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Full Length Research Paper

Acute and sub-chronic toxicological assessment of *Nannochloropsis oculata* in rats

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The aim of this study was to investigate the acute and sub-chronic toxicities of *Nannochloropsis oculata* biomass. In the acute toxicity study, twelve Sprague-Dawley rats of both sexes were gavaged with 12 g/kg body weight (bw) of *N. oculata* one time, and then tested for morbidity and mortality in 14 days. The oral LD_{50} of *N. oculata* in rats was greater than 12 g/kg bw, and no toxicity effects were observed on biomass in terms of morbidity signs, plasma biochemical parameters, organ tissue, or body weight gain in response to *N. oculata* doses up to 12 g/kg rat bw. In the sub-chronic toxicity study, thirty-six Sprague-Dawley rats of both sexes were chosen and divided into three groups and provided with a diet containing 0, 3, and 6 g of *N. oculata* per kg bw, respectively, early every morning and then allowed free access to normal food and water *ad libitum* for 60 days. No biologically significant effects of *N. Oculata* on organ weights, male body weight gain, or on the plasma biochemical parameters were observed in either treatment group. However, low creatinine and significant differences in body weight gain by female rats were noted in the treatment groups. These changes were not considered as toxicologically significant. The no observable adverse effect level (NOAEL) for *N. Oculata* under the conditions of this study was 12 g/kg bw/day for acute toxicity and 6 g/kg bw/day for sub-chronic toxicity for both male and female rats.

Key words: Toxicity, *Nannochloropsis oculata*, acute, sub-chronic, rats.

INTRODUCTION

Algae are photosynthetic organisms that are widespread in nature. They are capable of fitting to different environments ranging from aquatic environments to deserts. They are classified based on their size into two main classes: macro-, and micro-algae (Matsunaga et al., 2005). They have been used for centuries as food. For example, the Chinese people were the first users of algae as food, starting from around 2000 years ago. In fact, algae are a diverse group of organisms with valuable ingredients like proteins, carbohydrates, lipids, vitamins, pigments, polyunsaturated fatty acids, and fibres (Spolaore et al., 2006).

Nannochloropsis (also called marine chlorella) is a small green microalga genus, which is well known in aquaculture due to its nutritional value and potency to produce valuable materials. They have been extensively used in the aquaculture industry for growing small zoo-planktons such as rotifers and fish hatcheries (Rocha et al., 2003), and for producing green water (Spolaore et al., 2006). The biochemical composition of the six species makes them a valuable food source for animals and humans. They also constitute a good alternative source of Eicosapentaenoic acid (EPA, C 20:5 n3), which is a

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valuable polyunsaturated fatty acid for prevention of several human diseases. Experiments in hypertensive rats have demonstrated their beneficial effect in reducing blood pressure (Seto et al., 1992). In addition, examination of the effects of Eicosapentaenoic acid on rats fed with diets supplemented with *N. oculata* biomass showed an increased ω3/ω6 ratio in the rat livers and blood (Sukenik, 1999). Eicosapentaenoic acid has also been claimed to have excellent short-, and long-term skin-tightening effects (Spolaore et al., 2006). Nannochloropsis has also been used in human food and diet products. For instance, it has been incorporated in noodles to improve their nutritional profile (Schwartz et al., 1991).

However, despite all these advantages, not much is known about the safety of *N. oculata*. Andres et al. (1992) reported that *Nannochloropsis* sp. supplements of the diet of male Sprague-Dawley rats at concentrations up to 10% for four weeks did not produce toxicological effects in these rats. However, this study was an acute toxicity study, which only focused on male rats. Therefore, the objective of the present study was to examine the potential adverse effects of *N. oculata* on Sprague-Dawley rats of both genders in acute and sub-chronic toxicity studies.

MATERIALS AND METHODS

Microalgae

N. oculata was purchased from Reed Mariculture, USA, and was dried using a freeze-drying system (Labconco, USA), in the Institute of Bioscience, Universiti Putra Malaysia (UPM). Rat chow pellets were ground to a fine powder using an electric grinder and *N. oculata* was mixed with pellet powder and water. The experimental diet was cut to small cuboids and immediately dried overnight in an oven at 45°C. The dried pellets were kept in a closed container at 4°C. Food was prepared fresh every week. For the acute toxicity study, *N. oculata* was administered to the rats by needle gavage and the animals had free access to the normal chow pellets for two weeks. In the sub-chronic toxicity study, the *N. oculata* biomass was administered to the rats by incorporation in the food early in the morning. After the animals had finished the food including *N. oculata*, they were allowed free access to normal chow pellets.

