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Algicidal effects of aqueous leaf extracts of neem (*Azadirachta indica*) on *Scenedesmus quadricauda* (Turp.) de Brébission

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ABSTRACT

The application of synthetic algaecides for the control of algae produces by-products that are sometimes toxic to the environment. There is a need for natural and cheap alternatives to synthetic algaecides. In the present study, we investigated the potential of aqueous crude extract of *Azadirachta indica* to inhibit the growth of *Scenedesmus quadricauda*. Phytochemical screening of the extract revealed the presence of groups of bioactive compounds that are capable of inhibiting microalgal growth. Chlorophyll *a* concentration, dry weight production and cell density of microalga decreased with increasing crude extract concentration. After three days of exposure, the 1000 mg/L extract concentration resulted in complete growth inhibition and cell lysis. Furthermore, the ability of *S. quadricauda* to form multi-celled coenobial structures was compromised in a concentration dependent manner. In general, catalase and peroxidase activities of the microalga were upregulated with increasing extract concentration. These results imply that aqueous neem extract may provide a cheap and ecofriendly alternative for the control of microalgae in aquatic ecosystems.

Keywords: algaecide, growth inhibition, microalgae, Neem, oxidative stress, phenotypic plasticity

Introduction

The excessive proliferation of cyanobacteria and algae in aquatic ecosystems is controlled by factors such as nutrient composition and concentration, light quality, temperature, water residence time and interspecific competition between phytoplankton groups (Paerl & Huisman 2009). Chlorophytes on their own are not considered to be nuisance algae, however, nutrient enriched conditions favour the excessive proliferation of members of this group (Paerl *et al*. 2001). The risk to water quality deterioration is aggravated by the codominance of bloom forming cyanobacteria with members of the green algae. Specifically, there is environmental evidence that *Scenedesmus* and *Microcystis* co-dominate or alternate in nutrient enriched freshwater systems due to their vertical migration and ability to form blooms (Huang *et al*. 2012). Furthermore, the distinctive blooms formed by some members of the chlorophyta constitute serious environmental problems, especially for water treatment facilities and recreational water bodies, because they can cause clogging of filtration equipment, hypoxia, tastes and odours (Reynolds 1984; Paerl *et al*. 2001; Jeong *et al*. 2008; Su *et al*. 2011; Li *et al*. 2014). Algal management in swimming pools, fish ponds and large water bodies is usually carried out using physical, biological and chemical processes that are usually very expensive or potentially harmful to the environment (Anderson 1997).

The indiscriminate use of synthetic algaecides for chemical control of algae and cyanobacteria leads to environmental contamination, an increase in the level

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of toxic residues in aquatic organisms, as well as killing of untargeted aquatic flora and fauna (Su *et al*. 2011). This necessitates the exploration for cheap biological and environmentally friendly means of tackling this problem. A common approach is to search for bioactive compounds from plants that inhibit or prevent algal growth (Ghorbanian *et al*. 2008). For example, allelopathic compounds in rice and barley straws have been shown to limit the germination, growth, photosynthesis, respiration and metabolism of other plants including nuisance algae (Park *et al*. 2006a). While aquatic macrophytes, cyanobacteria and algae have been shown to produce allelochemicals that are capable of inhibiting both cyanobacteria and algae (Chia *et al*. 2012; Yang *et al*. 2014; Bittencourt-Oliveira *et al*. 2014), very little research has been carried out to investigate the algicidal activities of terrestrial plants (Xiao *et al*. 2011).

Neem extracts contain bioactive constituents that are potent against a large number of pests, and have minimal environmental toxicity because of their rapid degradation in the environment (Isman 2000). Despite the extensive data available on bioactive compounds from neem with pesticidal and medicinal potentials (Gualtieri *et al*. 2014; Hao *et al*. 2014), very little is known about their algicidal properties. This study explores the potential of aqueous leaf extract of *Azadirachta indica* as an eco-friendly and cost effective means of controlling algal growth, using *Scenedesmus quadricauda* as the test organism. The objective of this study was to determine the algicidal effect of aqueous neem extract on *Scenedesmus quadricauda*.

