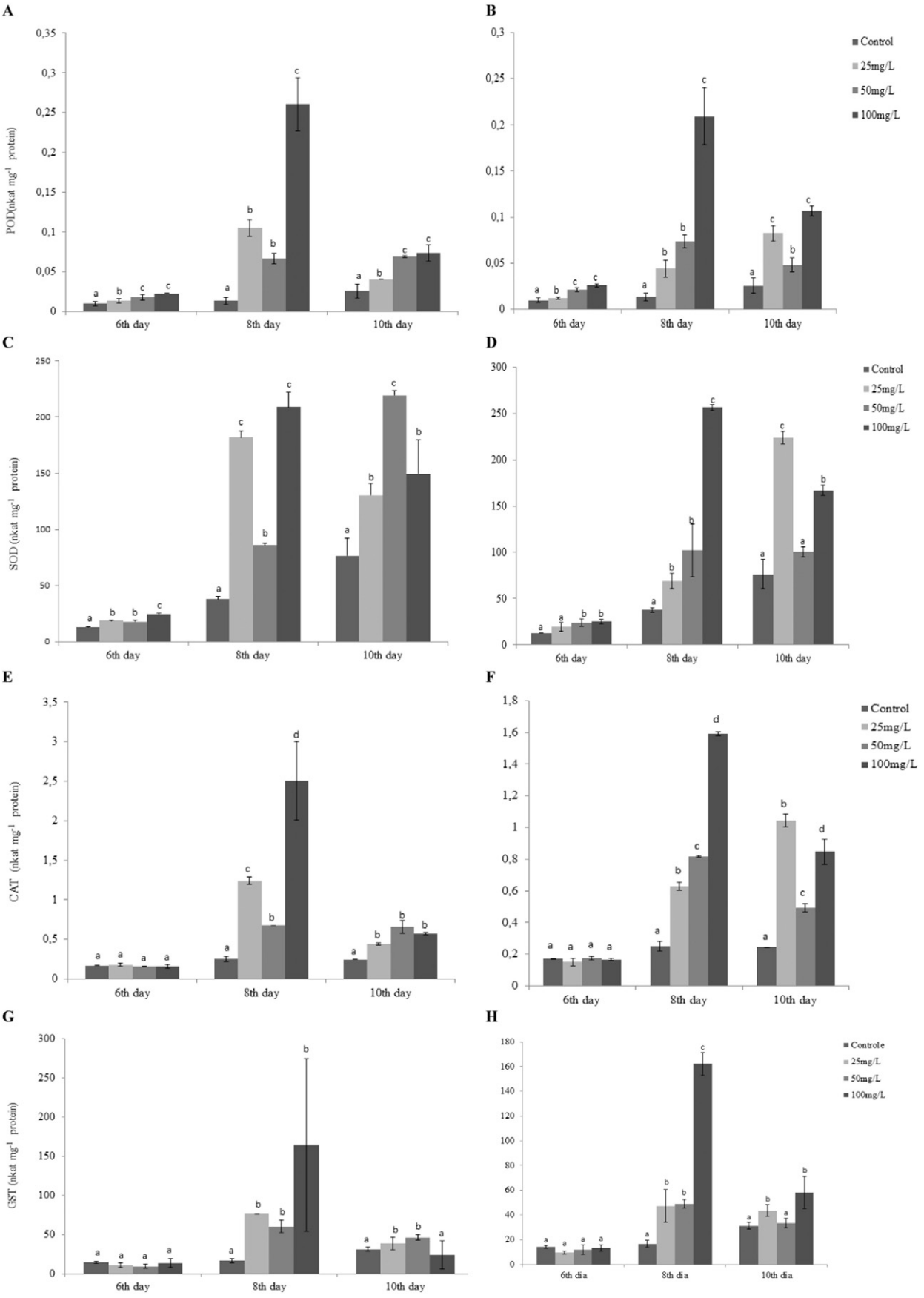

Fig. 7. Effect of different concentrations of fractions 1 and 2 of *H. vulgare* extract on POD (A and E), SOD (B and F), CAT (C and G) and GST (D and H) activities of *M. aeruginosa* BCCUSP232. Error bars represent standard deviation for $n = 3$. A–D represents results obtained with fraction 1, while E–H shows the results obtained during exposure to fraction 2 of *H. vulgare* straw extract. Means with different alphabets are significantly different ($p < 0.05$).

