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Analysis the Extracts of Microalgae *Nannochloropsis* sp. Bioactivity

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Abstract: Microalgae are known as a valuable protein resource, but also as producers of valuable compounds for the benefit of human health (eg. fatty acids polyunsaturated PUFA's, minerals, vitamins). Nannochloropsis is a green microalgae that completely processes chlorophylls b and c, and has the capacity to accumulate large amounts of carotenoids, known for their antioxidant properties. The goal of this research is to develop products or nutraceuticals from microalgae to application in human health. The various microalgae extracts will be analyzed for their beneficial effects of bioactivity and toxicity in human metabolic diseases. Different extraction methods will be carried out by project colleagues and tested in this search. The lipid metabolism assay of zebrafish larvae shows extracts that reduced lipid content: 4 ethanol extracts all with 15 minutes but N32 ($P < 0.05$) extracted at lower pressure (300 MPa); 3 with acetone all with timing 15 minutes and pressure 500 and 300 MPa ($P < 0.05$) and 1 with water which has a higher pressure of 500 MPa ($P < 0.001$). An anti-hepatic activity was evaluated by fattening HepG2 cells and only one extract (NC11 – acetone, timing 15 minutes) reduced the formation of lipid droplets in the cytoplasm ($P < 0.01$). In the analysis of the anti-inflammation activity, a test was carried out subdivided into NO and MTT and 6 extracts reduced cellular inflammation: 2 extracts with acetone ($P < 0.01$ and $P < 0.001$) influenced both by pressure (500 and 420 MPa) and by timing (15 and 9 minutes); 4 extracts with ethanol where $P < 0.05$ is verified in extracts with greater timing (15 minutes) and pressure (500 MPa). Finally, the anti-diabetes test was carried out, finding 4 extracts with bioactivity: 2 of them were in acetone with the same timing (15 minutes), but at a pressure of 300 MPa ($P < 0.05$); with ethanol we also found 2 extracts where found the same as in acetone but with $P < 0.05$ and $P < 0.01$. Toxicity was evaluated in the assays: *in vivo*, evaluating lethality or the presence of malformations in fish larvae zebra, and *in vitro*, through sulforhodamine B (SRB) and tetrazolium (MTT) cellular assay. Nannochloropsis extracts showed anti-obesity, anti-inflammation, anti-diabetes and anti-steatosis activity, which demonstrated its potential for the prevention and treatment of metabolic diseases.

Key words: Diabetes, steatosis, obesity, oceanica, extraction, liver.

1. Introduction

1.1 Obesity

Obesity is defined as a body weight parameter with a body mass index (BMI) greater than 30, due to positive energy balance, in different body zones [1, 2]. In fact, numerous studies have already revealed that some people have, in their genotype, a predisposition to obesity, but that this only manifests itself if exposed to external factors (eg. sedentary lifestyle), the distribution of fat influences the severity and the development of other pathologies [3]. Like hypertension, sleep apnea, sleep, some types of cancer, cardiovascular disease, 2 types of diabetes, high cholesterol and arthritis, associated with the musculoskeletal domain. Hence some evidence suggests that obesity will be more harmful than consumption of alcohol and tobacco and that attempts to change lifestyle through diet and exercise. So obesity is being considered such a high risk anomaly

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[4, 5]. Diet and physique exercise are not very effective in the long term, with a new substantial increase in weight being observed which often ends up exceeding the initial value [6, 7]. Allied to these two forms of combating obesity, we may have the use of anti-obesity drugs or the surgical procedures. However, the first ones can have numerous side effects, such as heart attacks, hypertension, strokes and even sudden deaths, and it is also very ineffective. In such a way that in December 2013, they would only have been approved, by the FDA and EMA, four types of medicine for long-term treatment of this disease. On the other hand, surgeries are much more efficient than the previously mentioned processes, but they are still extremely expensive, have a complicated postoperative period and intended only for the public with a BMI greater than 40. So, there is a great need for alternatives that will derive from macro and microorganisms, more specifically their secondary metabolites [2, 8].

1.2 Diabetes

Group of metabolic diseases characterized by an increase in blood glucose levels, known as hyperglycaemia. Chronic hyperglycaemia causes secretion disorders and/or insulin action and is associated with long-term damage and functional disturbances of severaltissues and organs (eyes, kidneys, nerves, heart and blood vessels) [9]. Type 1 diabetes is an absolute disturbance of insulin secretion due to the destruction of pancreas cells. Occurs in adulthood, it is an autoimmune disease and the loss of insulin secretion capacity is slower [10]. Type 2 diabetes is a decrease in the effect of insulin (insulin resistance). It starts with a lack of insulin, which is usually dependent on glucose [9]. Adipose tissue of obese patients usually has monocyte infiltration in the state of chronic inflammation [11]. This infiltration leads to insulin resistance of pro-inflammatory cytokines [12].

1.3 Microalgae

Microalgae are often photosynthetic organisms, mostly aquatic, from prokaryotes to eukaryotes, with great morphological and physiological diversity. Usually found in fresh waters of marine systems, these organisms are the responsible for the production of numerous compounds such as oxygen, fatty acids polyunsaturated, vitamins, minerals, fiber, peptides, enzymes, photosynthetic pigments such as chlorophylls and carotenoids and toxins. As photoautotrophic beings, they produce half of the atmospheric oxygen and consume carbon dioxide and, along with other organisms like bacteria, they can produce energy for all trophic levels above them [13]. It should be noted right away that the chemical composition of microalgae can vary according to internal and external factors. This composition is therefore dependent on the species, the conditions of the culture to which they were subjected, such as the incidence of light, pH, nutrients supplied, the temperature, the availability of CO_2 and salt [14, 15]. Over time it was becoming more and more noticeable that the marine environment is a reservoir of bioactivity. In turn, microalgae have aroused the curiosity of researchers for their antioxidant, anti-inflammatory, antimicrobial and anticancer potentialities in areas such as nutrition and pharmaceuticals. Some algae used by authors are *Spirulina maxima* (200 μg/mL) used in rats to suppress lipid accumulation [16], *Euglena gracilis* used, in varying concentrations, in adipose cells reducing or not the lipid accumulation depending on the concentration [17] and *Phaeodactylum tricornutum* used (3.5% to 6% (*w/w*)) also in mice with concentration-dependent lipid reduction of itself [18]. Obesity is one of the fields in which these organisms have been relevant, since, in other tests already carried out, using animal models, the benefits in terms of metabolism and body weight (decreased

lipogenesis, increased lipid oxidation, increased thermogenesis, increased lipolysis and decreased adipogenesis) [13].