Materials and methods

Microalgal strain

Experiments were carried out with cultures of the microalga *Scenedesmus quadricauda* ABU12. The microalga was isolated from a freshwater pond in Zaria, Nigeria (Chia *et al*. 2011; Chia & Musa 2014; Chia *et al*. 2014; 2015) and maintained in OECD medium (OECD 1984). Prior to inoculation with the microalga, the medium was autoclaved at 121°C for 30 min and its pH adjusted to 7.0. The cultures were maintained at 23 ± 2°C and continuous light (40 µmol photons m^{-2} s^{-1}) using cool white fluorescent lamps. The reagents used for culture medium preparation were of analytical grade, and were obtained from Sigma Aldrich (USA).

Plant collection and preparation

Fresh leaves of *Azadirachta indica* A. Juss were collected, identified and authenticated in the Herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria. The leaves were air-dried, crushed and pulverized into fine powder and stored in a glass container at 4°C. 250 g of the powdered sample was exhaustively extracted with distilled water using reflux method (Trease & Evans 1989). The mixture was filtered to remove debris. Thereafter, 200 mL of petroleum ether was added to the mixture, shaken vigorously and allowed to settle. The petroleum ether layer (on top) was removed and concentrated. Afterwards, 200 mL of chloroform was added to the aqueous layer and also vigorously shaken and allowed to settle. The crude aqueous layer was then concentrated until a brown coloured extract was obtained using mild heat. It was weighed, labelled, and sealed in a plastic container and stored at -20°C until required.

Phytochemical screening

The presence of bioactive secondary metabolites in the extract was determined using the methods outlined in Sofowora (1993) and Trease & Evans (2002) (Tab. 1). Furthermore, the presence of potential bioactive secondary metabolites in the extract was confirmed using Fourier

Table 1. Result of phytochemical screening of aqueous extract of *Azadirachta indica* leaves.

Phytochemicals	Test	Presence/absence	Reference
Flavonoids	Shinoda Sodium hydroxide	Present	Trease & Evans 2002
Saponins	Frothing	Absent	Trease & Evans 2002
Glycosides	Fehling	Present	Trease & Evans 2002
Anthraquinones	Borntrager's	Present	Trease & Evans 2002
Alkaloids	Meyer	Present	Sofowora 1993
Tannins	Ferric chloride	Absent	Trease & Evans 2002
Steroids	Lieberman Burchard's	Absent	Trease & Evans 2002
Terpenoids	Lieberman Burchard's	Present	Trease & Evans 2002
Carbohydrates	Molisch's	Present	Sofowora 1993
Phlobatannins	-	Absent	Trease & Evans 2002

Transform Infrared (FT-IR) analyses (Williams & Fleming 2005; Chia *et al*. 2012). A drop of the aqueous extract was squeezed between flat plates of sodium chloride and scanned from 4000 to 625 cm^{-1} using a FT-IR Model 8400s Spectrophotometer (Shimadzu, Japan).

Experiments

Experimental cultures were carried out in 250 mL Erlenmeyer flask containing 150 mL of culture media. Exponential phase growing cultures of the green microalga were exposed to different concentrations (1000 mg/L, 100 mg/L, 10 mg/L and 1 mg/L) of the aqueous extract at an initial density of 10^5 cells mL^{-1}. The control treatment contained *S. quadricauda* growing in BB medium without neem extract addition. The inhibition of *S. quadricauda* growth in relation to the control culture was monitored daily for four days. All experiments were carried out in triplicates.