A-5301 ASYS microplate reader (ASYS Hitech, Austria). Microcystins analysis per sample was carried out in triplicates. The ELISA method is one of the most common methods employed for cyanotoxins detection and quantification in field and laboratory studies, giving results that significantly correlate ($R = 0.96$; $p < 1.10^{-10}$) with those obtained using HPLC-DAD and LC-MS (see Metcalf et al., 2000; Babica et al., 2006; Bláhová et al., 2009). The Beacon ELISA kit had sensitivity of 0.10 μg L^{-1} MC-LR and cross-reactivity of 100% for MC-LR and 87% for MC-RR.

2.7. Statistical analysis

The cell density data was subjected to normality (Shapiro-Wilks) and homogeneity of variance (Levene) tests. Significant differences between the mean cell density data obtained from the different treatments were determined using one way analysis of variance (ANOVA). The Tukey's hsd post hoc test was used to separate significantly different means. Where the data was not normal and the variances not homogenous, the Kruskal-Wallis and Dunn tests were employed. All statistical analyses were carried out at 5% significance level.

3. Results

3.1. Phytochemical screening

The *H. vulgare* crude extract had 7.5 μg gallic acid equivalent/mg of extract for total phenols and 30 μg rutin equivalent/mg of extract for total flavonoids. The crude extract of *H. vulgare* and fraction 2 had similar HPLC profiles, and indicated that the fractionation technique was ineffective because both samples were primarily comprised of phenolic acids (Fig. 1A, B and C). However, PAD detector scan (200–600 nm) results gave peaks that are typical of phenolic acids absorption bands in the extract, demonstrating that the extraction method used was efficient for obtaining the lignin degradation products of interest (Fig. 2).

3.2. Oxidative degradation of albumin by barley extract

Compared to the negative and positive controls, the oxidative activity of the crude extract increased significantly at 500 and 250 μg mL^{-1} crude extract (Fig. 3). Furthermore, a clear increasing extract concentration dependent oxidative degradation of BSA can be observed.

3.3. Bioassay results of Microcystis aeruginosa BCCUSP232 exposed to H. vulgare extract and fractions

The bioassay results of *M. aeruginosa* exposed to *H. vulgare* extract and its fractions are given in Tables 1, 2 and 3. Throughout the experiment, *H. vulgare* extract significantly reduced the cell density of the cyanobacterium in a concentration dependent manner. There was complete growth inhibition at the highest extract concentration (500 mg L^{-1}), leading to 100% mortality at the end of the study (Table 1).

Among the *H. vulgare* extract fractions, fractions 1 and 2 significantly reduced the cell density of the cyanobacterium at 25 and 50 mg L^{-1}. Compared to the crude extract, the inhibitory effect of the fractions was lower (Tables 2 and 3).

Results are means ± standard deviation for $n = 3$. Means with different alphabets per column are significantly different ($p < 0.05$).

Results are means ± standard deviation for $n = 3$. Means with different alphabets per column are significantly different ($p < 0.05$).

3.4. Internal hydrogen peroxide (H_2O_2)

Internal H_2O_2 concentration of *M. aeruginosa* increased after exposure to the crude extract in a concentration dependent manner (Fig. 4A). From day 8 to 10, the highest internal H_2O_2 concentration was recorded at 500 mg L^{-1} crude extract. For fraction 1, only the 100 mg L^{-1} treatment significantly increased H_2O_2 concentration on days 6 and 8, while on day 10, there were no significant differences between the treatments and control (Fig. 4B). On the other hand, *M. aeruginosa* exposed to fraction 2 had a slight increase in H_2O_2 concentration at 25 mg L^{-1} on day 6, and significantly lowered levels at 50 and 100 mg L^{-1} on day 8 (Fig. 4C).