Animals

Forty-eight, eight week old Sprague-Dawley rats of both sexes were purchased from Asaphire-Enterprise (Serdang, Malaysia). During all experiments, the rats were housed individually in a well-ventilated, temperature-controlled room at 22 ± 2°C in stainless steel cages under a 12-h light/dark cycle. The rats were acclimatized for one week before initiation of the experiment. All procedures involving this animal experiment were approved by the Faculty of Medicine and Health Sciences Committee for Animal Care and Use, UPM.

Treatment

In a study aiming to determine the acute oral LD_{50}, a dose corresponding to 12 g *N. oculata* /kg bw was administered as a single dose via gavage to Sprague-Dawley rats (six males and six females in each treatment group). Rats were then examined for morbidity signs once a day in the morning and for mortality (twice a day; morning and afternoon) for 14 days (Moser et al., 1988). Animals were evaluated before and at the end of the study for body weight, blood chemistry and pathology analysis.

The sub-chronic toxicity study used 36 rats of both sexes for 60 days. The rats were divided into three groups: one control and two treatment groups. The first treatment group of rats (6 /sex/group) was fed with 6 g *Nannochloropsis oculata* /kg bw, the second group was fed with 3 g *N. oculata* /kg bw, and the control group was fed with normal pellets. The animal weights were recorded weekly. The animals were also evaluated for blood chemistry, antioxidant activity, and pathology analysis at the completion of the study.

Biochemical parameters

Blood was collected at the end of two studies and at the beginning of the acute study. The rats were fasted overnight and blood samples were collected from them through cardiac puncture under diethyl ether anaesthesia. Since the liver and kidney are prominent organs in detoxification, metabolism, storage, and excretion of xenobiotics and their metabolites, and are specifically susceptible to damage (Brzoska et al., 2003), the plasma biochemical parameters measured include: alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, urea, and creatinine. These biochemical parameters were determined by using Roche diagnostic kits and analysed using a Hitachi analytical machine.

Antioxidant activity

The total antioxidant activity of plasma was evaluated by Ferric Reducing Ability of Plasma (FRAP) (Benzie and Strain, 1996), and ABTS assay (Re et al., 1999) in sub-chronic study. The FRAP reagent was prepared by mixing 10 volumes of 300 mMol acetate buffer (pH 3.6) with 1 volume of 10 mMol 2,4,6 tripyridyl-s-triazine (TPTZ) in 40 mMol hydrochloric acid and 1 volume of 20 mM ferric chloride solution. Aqueous solutions of known Fe^{2+} concentrations in the range of 100-1000 µmol/L ($FeSO_4.7H_2O$) were used for calibration. All solutions were prepared fresh and used on the day of preparation. The fresh FRAP reagent (300 µl)was warmed to 37ºC and a reagent blank was reading at 593 nm by spectrophotometer, 10 µl of plasma/standard was then added along with 30 µl of de-ionized water. The mixture was incubated at 37ºC. Four minutes after adding the samples (plasma/standards), the absorbance was recorded as a final reading. Absorbance changes from blank to final reading were selected for the calculation of the FRAP value. For the ABTS assay, a 7 mM stock ABTS (Fluka, Germany) solution was prepared in distilled water. The radical cation of ABTS ($ABTS^{\cdot+}$) was prepared by mixing the ABTS stock solution with 2.45 mM potassium persulphate (ACROS, USA). This mixture was kept in the dark at room temperature for 12-16 h before use. The $ABTS^{\cdot+}$ will be stable for more than two days if it is kept in the dark at room temperature. The $ABTS^{\cdot+}$ was next diluted with phosphate buffer saline (pH 7.4) at 30°C to an absorbance of 0.70±0.02 at 734 nm. Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxilic acid) was prepared in a phosphate buffer saline (pH 7.4) to a final concentration of 250-4000 µM for later use as a stock standard. 1 ml of the diluted $ABTS^{\cdot+}$ was mixed with 10 µl of plasma/trolox. The mixture was incubated at 30ºC for 6 min. Thereafter, the changes in absorbance at 734 nm were recorded after 6 min of initial mixing. The absorbance of the diluted $ABTS^{\cdot+}$, which served as control, was recorded daily at 734 nm. All measurements were performed in duplicates. The percentage inhibition of $ABTS^{\cdot+}$ production by the sample was calculated according to the following formula:

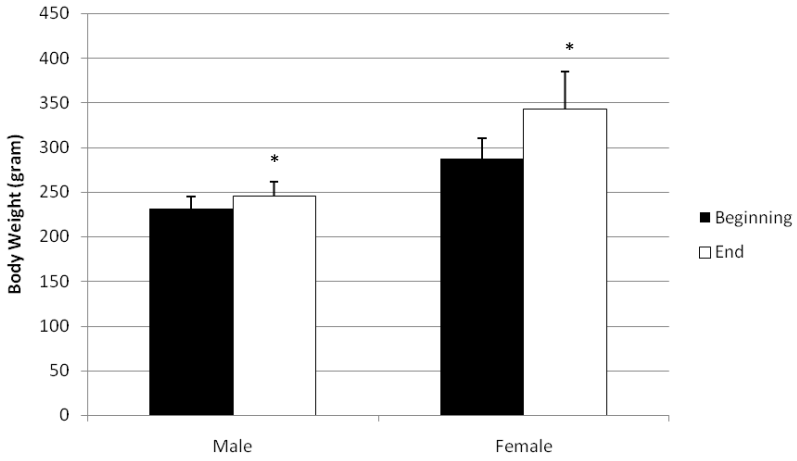

Figure 1. Effect of *N. oculata* on body weight (gram) in male and female rats. *indicates significant different at p<0.05.

$$\%\ inhibition = \left[\left(\frac{A_{c(0)} - A_{a(t)}}{A_{c(0)}}\right)\right] \times 100$$

$A_{c(0)}$= Absorbance of the control at t= 0 minute, $A_{a(t)}$ = Absorbance of the antioxidant (plasma) at t=6 min.

The free radical scavenging capacity of the plasma was calculated as the percentage inhibition of $ABTS^{\cdot+}$.

Macroscopic and Microscopic examination

All rats were anesthetized by diethyl ether and sacrificed using a surgical set. The liver and kidneys were washed using normal saline and weighed immediately. They were then fixed in 10% neutral-buffered formalin. For histopathological examination, tissue was processed into paraffin blocks, sectioned at nominal 4 µm, mounted on glass microscope slides and stained with Hematoxylin and Eosin (Bancroft and Gamble, 2008).

Mineral quantification of *N. oculata*

Mineral analysis was done based on the method proposed by Dawczynski et al. (2007). About 0.5 g of *N. oculata*, in homogenized and pulverized form was digested with 2 ml hydrogen peroxide and 5 ml of nitric acid in the closed vessels of a microwave sample digestion apparatus (Milestone, ETHOS 1, USA) for 30 min at 160ºC. The extract was cooled at room temperature, transferred to polypropylene tubes, made to 50 ml volume with pure water and kept at 4ºC until used. Macro and trace elements were determined by Flame atomic absorption spectrometry (NOVAA 400, Germany), and ultra trace elements were determined by means of inductively coupled plasma mass spectrometry (ICP-MS) (Elan, Perkin Elmer, USA).

Statistical analysis

The data were analysed using the SPSS program, version 16. The one-way analysis of variance (ANOVA) was used to test for significant differences between the experimental groups and followed by the Tukey post hoc pair-wise comparisons. The Welch test was used whenever the Levene's test of homogeneity of variances revealed significant variance differences between the three experimental groups. A paired-sample t-test was employed in statistical testing for significant differences for the two groups. Non-parametric methods were used when the distribution of certain variable(s) differed from normal. The non-parametric methods employed were the Kruskal-Wallis test and the Wilcoxon rank sum test for pair-wise comparisons. For all tests the significance level was set at p<0.05. The results were expressed as mean ± standard deviation.

RESULTS

Acute study

No mortality was seen in the rat used in this study. Therefore the oral LD_{50} of *N. oculata* biomass was higher than 12 g *N. oculata* /kg bw, thus, suggesting that Nannochloropsis is practically non-toxic at this, or any lower dose. The rats were also assessed for clinical signs once a day. No treatment related biological changes were observed during the acute study, and no adverse effects were noted in their body weight (Figure 1). There were also no treatment-related biologically significant adverse effects of *N. oculata* on the tested plasma chemistry parameters in the male and female rats (Table 1).