Growth and Biomass determination

Aliquots were taken daily for biomass and growth kinetics determination. Growth monitoring was done using microscopic cell counts. Growth inhibition (GI) was calculated as:

$GI = (N_c - N_t)/Nc \times 100\%$ .....Equation 1

Where N_c is the cell density for the control cultures, and N_t is the cell density of the treatments.

Dry weight was obtained by filtering 5 mL of culture on a pre-dried and weighed Sartorius 0.22 *u*M pore size membrane filter. The residue was weighed to the nearest mg using a digital weighing balance. The extraction and analysis of chlorophyll *a* were carried out following the procedures outlined by Shoaf & Lium (1976). Chlorophyll *a* concentration was determined using the equation given by Németh (1998):

Chlorophyll *a* (mg/L) = $(17.12 * A_{663nm}) - (8.86 * A_{647nm})$.....Equation 2

Where A_{663nm} and A_{647nm} represent absorbance at 663 and 647 nm, respectively.

Microscopic analyses

The Improved Neubauer counting chamber was used to determine algal cell density per mL.

Morphological analysis was done by quantifying the number of cells per coenobium per experimental treatment and control using the procedures described in Lombardi *et al*. (2007). Coenobia were classified into four categories: four-celled coenobium, three-celled coenobium, two-celled coenobium and free or uni-cells (absence of coenobium).

Antioxidant enzyme activities

Total protein extraction for antioxidant enzyme assays was done in 0.05 M phosphate buffer (pH 7.8). Catalase (EC 1.11.1.6) activity was determined following the method of Luck (1974). One enzyme unit was calculated as the amount of enzyme needed to change the absorbance at 240 nm by 0.05 units. Peroxidase (EC 1.11.1.11) activity was assayed according to the method of Reddy *et al.* (1995). The change in absorbance was recorded every 30 seconds for three minutes, and one unit of peroxidase was defined as the change in absorbance per minute at 430 nm.

Data analyses

Two-way ANOVA was used to determine the differences in mean growth, morphological characteristics and biomass production of *S. quadricauda* under the different experimental conditions tested. Where significant differences were detected, separation of means was done using Tukey's HSD Post Hoc test. ANOVA and Tukey's HSD test were done using Statistica version 8.0 for windows.

Results

The results of phytochemical screening showed the presences of flavanoids, glycosides, anthraquinones, alkaloids, terpenoids and carbohydrates, while steroids, saponins, phlobatannins and tannins were absent (Tab. 1). FTIR analysis confirmed the presence of different functional groups based on their peak characteristics. These functional groups served as signatures for identifying the presence of bioactive compounds that tested positive in the initial qualitative phytochemical tests. Stretching bond that confirms the presence of primary and secondary amines (-NH) and hydroxyl (-OH) groups (3396.76 cm^{-1}), stretching vibrations specific to C-H (1399.40 cm^{-1}), C-O stretching for alcohol ethers, esters, carboxylic acids, anhydrides (1066.67 cm^{-1}) and C=O carbonyl stretching (with wave numbers between 1800 and 900 cm^{-1}) were detected.

Chlorophyll *a* concentration and cell density decreased with increasing concentrations of aqueous neem extract (Fig. 1A, B). The lowest chlorophyll *a* concentration and cell density were observed at the highest extract concentration. Dry weight production on the other hand generally decreased with increasing concentrations except for the highest extract concentrations on days 1 and 2. The lowest dry weight production was observed at the 100 mg/L extract treatment (Fig. 1C). These observed differences were statistically significant ($P<0.05$) between the treatments for chlorophyll *a* and cell counts, while for dry weight they were not ($P>0.05$) (Tab. 2). From day 3 (72 h) after exposure to the extracts, the highest concentration resulted in complete growth inhibition (Fig. 2).