3.5. Lipid peroxidation

The lipid peroxidation results of *M. aeruginosa* exposed to *H. vulgare* crude extract, and fractions 1 and 2 are given in Fig.5. Exposure to the crude extract significantly increased lipid peroxidation on day 6, while

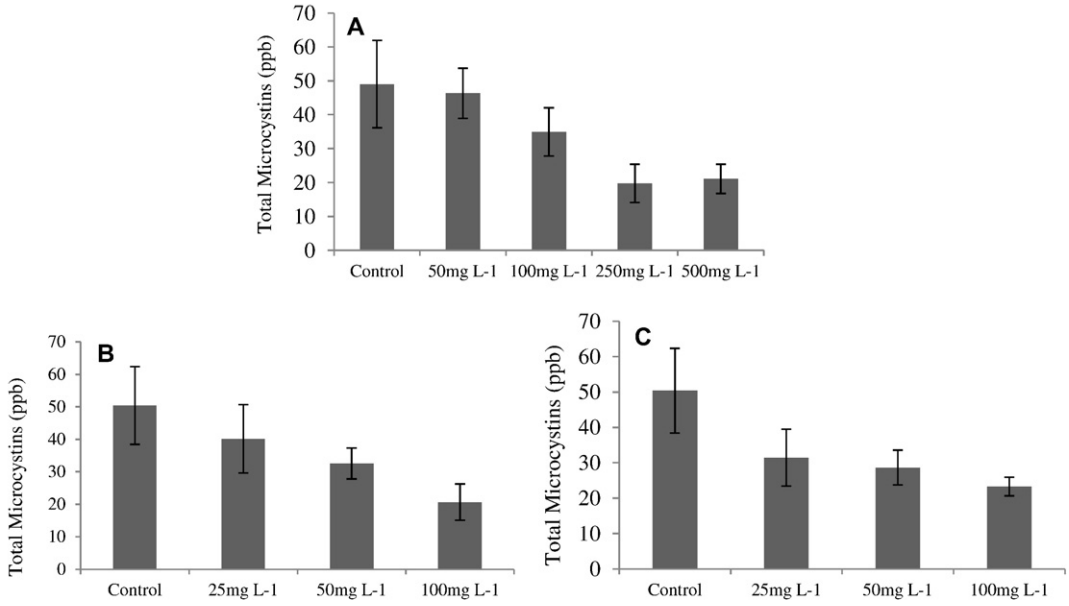

Fig. 8. Changes in total microcystins concentrations of *M. aeruginosa* BCCUSP232 as a function of different exposure concentrations of *Hordeum vulgare* extract (A), fraction 1 of *H. vulgare* extract (B), and fraction 2 of *H. vulgare* extract (C). Error bars represent standard deviation for $n = 3$.

on day 8 only the 50 mg L^{-1} treatment significantly increased it (Fig. 5A). The most significant increase in lipid peroxidation of the cyanobacterium after exposure to fractions 1 and 2 occurred on day 10 at 100 mg L^{-1} treatment (Fig.5B and C).

3.6. Antioxidant enzyme activities

POD activity significantly increased at 250 and 500 mg L^{-1} crude extract on day 6, and on day 8, all crude extract concentrations resulted in significantly higher POD activity than the control (Fig. 6 A). On day 10, only the 100 mg L^{-1} crude extract concentration caused a significant increase in POD activity of *M. aeruginosa*. There was a general significant ($p < 0.05$) increase in SOD activity in the presence of the crude extracts from day 6 to 8 of the experiment (Fig. 6 B). However, at 500 mg L^{-1} crude extract, SOD activity was significantly inhibited on days 8 and 10 of the assay. The most significant changes in GST activity were recorded on days 8 and 10 of the experiment. Specifically, GST activity increased at 50 and 100 mg L^{-1} crude extract on day 8, while at 500 mg L^{-1} crude extract it was inhibited on day 10.