Sub-chronic study

All rats survived the scheduled sacrifice and no test material related clinical observations or ocular abnormalities noted during the treatment period.

Differences in body weight gained between the treatment groups and the control group were not significantly different in the male rats. However, a significant difference in body weight gain was observed between the female rats receiving treatment and the control group in

Table 1. Effect of *N. oculata* on plasma chemistry parameters in acute study.

Rats	Bili (µmol/L)	AST (UKat/L)	ALT (UKat/L)	Urea (mmol/L)	Creat (mg/dl)
Male					
Before	0.06 ± 0.03	1.40 ± 0.20	0.90 ± 0.06	5.30 ± 1.60	0.24 ± 0.02
After	0.07 ± 0.02	1.95 ± 0.45	0.88 ± 0.27	6.16 ± 1.01	0.27 ± 0.04
Female					
Before	0.05 ± 0.03	1.58 ± 0.52	0.80 ± 0.06	4.99 ± 0.91	0.19 ± 0.02
After	0.08 ± 0.08	1.40 ± 0.40	0.88 ± 0.20	5.46 ± 0.74	0.22 ± 0.02

Results are expressed as mean ± SD (n=6). No significant differences were observed (p>0.05) Bili = total bilirubin, AST = aspartate aminotransferase, ALT = alanine aminotransferase, Creat = creatinine.

Table 2. Effect of *N. oculata* on body weight gain of rats.

Week	Male			Female		
	control	3 g/kg	6 g/kg	control	3 g/kg	6 g/kg
1	45.17 ± 18.81^{a}	58.50 ± 14.38^{a}	54.00 ±20.91^{a}	17.33 ± 8.48^{a}	31.33 ± 6.77^{b}	26.00 ± 6.63^{ab}
2	33.33 ± 12.72^{a}	38.17 ± 11.28^{a}	34.50 ± 9.40^{a}	10.50 ± 4.97^{a}	19.67 ± 4.46^{b}	17.67 ± 4.13^{b}
3	32.83 ± 29.03^{a}	34.67 ± 6.97^{a}	30.50 ± 9.01^{a}	19.50 ±10.60^{a}	19.00 ± 3.80^{a}	13.00 ± 3.52^{a}
4	19.33 ± 13.15^{a}	34.00 ± 11.78^{a}	20.67 ± 7.01^{a}	8.67 ± 5.99^{a}	8.67 ± 3.61^{a}	11.67 ± 5.95^{a}
5	49.00 ± 20.93^{a}	43.50 ± 9.48^{a}	31.17 ± 9.41^{a}	10.83 ± 3.49^{a}	15.50 ± 7.23^{a}	11.50 ± 4.08^{a}
6	32.83 ± 21.60^{a}	19.83 ± 8.01^{a}	11.50 ± 6.77^{a}	2.83 ± 7.14^{a}	1.16 ± 6.30^{a}	-0.66 ± 2.94^{a}
7	28.17 ± 15.25^{a}	33.17 ± 8.18^{a}	30.00 ± 7.59^{a}	8.83 ± 2.23^{a}	16.67 ± 8.95^{a}	13.50 ± 3.98^{a}
8	15.33 ± 9.14^{a}	21.50 ± 8.48^{a}	16.00 ± 6.00^{a}	3.00 ± 3.28^{a}	8.50- ± 6.22^{a}	3.83 ± 3.12^{a}
9	3.83 ± 6.61^{a}	2.67 ± 9.13^{a}	7.66 ± 8.43^{a}	5.17 ± 5.11^{a}	3.67 ± 2.73^{a}	7.83 ± 4.57^{a}

Results are expressed as mean ± SD (n=6). Data with different superscripts in the same row and same gender are significantly different at p<0.05.

Table 3. Effect of *N. oculata* on plasma chemistry parameters in sub-acute study.