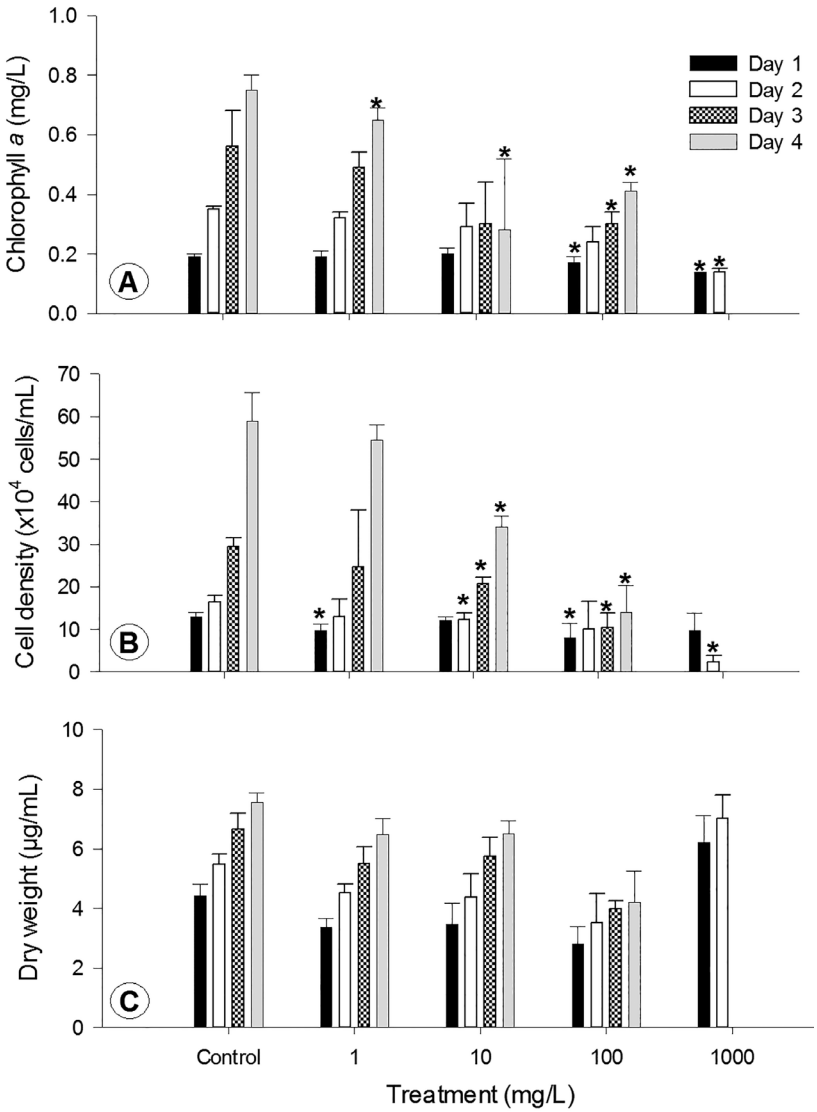

Figure 1. The production of **A.** chlorophyll *a* (mg/L), **B.** cell counts (x10^4 cells/mL), **C.** dry weight (µg/mL). Asterisk means the value is significantly different from the control.

Table 2. Analysis of variance summary results for biomass production, phenotypic plasticity and antioxidant enzyme activities of *Scenedesmus quadricauda* exposed to different concentrations of aqueous neem leaf extracts.

	Treatment		Time (days)		Treatment vs Time (days)	
	F value	P value	F value	P value	F value	P value
Cell count	27.17	0.01	25.57	0.01	5.96	0.01
Dry weight	1.18	0.3342	2.04	0.1236	1.08	0.4014
Chlorophyll a	60.44	0.01	1.42	0.251	1.62	0.1236
Peroxidase (POD)	0.45	0.7724	3.82	0.017	0.75	0.6915
Catalase (COD)	5.11	0.002	19.76	0.01	3.93	0.0005
Free cells	21.38	0.01	17.68	0.01	3.24	0.0025
2 celled-coenobium	13.37	0.01	11.9	0.01	3.48	0.0014
3 celled-coenobium	10.04	0.01	6.99	0.0007	3.14	0.0032
4 celled-coenobium	14.11	0.01	7.54	0.0004	6.79	0.01