All concentrations of both fractions significantly increased POD and SOD activities of the cyanobacterium from day 6 to 10 of the assay (Fig. 7A, B, E and F). Furthermore, the most significant fraction concentration dependent changes in POD, SOD and CAT activities were recorded on days 8 and 10 of the assay (Fig. 7A, B, C, E, F and G). On day 6 of the assay, the change in GST activity was not significant between the different concentrations of the fractions (1 and 2) and the control (Fig. 7D and H). However, from day 8 to 10 of the assay, the different concentrations of fractions 1 and 2 significantly increased the GST activity of *M. aeruginosa*. Furthermore, the highest GST activity was recorded at 100 mg L^{-1} of fractions 1 and 2 on days 8 and 10 of the assay.

3.7. Total microcystins

Increasing concentrations of the crude extract, fraction 1 and fraction 2 lowered total microcystins concentrations in a concentration dependent manner on day 10 of the experiment (Fig. 8A, B and C). The most significant decline in total microcystins was observed at 250 to 500 mg L^{-1} of the crude extract, and 100 mg L^{-1} of fractions 1 and 2.

4. Discussion

Initial analysis of the extract revealed the presence of phenolic acids, which is in agreement with the findings of Merken and Beecher (2000), and Sticher (2008). Furthermore, Pillinger et al. (1992), Everall and Lees (1997), and Martin and Ridge (1999) demonstrated that the degradation products of barley straw such as polyphenolic compounds (e.g. cinnamic acid, p-coumaric acid, sinapic acid, ferulic acid, caffeic acid) and quinones inhibited the growth of algae and cyanobacteria. This means that the extraction method was efficient for obtaining the lignin degradation products of interest (Fig. 2).

As a means of understanding the mechanism of action of barley straw extract and its fractions, physiological and biochemical changes of *M. aeruginosa* were investigated. This is because most studies on the effect of barley straw extracts have been restricted to its growth inhibitory effects (Martin and Ridge, 1999; Waybright et al., 2009; Iredale et al., 2012), while the mechanisms of action have rarely been given consideration. An assessment of the pro-oxidative activity of the extract demonstrated that increasing extract concentration increased the oxidative damage caused by barley straw. The increase in pro-oxidative activity of the extract correlated with increased presence of polyphenols. Aguiar et al. (2007) demonstrated that polyphenolic compounds are potent pro-oxidative agents; and their activity is directed at reducing $Fe^{+3}$and Cu^{+2}, and promoting reactive oxygen species (ROS) production primarily in the form of OH from Fenton and Fenton cuprous reactions. According to Torres et al. (2008) and Chia et al. (2015a, 2015b), exposure to toxic agents and/or stressful conditions directly interferes with the production and concentration of ROS in algae, by significantly increasing their levels, and consequently, destroying intracellular components. In addition, Grant and Loake (2000) reported that increased ROS content in the tissues of target organisms leads to their death.

Based on the pro-oxidative activities recorded, changes in intracellular H_2O_2 of *M. aeruginosa* during exposure to *H. vulgare* extract and fractions were investigated. This revealed a significant increase in H_2O_2 formation after exposure to the extract and fractions of *H. vulgare*, which is in agreement with the findings of Dummermuth et al. (2003), Pinto et al. (2003) and Halliwell (2007). The authors showed that the induction of oxidative stress may accelerate oxidative damage, leading to substantial changes in the conformation of important biomolecules such as membrane lipids, proteins and nucleic acids. In addition, Torres et al. (2008) and Mikula et al. (2012) demonstrated that oxidative stress damages photosynthetic structures, reduces pigment levels and photosynthetic potential, and inhibits biomass production, which explains the decline in cell density of *M. aeruginosa* during exposure to *H. vulgare* extract and fractions in the present study.