1.4 Nannochloropsis sp.

The little variable morphology makes the systematics of Nannochloropsis very difficult. These cells are characterized by a continuum between the outer nuclear envelope and the nuclear membrane outer cytoplasm, are spherical and immoveable. During cell replication, these cells are duplicated and segregated coordinately. The chemical composition of Nannochloropsis, as well as that of all microalgae as previously mentioned, depends on the conditions environmental (eg. quality and quantity of light, temperature, composition of the culture). Looking for the lipid profile of this strain, we see that it includes mono and polyunsaturated fatty acids with high nutritional value. Oleic acid, palmitoleic acid and palmitic acid are also emphasized. In terms of nutritionally active compounds, we see that these microalgae have a significant percentage of carotenoids that contribute to light gathering and function as secondary pigments in photosystem II of photosynthesis. Besides, these compounds are of great importance with regard to the protection of the membranes of thylakoids in the photo-oxidative process. After discovering all these benefits of species, it was imperative to establish a way to preserve the biomass nutrition after specimen collection. This need is associated with the fact that the individuals undergo decomposition and oxidation, which directly interfere with the stability of its components [19].

1.5 Zebrafish

In terms of classification of ovarian development, fish can be of two types: synchronous or asynchronous. In the first type, we see the oocytes being formed and the spawning happen once a year or once in a lifetime. The gonads of this type of fish pass through multiple phases and spawning occurs during the breeding season. Already the asynchronous fish, they spawn throughout the year and the stages of development of the oocyte are present always and at the same time. The specimen used in this work, the fish zebra, belongs to the asynchronous group, having the 5 phases of the oocyte present in each ovary, in simultaneous [20]. In terms of research, zebrafish have often been used as model since it combines its excellent embryology with ease of manipulation genetics. In terms of embryology, it relates to the fact that it is an asynchronous fish, as mentioned and explained before and also because their embryos have relatively large dimensions and its development is easy to observe through the chorion due to its transparency. This development process is fast, after 5 days of fertilization, their larvae are already able to swim and feed themselves. In terms of genetic data, this species proves to be very useful, because it facilitates the analysis and identification of genes through mutations, the establishment of inbreeding and the formation of stocks. In recent years, we have witnessed the development of specific methodologies for be applied to zebrafish, which has allowed new experimental approaches [21]. Zebrafish show advantages in being an in vivo model due to its biological conservation, qualified for imaging, availability of genetic tools and disease models, as well as various applications in the development of nano medicine [22].

1.6 Goals

The global objective of this research is to develop products or nutraceuticals from the microalgae Nannochloropsis for application in human health, reversing or attenuating the obesity and associated diseases. The various microalgae extracts will be analyzed for their beneficial effects of bioactivity and toxicity in human metabolic diseases. Different extraction methods will be carried out by project colleagues and tested in this tracking. For all the reasons mentioned above, we will carry out this study

that will use, as a means of analysis of the lipid quantity, the zebrafish (*in vivo*) in the anti-obesity and anti-diabetes assay, cells (*in vitro*) such as macrophores in anti-inflammation and fattened cells in steatosis, trying to observe a decrease in lipid mass.

2. Materials and Methods

2.1 Nannochloropsis Oceanica Extracts Used

The extracts used in this project come from the University of Aveiro from the Prof. Jorge Saraiva, within the scope of the AlgaValor project, and therefore were not obtained by me. These samples were obtained by extraction by HPP (high pressure processing) (Table 1).

2.2 Zebrafish Trial with Nile Red

The zebrafish Nile Red assay analyzed the lipid-reducing activity of compounds as described in [23]. Since the chosen procedures are not considered animal experiments in accordance with the EC Directive 86/609/EEC, it was not necessary to seek the consent of an ethics committee. In short like you can see in Fig. 1, fish embryos zebra were raised from 1 DPF (days post fertilization) in egg water (60 ug/ml of water sea salt in distilled water) with PTU (1-phenyl 226 2-thiourea) for a final concentration of 200 μM, to inhibit pigmentation. From 3 DPF to 5 DPF, the exposure of zebrafish larvae to compounds at a final concentration of 10ug/ml. There was a daily renewal of water and compounds in the 48-well plate with a density of 5-7 larvae/well. In the assay, we also included a negative control (REV, resveratrol, 50 mM stock solution) and another positive control (0.1% DMSO (dimethyl sulfoxide)). The plate was left with Nile Red (500 ng/ml working solution) overnight for the lipids to incorporate the dye. In order to prepare larvae, for the observation of fluorescent microscope, anesthesia microscope MS-222 (Tricain, 1.5 % of the reserve solution) is available in each well. With the help of the ImageJ software, it was possible to quantify the intensity of fluorescence detected by the microscope [24].

Table 1 Extract mass (mg) and respective yield (mg/mg) for each extraction condition (Pressure, Time, Solvent).