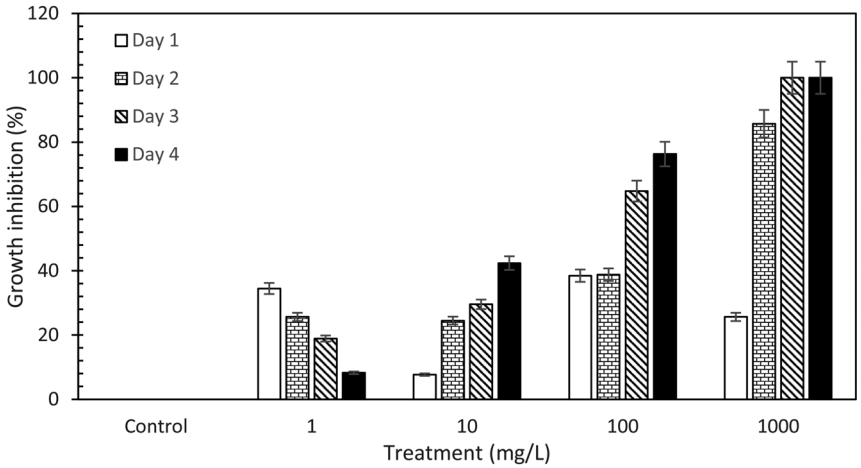

Figure 2. Growth inhibition rate of *Scenedesmus quadricauda* as a function of different aqueous neem extract concentrations (mg/L).