In order to determine the extent of oxidative damage caused by increased ROS production in *M. aeruginosa* during exposure to *H. vulgare* extract and fractions, MDA levels were measured. The observed increased production of MDA with increasing *H. vulgare* extract and fraction concentrations was a clear indication of membrane destabilization by lipid peroxidation. Lipid peroxidation is commonly caused by increased ROS production (Meng et al., 2015), which generates an imbalance in the cellular redox state and is accompanied by increased MDA content (Bhandari and Sharma, 2006). Similar to our results, several researchers have observed increased lipid peroxidation in photosynthesizing organisms exposed to different bioactive compounds (He and Häder, 2002; Yu et al., 2003; Mishra et al., 2008; Li et al., 2016).

The determination of antioxidant enzyme activities was also used to evaluate the mechanisms of action of *H. vulgare* straw extract and fractions on *M. aeruginosa*. These enzymes participate in the elimination and/or reduction of the damage caused by ROS (Zhang et al., 2013; Lozano et al., 2014; Chia et al., 2015b). As a first line of defense, POD and CAT are involved in the sequestration and conversion of H_2O_2 to H_2O and O_2 (Qian et al., 2012). This explains the increase in activities of both enzymes recorded during exposure of *M. aeruginosa* to crude extract and fractions of *H. vulgare* straw. These results corroborate with those reported by Chia et al. (2015b). The authors observed an up-regulation of POD and CAT activities of *M. aeruginosa* exposed to different anatoxin-a concentrations. The changes in SOD activity recorded in the present study are indicative of increased production of the O_2^- molecule. Superoxide dismutase is responsible for catalyzing the dismutation of O_2^- to H_2O_2 and O_2 (Gratão et al., 2005; Halliwell, 2006; Miller, 2012). The inhibition of SOD activities observed in the present study has been linked previously to a rapid substrate overload of the enzyme during oxidative stress (Blokhina et al., 2003). In addition, our results demonstrated that increased ROS content can be characterized by a fall in the efficiency of the enzymes or molecules sequestering these molecules, vis-à-vis the antioxidant defenses, thereby resulting in a breakdown of the balance between the production and removal of ROS (Halliwell and Whiteman, 2004; Scandalios, 2005). The increased GST activity observed during exposure of the cyanobacterium to *H. vulgare* straw extract and fractions is directly related to the role it plays in the biotransformation and detoxification of bioactive substances in different organisms (Ziglari et al., 2008; Vestena et al., 2011; Yamuna et al., 2012). The enzyme catalyzes the conjugation of bioactive secondary metabolites with the reduced form of glutathione (GSH), leading to the transformation, and finally, detoxification of the molecules (Salinas and Wong, 1999; Vestena et al., 2011).

The environmental and human health effects of microcystins require that algicidal investigations consider changes in the levels of these toxins. In the present study, crude extract and fraction treatments had a dose dependent reduction effect on total microcystins content of

M. aeruginosa. A number of studies have shown that bioactive substances such as cylindrospermopsin (Rzymski et al., 2014) and anatoxin-a (Chia et al., 2015a) inhibit the ability of *M. aeruginosa* to produce microcystins, which is in agreement with our results. While the physiological functions of microcystins are still debated, the molecules act as allelopathic agents in intra- and inter-specific interactions, and enhance colony formation and maintenance by cyanobacteria (Pimentel and Giani, 2014). According to Dziallas and Grossart (2011), microcystins play important protective roles against oxidative stress. Therefore, the ability of *H. vulgare* extract and fractions to inhibit the production of microcystins enhances its algicidal potential.

5. Conclusion

The results obtained in the present study confirm that *H. vulgare* (barley) straw extract and fractions are capable of controlling *M. aeruginosa* growth. As a contribution to understanding the mechanism of action of *H. vulgare* degradation extract and its fractions on the cyanobacterium, our study revealed a decline in total microcystin concentrations, while internal H_2O_2 content and lipid peroxidation were increased. In addition, the activities of important antioxidant enzymes were significantly altered.

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