Conditions (Pressure/Time)	Sample No.	Solvent	m extract obtained (mg)	Performance % (mg/mg)
500 MPa/15 min	N3	Water	40.50	32.36
	N5	Ethanol 48%	41.07	32.67
	N8	Ethanol 96%	26.10	20.75
	N11	Acetone	2.60	2.06
420 MPa/9 min	N15	Water	32.42	25.67
	N17	Ethanol 48%	31.44	24.85
	N20	Ethanol 96%	31.29	24.83
	N24	Acetone	-	2.30
300 MPa/15 min	N27	Water	42.61	34.14
	N29	Ethanol 48%	44.07	34.79
	N32	Ethanol 96%	21.70	17.42
	N35	Acetone	3.10	2.47
15 min controls	NC5	Ethanol 48%	39.22	31.59
	NC9	Ethanol 96%	13.20	10.54
	NC11	Acetone	2.60	2.05
9 min controls	NC14	Water	39.74	31.34
	NC17	Ethanol 48%	39.59	31.85
	NC23	Acetone	3.98	3.21

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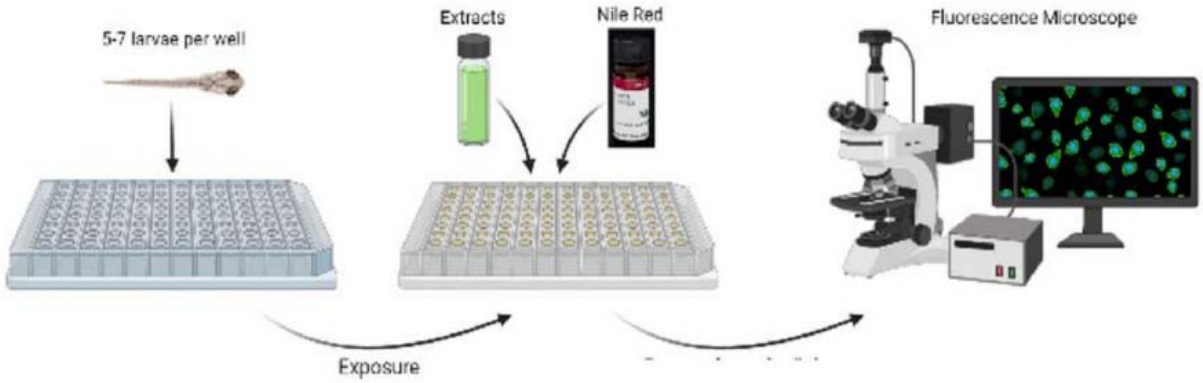


Fig. 1 Simplified representation of the Zebrafish test with Nile Red.

2.3 Anti-diabetes Trial

A 96-well plate was planted with 5 zebrafish larvae (3 DPF) in each for a total volume of 200 µL. On the plate we had three replicates of the 0.1% DMSO control (control solvent), two replicates of the Emodin 10 µM positive control (shows increased uptake) and the extracts. After 1 hour of exposure, 2-NBDG (2-(N-(7-Nitrobenz-2-xa-1,3diazol-4-yl)Amino)-2-Deo xyglucose) in 100 µL of Ethanol :Egg Water (1: 2) for a concentration of 10 mM (33.3% Ethanol). A new exposure is made with 170 µL of 2-NBDG, a fluorescent analogue of glucose, with Egg Water + PTU (200 µM), for 3 hours (required protect from light due to the high sensitivity of 2-NBDG; final concentration of 2-NBDG: 100 µM). At the end, we analyzed the plate under an Olympus microscope. In this test it was necessary to dilute the stock solution of Emodin (10 mM). First use Egg Water (1: 10) to 1 mM (10% DMSO), then again with Egg Water (1:2) to 500 µM (5% DMSO). We add 4 µL to the respective wells (1: 50 dilution) for the concentration (10 µM, 0.1% DMSO). The same was done with the extracts, initially with a concentration of 10 mg/mL. diluted in Egg Water + PTU (1: 20) in DMSO to 500 µM (5% DMSO). We add 4 µL to the wells (1:50 dilution) to obtain a final concentration of 10 µg/mL (0.1% DMSO). This process is presented in Fig. 2.

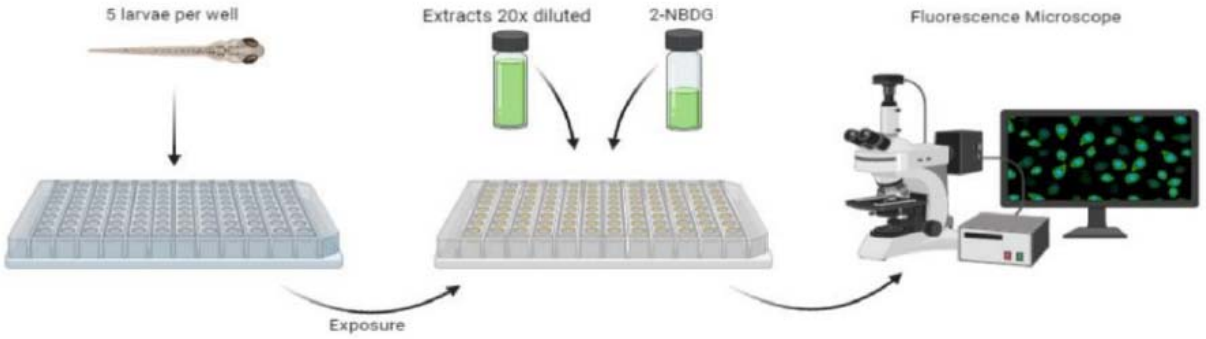


Fig. 2 Simplified representation of the anti diabetes trial.

2.4 Steatosis Inhibition Assay

HepG2 cells used in the steatosis inhibition assay, as they are cancer cells with high multiplication rates, having similarities with the epithelial tissue at the morphological, therefore performing different liver functions. The HepG2 are a human hepatoma frequently used in drug metabolism assays and hepatotoxicity [25]. As shown in Fig. 3, a 96-well plate was seeded with 1 x 10^5 cells/ml in DMEM

(Dulbecco's modified Medium Eagle) which grew for 24 hours under 37 °C and 5% CO_2 conditions. The cells are treated with SO (sodium oleate), concentration 62 μM, to induce lipid accumulation. This exposure is done on all wells except controls. Subsequently, there was exposure to microalgae extracts, concentration 25 ug/mL, to try to revert the lipid accumulation induced and remained in an incubator for 6 hours. The addition of 100 μL of HBSS (Hank's Buffered Salt Solution) with HO-33342 (1: 100) and Nile Red (1: 400) to the different wells will allow lipid quantification in a fluorescence plate reader, through the quantification of fluorescence in the images, after 15 minutes of incubation in the dark and 3 washes with HBSS. Finally, we added 100 μL of 0.4% (*w/v*) SRB in 1% acetic acid for cell viability analysis [26].