Parameter	Unit	Male			Female		
		Control	3 g/kg	6 g/kg	control	3 g/kg	6 g/kg
Bili	µmol/L	0.07 ± 0.07^{a}	0.04 ± 0.04^{a}	0.08 ± 0.06^{a}	0.08 ± 0.03^{a}	0.11 ± 0.06^{a}	0.08 ± 0.06^{a}
AST	Ukat/L	1.20 ± 0.24^{a}	1.13 ± 0.17^{a}	1.12 ± 0.23^{a}	1.42 ± 0.75^{a}	1.12 ± 0.12^{a}	1.46 ± 0.84^{a}
ALT	Ukat/L	0.75 ± 0.14^{a}	0.81 ± 0.13^{a}	0.72 ± 0.17^{a}	0.68 ± 0.10^{a}	0.76 ± 0.12^{a}	0.93 ± 0.49^{a}
Urea	mmol/L	4.45 ± 2.67^{a}	4.16 ± 0.45^{a}	4.97 ± 1.04^{a}	6.28 ± 1.45^{a}	5.86 ± 2.14^{a}	5.50 ± 0.96^{a}
Creat	mg/dl	0.72 ± 0.12^{a}	0.76 ± 0.26^{a}	0.26 ± 0.05^{b}	0.68 ± 0.06^{a}	0.31 ± 0.09^{b}	0.28 ± 0.05^{b}

Results are expressed as mean ± SD (n=6). Data with different superscripts in the same row and same gender are significantly different at p<0.05. Bili = total bilitubin, AST = aspartate aminotransferase, ALT = alanine aminotransferase, Creat = creatinine.

the early weeks of the study. These results showed no adverse effect of the biomass on clinical observations and body weight gain (Table 2).

Values of plasma chemistry determined at the termination of the study are presented in Table 3. No significant treatment-related biological adverse effects were observed in the plasma chemistry parameters, such as total bilirubin, AST, ALT, and urea in comparison with control group in male and female rats. However, the treatment groups showed significantly reduced levels of creatinine (p<0.05), in comparison with the control group. We believe the reduction seen in creatinine could be due to the treatment received but we do not considered it to be of toxicological significance.

Organ weights and histological qualitative assessment of organs

The study results demonstrated that *N. oculata* biomass

Table 4. Effect of *N. oculata* on the organ weights (gram) in male and female rats.

Organ	Male			Female		
	Control	3 g/kg	6 g/kg	control	3 g/kg	6 g/kg
Liver	15.83 ± 2.14	16.00 ± 0.63	14.17 ± 2.64	8.67 ± 1.03	9.83 ± 1.72	9.67 ± 1.37
Kidney	3.50 ± 0.55	3.00 ± 0.63	2.67 ± 0.52	2.33 ± 0.52	1.83 ± 0.41	2.33 ± 0.52
FBW	446 ± 49.13	420 ± 52.90	390 ± 42.75	259 ± 15.47	276 ± 24.58	253 ± 14.21
RLW	3.53 ± 0.18	3.86 ± 0.62	3.60 ± 0.35	3.33 ± 0.22	3.56 ± 0.59	3.80 ± 0.38
RKW	0.77 ± 0.05	0.70 ± 0.08	0.67 ± 0.07	0.89 ± 0.17	0.66 ± 0.15	0.92 ± 0.22

Results are expressed as mean ± SD (n=6).No significant differences were observed (p>0.05), FBW: Final Body Weight, RLW: Relative Liver Weight, RKW: Relative Kidney Weight.

Table 5. Antioxidant activity of *N. oculata* in plasma (mmol/L) by FRAP test.

Rat gender	Treatment group		
	Control	3 g/kg	6 g/kg
Male	0.18 ± 0.03	0.17 ± 0.02	0.19 ± 0.02
Female	0.22 ± 0.05	0.23 ± 0.03	0.24 ± 0.02

Results are expressed as mean ± SD (n=6). No significant differences were observed (p>0.05).

Table 6. Antioxidant activity of *N. oculata* in plasma by ABTS (percent of inhibition).

Rat gender	Treatment group		
	Control	3 g/kg	6 g/kg
Male	84.67 ± 1.59	84.39 ± 1.44	84.21 ± 1.04
Female	87.62 ± 0.95	84.80 ± 3.68	86.25 ± 0.92

Results are expressed as mean ± SD (n=6). No significant differences were observed (p>0.05).

Table 7. Mineral content of *N. oculata*.

Mineral	Concentration (of dry weight)
Na	64.0 ± 19.34 g/kg
K	10.75 ± 0.56 g/kg
Mg	2.76 ± 0.03 g/kg
Ca	0.84 ± 0.19 g/kg
Fe	0.81 ± 0.004 g/kg
Zn	0.03 ± 0.001 g/kg
Cu	0.005 ± 0.0003 g/kg
Se	0.04 ± 0.025 mg/kg
As	0.38 ± 0.1 mg/kg
Pb	0.14 ± 0.005 mg/kg
Cd	0.0068 ± 0.0014 mg/kg
P	ND

The results are expressed as mean ± SD, ND = not detected.

supplementation of rat feed did not cause biologically significant changes in kidney and liver weights, or relative weights in either the male or the female rats (Table 4).