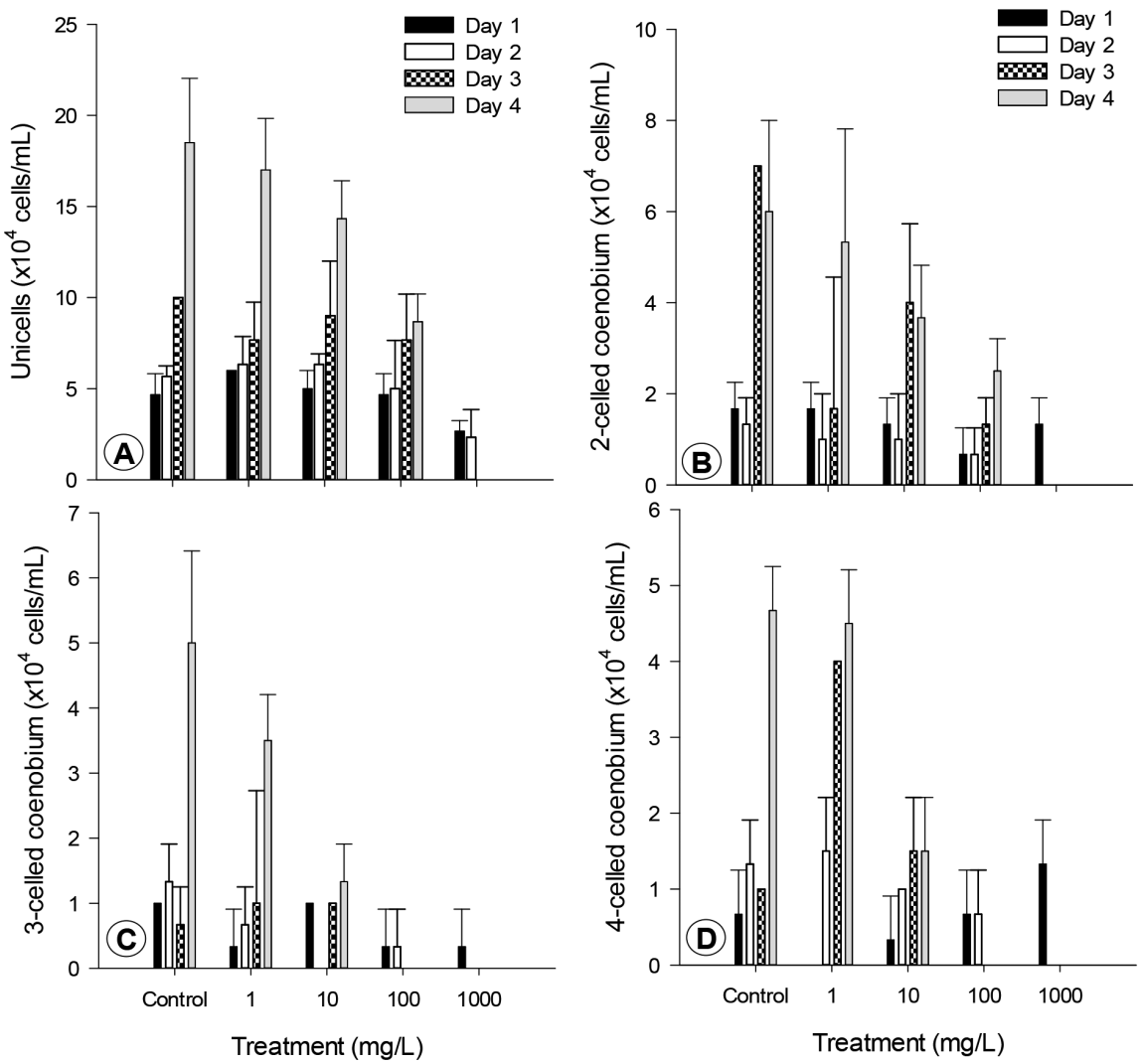

Figure 3. Morphological variation (x10⁴ cells/mL) of *Scenedesmus quadricauda* after exposure to aqueous extract of *Azadirachta indica* **A.** unicells (single cells) **B.** 2-cells per coenobium **C.** 3-cells per coenobium **D.** 4 cells per coenobium**.** Asterisk means the value is significantly different from the control.

At the end of the experiment, the highest number of free cells was found in the 1 mg/L aqueous extract and control treatments (Fig. 3A). Furthermore, the highest extract concentration resulted in almost 100% growth inhibition with respect to the presence of unicells (free cells) and 2 celled coenobial structures (Fig. 3A-B). The production of 3- and 4-celled coenobia was highest in the control at the end of the experiment (Fig. 3C-D). However, with increasing extract concentration, the number of 3 and 4 celled coenobial structures decreased. The changes in all morphological variants between the treatments and the control were significant ($p<0.05$). Significant interactions were also observe between treatments and exposure time ($p<0.05$) (Tab. 2).

After the first 24 h of exposure, CAT activity of *S. quadricauda* was higher at all extract concentrations than the control (Fig. 4A). However, at the end of the experiment, almost all the cultures exposed to neem aqueous extracts recorded significant decreases in CAT activity. Peroxidase activity on the other hand increased within the first 72 h of the exposure of *S. quadricauda* to the extract. However, at the end of the experiment, a general decrease in POD activity was observed in all treatments except the 10 mg/L treatment (Fig. 4B).