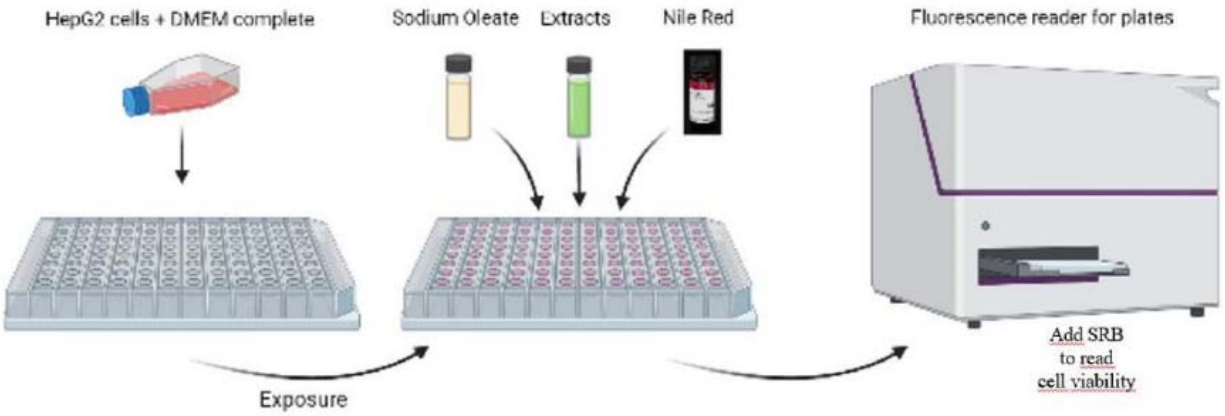


Fig. 3 Simplified representation of the steatosis inhibition assay.

2.5 Inflammation Assay

Raw 264.7 cells used in the inflammation assay as they are a reliable model macrophage capable of phagocytosis and pinocytosis. When stimulated with LPS (cell wall lipopolysaccharides that stimulate inflammation), these cells produce, more abundantly, NO (nitric oxide) [27]. This test is composed of two complementary tests like in Fig. 4: the NO test and the MTT test (3-(4,5-Dimethyithiazol-2-yl)-2,5-Diphenyltetrazolium Bromide). In the NO assay, it is possible measure cellular inflammation, induced by LPS, and in the MTT test, information is obtained on cell viability. These two tests are performed, because if cell viability is low (great cell death) and the anti-inflammatory activity is high, we can be have been misled into concluding that the inflammation has decreased due to the extracts, and yet this decrease was due to the fact that LPS does not act on dead cells.

We used the cell pellet obtained with 1 mL of complete DMEM, 10 μL of this mixture with Trypan Blue (dye for living cells that appear bright under the microscope while cells are dead cells turn dark blue) will go to the automatic cell counter. From here we calculate the volume needed to obtain a final concentration of 3.5 x 10^5 in a final volume of 12 mL. This final volume will be distributed among the wells (100 μL per well) and incubated for 24 hours at 37 °C. Subsequently, the old medium is removed and 100 μL of DMEM is added complete solution, 0.25 μL of extracts and DMSO and 5 μL of LPS stock solution (50 μL of LPS in PBS 0.1 mg/mL diluted in 200 μL complete DMEM; final concentration 1 ug/mL) to the respective wells. Again, 24 hours of incubation at 37 °C. For the NO assay, we placed 75 uL of starter plate in a new plate with 75 μL of Griess reagent (for 20 mL: 20 mL of 2% H_3PO_4 + 200 mg Sulphanilamide (1%; 10 mg/mL) + 20 mg N-[1-naphthyl]-ethylenediamine dihydrochloride (0.1%; 1 mg/mL)). Incubate at room temperature, in the dark, for 15 minutes and then we measured the absorbance. In the MTT assay, we

removed the medium remaining in the initial plate, we add 100 μL of MTT 0.5 mg/mL (for 11 mL: 5.5 mg of MTT in 11 mL of complete DMEM) and incubated at 37 °C for 45 minutes. Then we remove the supernatant again, we add 100μL of DMSO (DMSO for the MTT assay) and we read the absorbance.

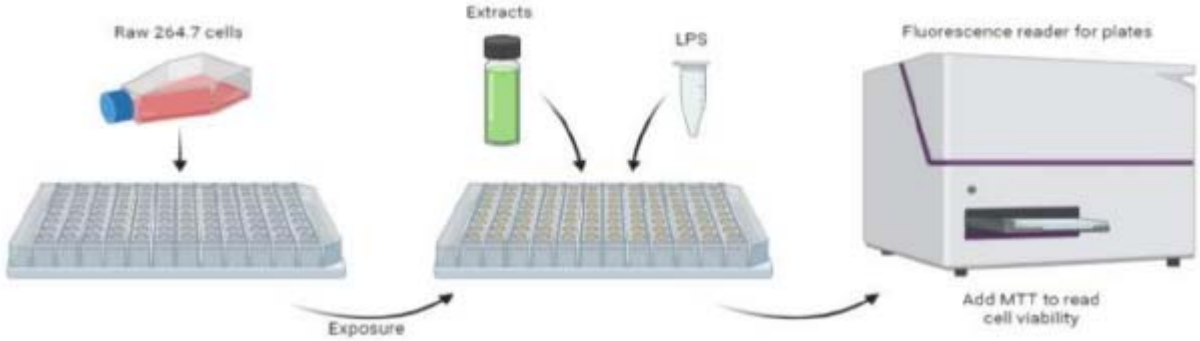


Fig. 4 Simplified representation of the inflammation assay.