The rat livers and kidneys in both the acute and sub-chronic studies were assessed for possible toxic effects of *N. oculata.* The liver and kidney tissue of the rats of the treatment groups showed normal architecture and no significant pathological changes were observed in the different parts of the examined tissue. These results support the conclusion that feeding rats with *N. oculata* at doses equal to, or less than12 g/ kg bw had no acute or sub-chronic adverse pathological effects.

Antioxidant status of plasma

The antioxidant activity of *N. oculata* in plasma was evaluated by the ferric reducing ability of plasma (FRAP) and ABTS tests. The results of these studies indicated that there were no significant differences in the antioxidant activities between the treatment groups and the control one (Tables 5 and 6).

Mineral quantification of *N. oculata*

The heavy metals content of *N. oculata* biomass was negligible. However, it was rich in sodium, potassium, magnesium, and calcium (Table 7).

DISCUSSION

One previous study reported on the acute administration of Nannochloropsis sp. to rats (Andres et al., 1992). They exposed male Sprague-Dawley rats to fresh form of Nannochloropsis sp. at dosages of up to 10% of the daily food consumption for up to four weeks. Results of their study illustrated that Nannochloropsis did not affect the haematological indices, body growth, or the organ weights of the rats. In our study, the acute and sub-chronic administrations of *N. oculata* for 14 and 60 days, respectively, at dietary levels up to 12 g/kg bw were well tolerated and did not affect the health parameters of Sprague-Dawley rats.

A comparison between the weights of rats in the acute

study before and after treatment unveiled significant differences. The females exhibited higher weekly weight gain than the males. This finding mostly corresponds to a normal physiological increase in body weight due to normal growth and, hence, the increases in rat body weights were not considered as adverse treatment-related effects. Body weight gain by the female rats in the sub-chronic toxicity study differed significantly from those of the control group in the first and second weeks. Since no adverse treatment effects were observed in the other examined parameters, this significant difference could be due to other reason(s), but not necessarily due to adverse *N. oculata*-related effects.

Furthermore, there were no adverse changes in clinical chemistry in the male or female rats that could be attributed to treatment with *N. oculata*. In the sub-chronic toxicity study a significant reduction of the plasma creatinine was observed in both genders of the treatment groups in comparison with the control group. This finding was not considered to be of toxicological significance because in nephrotoxicity an increase in the levels of plasma creatinine is expected due to the attendant damage to the renal function. In addition, the observed levels of plasma creatinine were still within the lower limits of the normal range. The non-reduced levels of creatinine in the plasma of the male rats relative to the female rats under the low dose treatment may be due to their muscle masses, which are higher than those of the female rats. No macroscopic or microscopic changes were observed after administration of *N. oculata* for 60 days. With respect to mean and relative organ weights, no significant differences were observed between the treatment and control animals. Thus, incorporation of *N. oculata* into rat feed at levels up to 12 g/kg of bw can be considered as safe.

A recent study has shown Nannochloropsis to be a potentially good source of antioxidant (Goh et al., 2010). However, in our study the antioxidant activity of plasma by ferric reducing ability of plasma (FRAP), and ABTS tests showed that there were no significant differences in the antioxidant activities between the treatment groups and the control. One reason for this, might be the role of the antioxidant in protecting the elevated demand on long-chain polyunsaturated fatty acids, which is abundant in *N. Oculata,* against oxidation (Pal et al., 2011).

Bioaccumulation of heavy metals by microalgae is common as microalgae have a strong affinity towards heavy metals and they are frequently employed as a biosorbent material (Doshi et al., 2008). Our findings showed that there is no high accumulation of heavy metals in the biomass that we used, hence, indicating that the microalga is safe from the heavy metals content point of view.

In summary, the results of the present study demonstrate a safety profile for *N. oculata*. There were no treatment-related effects noted at any dose level tested. Therefore, based on these results, the no observable adverse effect level (NOAEL) for *N. oculata* under the conditions of this study was considered to be 12 g/kg of bw per day in the acute toxicity study and 6g/kg of bw in the sub-chronic toxicity study.

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