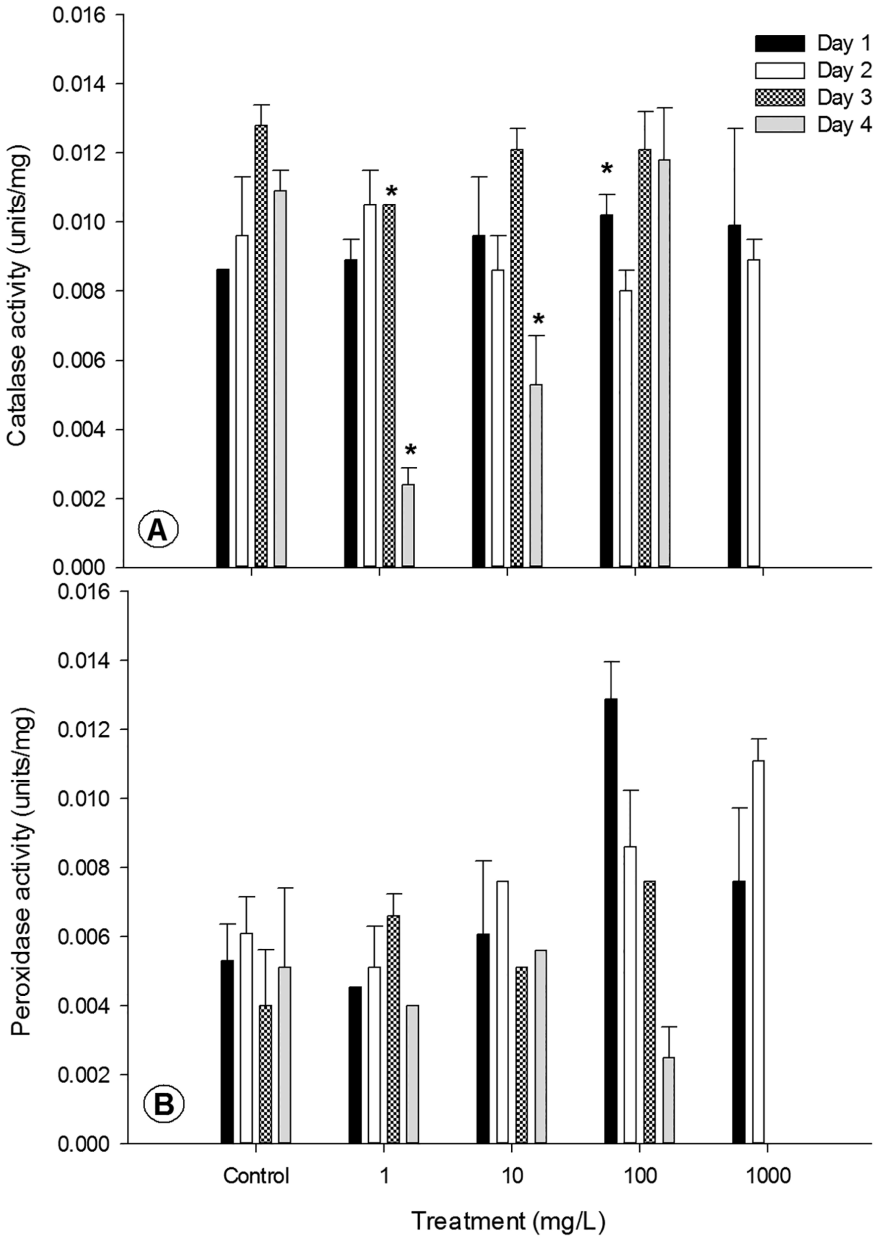

Figure 4. Antioxidant enzyme activities of *Scenedesmus quadricauda* exposed to different concentrations (mg/L) of aqueous neem leaf extract **A.** catalase (CAT, units/mg) **B.** peroxidase (POD, units/mg). Asterisk means the value is significantly different from the control.

Discussion

Neem leaf has been reported to possess bioactive compounds which have antiplasmodial, antibacterial, antifungal and antiviral properties (Pillinger *et al*. 1994; Ferrier *et al*. 2005). The presence of compounds with potential inhibitory properties was confirmed on the basis of qualitative phytochemical screening. FTIR analysis showed the presence of C-O, C=O, and –OH in the extract which inferred the presence of carboxylic and terpenoid compounds. Terpenoids are made up of several compounds having varied structural diversity and biological activity (Einhellig 2004). The presence of alkaloids in the aqueous extracts may also be implicated for inhibitory effects. This is because alkaloids have been shown to have some level of allelopathy on plants (Macias *et al*. 2007). Furthermore, a recent study showed the prospect of amides and amino acids with biological activity in drug synthesis (Lazareva 2011), which means their detection in in our study may have contributed to the algicidal effects observed.