2.6 Statistical Study

After obtaining the data for each test, they were analyzed using the GraphPad software.

Data normality test and identification of outliers were performed using the ROUT method

with Q = 1%. Depending on the nature of the data (parametric or non-parametric) it waschosen the appropriate ANOVA. For normal data, ANOVA followed by Dunnet's posthoc test,for nonparametric data, the Kruskal Wallis test with Dunns. Significance was considered as follows: $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***.

3. Results and Discussion

3.1 Zebrafish Trial with Nile Red

Looking at Graph 1, it is possible to verify that there were extracts with a decrease substantial amount of lipid (depicted in Fig. 5) compared to the control Extracts with activity, dissolved in ethanol, all have an extraction timing of 15 minutes, with greater significance being the one that was extracted at the lowest pressure of 300 MPa (N32). With acetone, the timings are the same (15 minutes) changing only the pressure (500 and 300 MPa), however, this did not affect the significance of the results. With water we only have one extract with activity, the one with the highest extraction pressure (500 MPa). These results are in line with those obtained in [13] with tests in mice and cells, using 5 microalgae many different. In this study, lipid reduction was verified according to the microalgae used, applied concentration and respective model, highlighting the role of microalgae in the treatment of obesity in addition to its prevention.

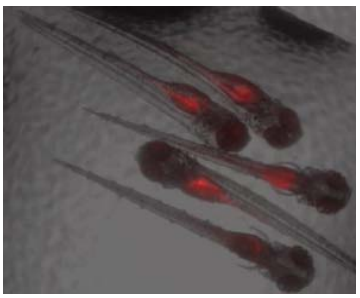
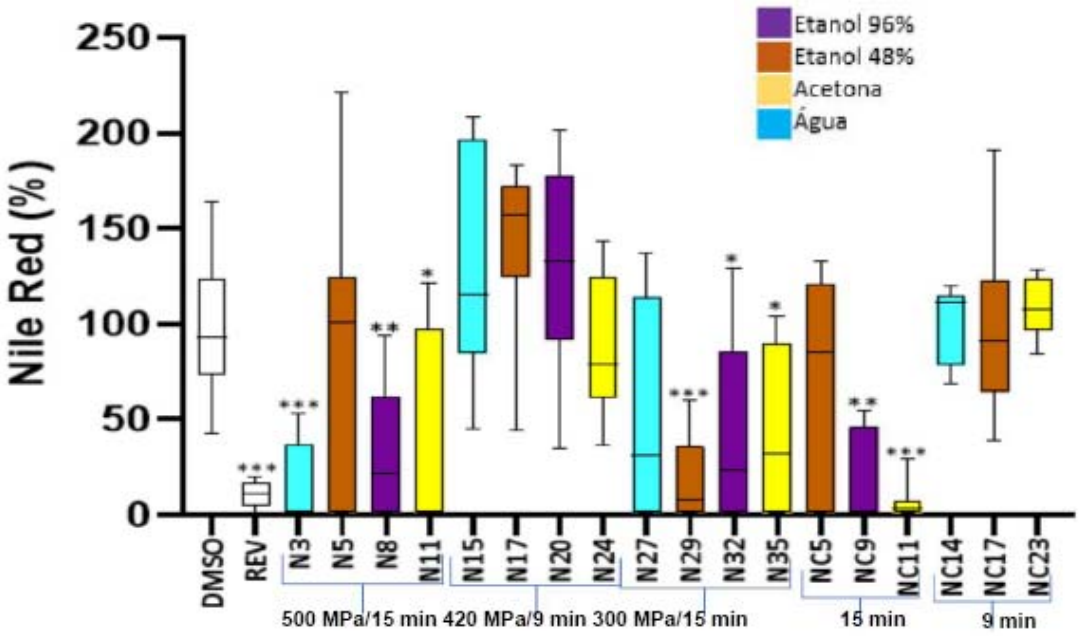


Fig. 5 Bright field and fluorescence channel overlay of zebrafish larvae stained with Nile Red for visualization of neutral lipid content.



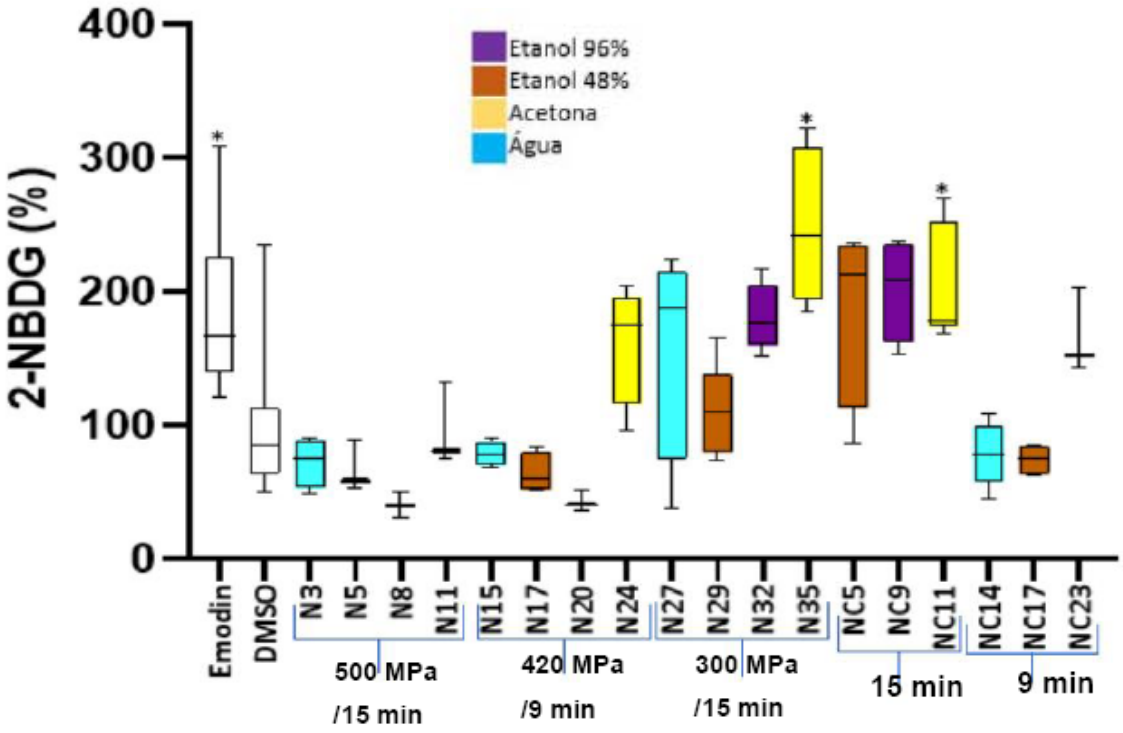
Graph 1 Lipid quantification for each of the extracts used.

3.2 Anti-Diabetes Trial

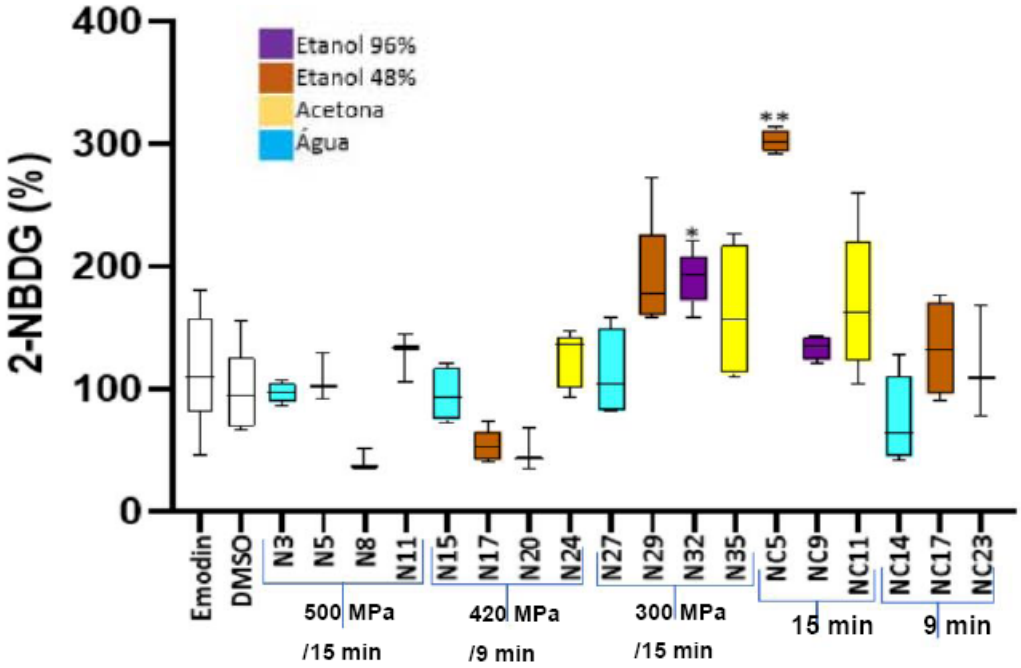
Looking at graphs 2 and 3 and comparing each extract, either in terms of the eye or the vitellin bag, with the DMSO control we can conclude that we had 4 extracts with activity. Two of them were in acetone (N35 and NC11), both of 15 minutes and with benefit if the extraction for a lower pressure (300 MPa). In terms of ethanol, we had two extracts (N32 and NC5), extracted in 15 minutes and again with more activity if lower pressure (300 MPa). Fig. 6 shows an example of the obtained images at the fluorescence microscope.

Compared to what was done in the present work, in [28] the induction of the diabetes in rats and, through the oral administration, for 3 weeks, of extracts of *Nannochloropsis oculata* (concentration between 10 and 20 mg/kg), there was a significant reduction, in the order of 20%, of the concentrations of glucose, triglycerides, cholesterol, LDL and an increased concentration of HDL-C and insulin in the blood.

Fig. 6 Bright field and fluorescence channel overlay of zebrafish larvae stained with 2-NBDG for visualization of neutral lipid content.



Graph 2 Mean fluorescence intensity of 2-NBDG (%) in zebrafish yolk sac.



Graph 3 Mean fluorescence intensity of 2-NBDG (%) in the zebrafish eye.

3.3 Steatosis Inhibition Assay

Analyzing graph 4, we can conclude that cell viability is around the range of 100%; therefore it shows that the extracts in the majority do not have adverse effects.

This finding is essential to be done since the wells with low cell viability and promising anti-steatosis

activity may be compromised. In Fig. 7, it's possible to see the cells conformation. The low percentage of steatosis may be due to the fact that the cells are dead and not to the properties of the extracts. However, in another study [29] with microalgae they showed no adverse effects on mice above 25%, which indicates that they can be used as a source of protein and acids fat.