The results of this study showed that aqueous neem extract has algicidal potential because it significantly reduced the biomass (cell number and chlorophyll a) of *S. quadricauda*. The observed decrease in chlorophyll *a* content with increasing extract concentration can cause a decrease in the rate of photosynthesis and hamper CO_2 assimilation. Consequently, this will negatively affect cell division as there will be no substrate for energy processes and biomolecules synthesis that maintain life processes of the cell. Similar to our results, Ball *et al*. (2001) and Park *et al*. (2006b) demonstrated that barley and rice straw inhibited microalgal growth, by limiting germination and normal cell metabolism.

The release of new colony from *S. quadricauda* mother cells is done in the form of daughter cells with varying number of cells per colony, after unrolling during asexual reproduction (Lürling 2003). Recent studies have shown that this process can be altered when members of the *Scenedesmus* genus are exposed to stress conditions (Pena-Castro *et al*. 2004; Lombardi *et al*. 2007; Chia & Musa 2014; Chia *et al*. 2015). The resultant decrease in number of cells per coenobium with increasing aqueous neem extract concentration is a demonstration of its bioactivity. The lack of formation multi-celled coenobia at the end of the experiment after exposure to the extract confirms its significant negative effects on the fitness of this microalga.

The excessive production of oxygen radicals during algal metabolism is well reported, especially when they are exposed to toxicants or stress conditions (Zhang *et al*. 2013). As a result of increased reactive oxygen species (ROS), microalgae tend to up-regulate the biosynthesis and activities of ROS combating enzymes (Chia *et al*. 2015). The exposure of *S. quadricauda* to neem aqueous extract was not an exception, as it altered the activities of CAT and POD. However, the changes in POD activity were not significant. On the other hand, CAT activity was significantly increased in a concentration dependent manner within the first 48 h of exposure. This is indicative that the microalga suffered severe oxidative stress with increasing aqueous neem extract concentration. The enzyme represents the first line of defence in plants because it is involved in the decomposition of hydrogen peroxide (H_2O_2) and superoxide (O_2^-) to water and oxygen, by the interaction of the amino acids asparagine at position 147 and histidine at position 74, which causes a proton transfer between the oxygen atoms (Torres *et al*. 2008). However, we observed that by the 4th day after exposure to the extract, the microalga was no longer able withstand the effect of the extract, as it inhibited CAT activity. The decline in CAT activity may be related to its inactivation via the binding of thiol groups with the bioactive components of the extract investigated (Cordeiro-Araújo *et al*. 2015). The presence of toxic or bioactive substances in plants induces high production of compounds like nitric oxide and H_2O_2, which have the potential to inhibit CAT activity (Clark *et al*. 2000; Qiao *et al*. 2014).

In conclusion, the present study showed that aqueous extract of neem is capable of significantly inhibiting the growth of *S. quadricauda*. Cell density and chlorophyll *a* production were inhibited in the neem extract treatments. However, at the highest extract concentration, there was slight dry weight increase within the first 2 days. Furthermore, aqueous neem extract reduced the ability of *S. quadricauda* to form multi-celled coenobia or colonies. The extract altered antioxidant enzyme activities of the microalga, indicating the induction of oxidative stress. The presence of potential algicidal groups was confirmed using phytochemical screening. Further studies are needed to isolate and confirm specific molecules from this plant with algicidal properties. However, even without the isolation of specific molecules, these results show that aqueous extract of neem may provide a cheap and safe alternative for preventing excessive microalgal growths in recreational waters like swimming pools, ponds and other aquatic ecosystems. Furthermore, investigations are required to determine the toxicity of these extracts to humans and other organisms.

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