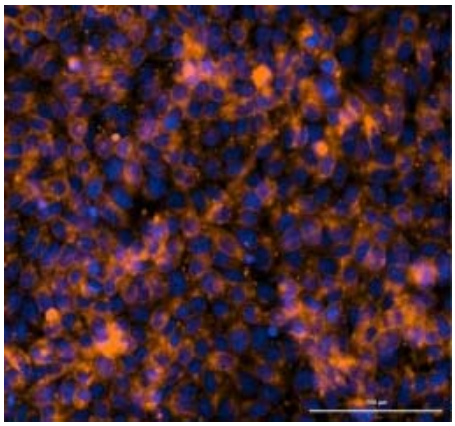
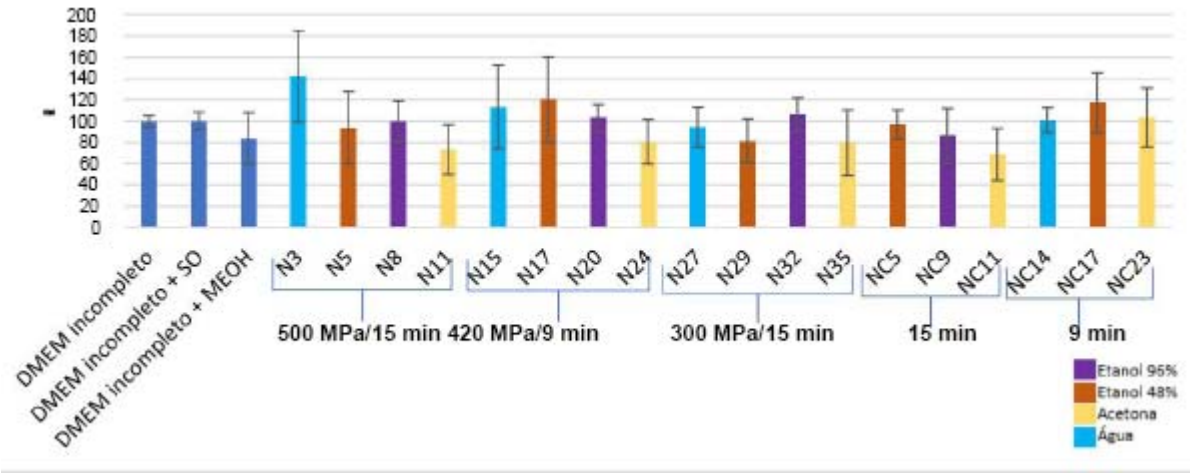


Fig. 7 HepG2 cells stained with Nile Red. Blue: Core; Orange: Lipids.

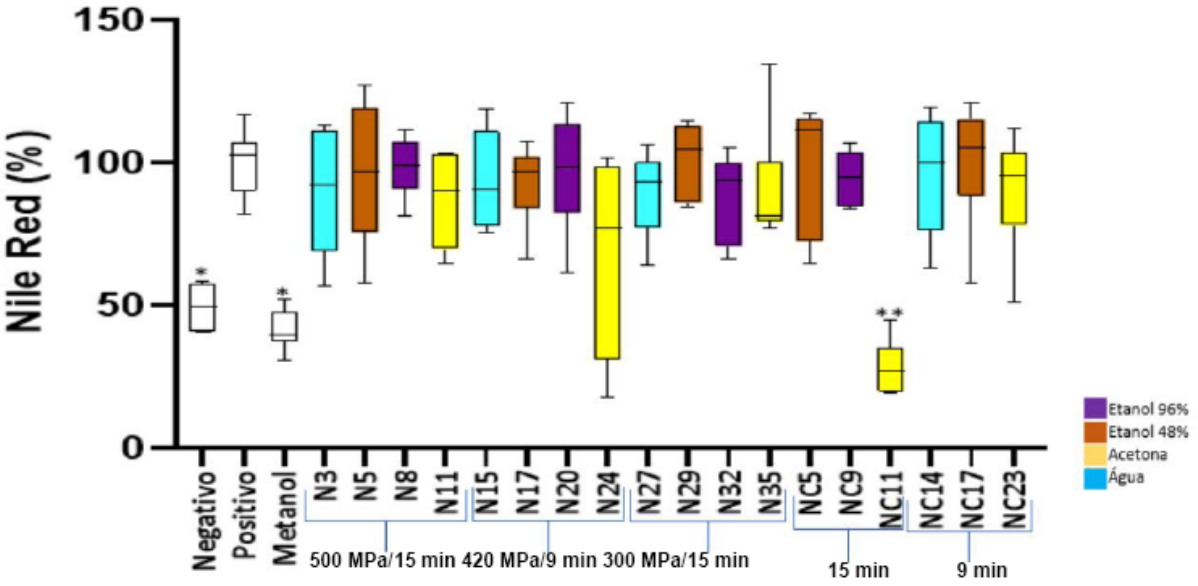


Graph 4 Analysis of cell viability through in vitro SRB.

Now analyzing graph 5 and making a parallel with graph 4, we can conclude that the NC11 extract (15 minutes of extraction) was the only steatosis inhibitor.

NOTE: If we wanted to discard any of the controls, we could do so with the control methanol, as the sodium oleate control already has methanol as a solvent of the oleate. This control is only carried out to prove that, in fact, methanol does not influence in no way the cells and their fattening.

In [30], studies done with *Nannochloropsis oculata* in rats supplemented with oil (5 mL) homogenized with 5 mL of olive oil, every 7 days, show that there were no changes in the lipid quantity of the liver of the individuals in relation to a normal diet of the same ones by NMR analysis.

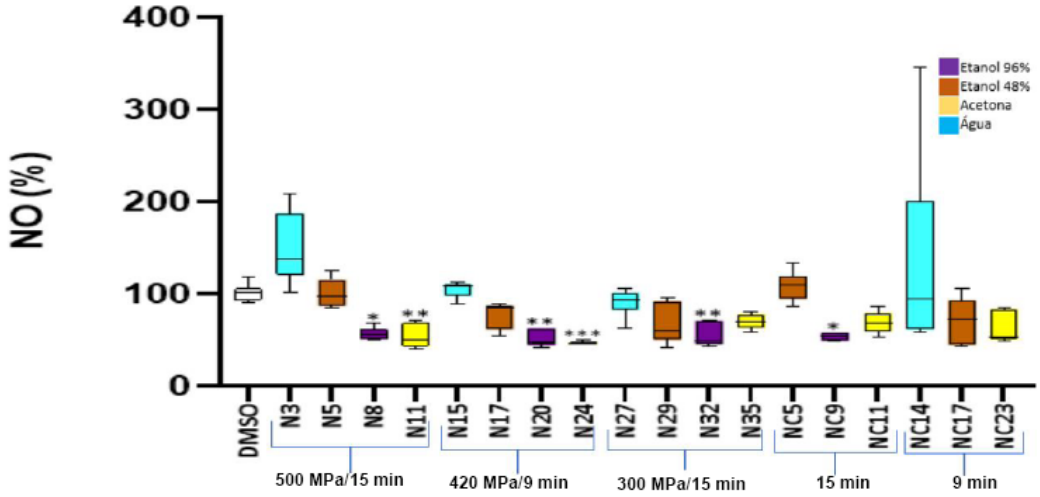


Graph 5 Lipid quantification and consequent anti-steatosis activity.

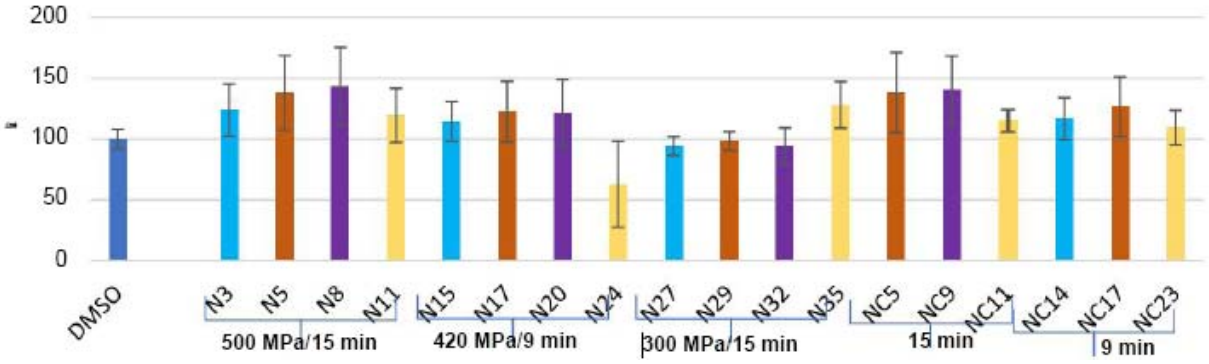
3.4 Inflammation Assay

In Graph 6, we have the DMSO which is a control that represents the maximum % of NO in the assay. Therefore, by comparison, we have 6 extracts with anti-inflammatory activity. This activity it was only observed in extracts with acetone and 96% ethanol solvents.

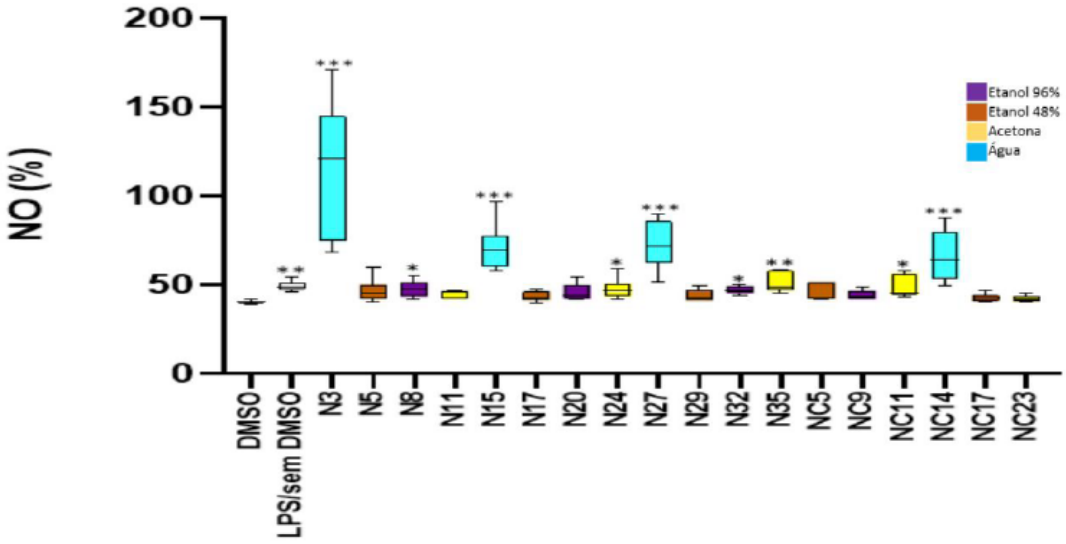
In the first case, the significance was affected by both timing (15 and 9 minutes) and pressure (500 and 420 MPa), because from one extract to the other, both change. In the second case, we see that the timing of 15 minutes with high pressure (500 MPa) is pro anti-inflammatory activity, because keeping the time and decreasing pressure to low significance. Analyzing Graph 7, we can verify that despite this bioactivity, the N24 extract was toxic to the cells because it had a significant decrease in cell viability.



Graph 6 Anti-Inflammatory Activity.



Graph 7 Anti-Inflammatory Cellular Viability.



Graph 8 Pro-Inflammatory Activity.

As described in [31], *Nannochloropsis* is especially rich in PUFA's, which have anti-inflammatory activity.

As obtained in this study and showed in Graph 8, in [32], a trial with *Nannochloropsis oceanica* included in the diet for Atlantic salmon (10 or 20%, 84 days) revealed that this microalgae have no pro-inflammatory activity significant with 10% of the diet, although negative effects were found, in intestinal physiology, with 20% of the diet.

4. Conclusions

Obesity, as previously described, is a metabolic disease that increasingly has been taking worrying proportions in the day to day and in the life of the human being, because it entails carry harm that seriously increases the risk for other diseases.

With the need to revert or alleviate this situation and, as the properties of microalgae, I submitted the microalgae Nannochioropsis oceanica to four types tests (obesity, diabetes, steatosis, inflammation) to then check if she has also beneficial properties.

From the tests carried out, I conclude that, in fact, the microalgae used presented anti-obesity, anti-diabetes, anti-steatosis and anti-inflammatory bioactivity. This way, could be used in the future as a nutraceutical or functional food. However, more studies would be needed to understand the mechanism of action and to identify the compounds responsible for bioactivity.

The article was originally written in Portuguese

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