

BIOSCREENING OF THE METHANOLIC CRUDE EXTRACT OF *ULVA LACTUCA* AND ITS WATER FRACTION

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ABSTRACT

Marine algae have been used as a novel food with potential nutritional benefits, and in industry and medicine for various purposes. Recently, aquatic habitats have increasingly been shown to provide a rich source of natural bioactive compounds. Green algae *Ulva lactuca* is a widespread macroalgae occurring at all levels of the intertidal zone, in calm and protected harbours as deep as 10 meters and in northern climates. Many of its nutrients include iron, protein (15%), iodine, vitamins (A, B1, and C) and trace elements. The present study investigated the bioactivity of *Ulva lactuca* methanolic crude extract and its water fraction. Also we identified on their different bioactive constituents using high performance liquid chromatography technique. Our results showed that methanolic crude extract of *Ulva lactuca* and its water fraction had phenolic and flavonoid active constituents acting in synergism to increase their bioactivity such as antioxidant, anti-inflammatory and antihemolytic activities. Through 15 days, there was no mortality detected among mice injected with *Ulva* water fraction different doses. Therefore, we could not calculate the LD₅₀ and accounted it as safe agent. Finally we concluded that *Ulva* water fraction safe to be used in the treatment of oxidative stress and inflammation related disorders.

Keywords: green algae *Ulva lactuca*, *Ulva* water fraction.

INTRODUCTION

Marine algae have been used as a novel food with potential nutritional benefits, and in industry and medicine for various purposes (Santoso *et al.*, 2004). Recently, aquatic habitats have increasingly been shown to provide a rich source of natural bioactive compounds with hypocholesterolemia, anti-inflammatory, antiviral, antineoplastic, antimicrobial and hypertensive properties. According to their chemical structure most of isolated compounds belong to sulfated polysaccharides, phenolics, terpenoids, lactones, sterols and fatty acids (McDermid and Stuercke, 2003; Qi *et al.*, 2006 and Duan *et al.*, 2006). On the other hand, the antioxidant activity is one of the most important active in marine bioactive substances and lots of algal and algae-derived compounds exhibited potent antioxidant such as carotenoids,

phenolics, terpenoids and sulphated polysaccharides. The antioxidant activity of these compounds are mainly attributed to scavenging activity against free radicals such as superoxide and hydroxyl radicals, chelating ability, quenching singlet and triplet oxygen, and reducing power (Ruberto *et al.*, 2001 and Athukorala *et al.*, 2006).

Natural antioxidant such as α -tocopherol, phenols and β -carotene found in higher plants are being used in the food industry to inhibit lipid peroxidation and they can protect the human body from free radicals and retard the progress of many chronic diseases (Matsukawa *et al.*, 1997 and Qi *et al.*, 2005). However, Gey, (1990) have reported that the synergistic action of wide spectrum of antioxidant is better than the activity of a single antioxidant, and that antioxidants from

natural source (primarily foods) have a higher bioavailability and therefore higher protective efficacy against oxidative stress than synthetic antioxidants.

ROS and free radical capable of causing damage to biomolecules, such as nucleic acids, lipids, proteins and other cellular constituents (Kubo *et al.*, 1992 and Halliwell 1996). The degradation and/ or modification of these molecules have been associated with various chronic diseases, such as cancer, coronary heart disease atherosclerosis, cataracts, ageing muscular dystrophy and some neurological disorders (Finkel and Holbrook, 2000; Cooke *et al.*, 2002). For these reasons, many products with antioxidant properties are widely used in order to minimize oxidative damage to living cells and to prevent oxidative deterioration of food. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commonly used to inhibit lipid peroxidation (Farg *et al.*, 1989). Now a day, BHT and BHA need to be replaced with natural antioxidant, however they were found to be toxic, responsible for liver damage, promoters of carcinogenesis and alteration the enzyme activities, as well as general consumer rejection of synthetic food additives (Gulcin *et al.*, 2002). Thus, it is important to develop, identify and utilize of new source of safe and effective antioxidants of natural origin (Li *et al.*, 2007; Qi *et al.*, 2005). Tumor Necrosis Factor Alpha (TNF- α), is a pro-inflammatory cytokine produced by monocytes/macrophages and activated by mast cells, endothelial cells, fibroblasts, neurons and glial cells during acute inflammation and it is responsible for a wide range of cell signals about cell viability, gene expression, homeostasis control and synaptic integrity (Black *et al.*, 1997).

TNF- α was described for the first time by Carswell *et al.* in 1975 as a protein component of serum of mice stimulated with bacterial antigens, and was brought to light the ability to induce death in cancer cell lines *in vitro* and *in vivo* to destroy transplanted sarcomas.

Characteristically, this cytokine was able to cause tumour cells death without compromising the viability of healthy cells. The subsequent isolation and molecular characterization of the gene have provided information on the structure and functioning of this molecule (Cereda *et al.*, 2012).

The aim of this study was to analyze the crude methanolic extract obtained from *Ulva lactuca* and its different fractions using HPLC technique and evaluate their antioxidant, anti-inflammatory and antihemolytic properties *in vitro*.

MATERIALS AND METHODS

Algae selection and collection

The green algae (*Ulva lactuca*) were collected from Abu Kir beach, Alexandria coast, identified and authenticated in Oceanography department, faculty of Science, Alexandria University.

Preparations of the methanolic crude extract and water fraction of green algae (Yacout *et al.*, 2011)

The algae well washed with tap water then left to dry in air for two days. 500 g of dried algae were grinded, completely flooded with 1.5L absolute methanol over night, and then filtrated. The filtrate was taken (methanolic extract) and algae were flooded again in absolute methanol over night then filtrated. The filtrates were collected and evaporated using rotary evaporator. The obtained methanolic extract was lyophilized (120 g). 110 grams of the dried extract were fractionated using distilled water (0.5L \times 2). The fraction was lyophilized and weighed 75 g. The powder was collected and kept in refrigerator till used.

Quantitative determination of *Ulva* water fraction contents by high performance liquid chromatography (HPLC) (Qadir *et al.*, 2009)

The analysis of water fraction of *Ulva Lactuca* extract was carried out on an Agilent-1100 HPLC system equipped with a quaternary gradient pump unit, ultra violet (UV) detector

at a wave length of 320 nm and the analytical column was Zorbax Eclipse XDB-C18 (150 x 406 mm , 5 µm particle size) column (Agilent, Santa Clara, CA, USA) was used. Elution was carried out at a flow rate (retention time) of 0.075 ml/ min at 23°C. The mobile phase consisted of 8 % acetonitrile, 22 % isopropyl alcohol and 70 % formic acid solution (1%). All standards (Chlorogenic acid, Caffeic acid, 3,4-Dicaffeoyl quinic acid, 3,5-Dicaffeoyl quinic acid, 4,5-Dicaffeoylquinic acid, Gallic acid, Rutin, Tanic acid, Quercetin and Retinol) and sample were dissolved in distilled water (1 mg/ml) and were filtered through 0.22 µm syringe filter prior to HPLC analysis. The injection volume was 20 µl of either sample or standard. The results are calculated by internal normalization of the chromatographic peak area. *Ulva* water fraction identification was made by comparing the relative retention times of sample peaks with standards.

Determination of lethal dose 50 (LD₅₀) of the water fraction of the methanolic extract of *Ulva Lactuca* crude in mice (Oukkache *et al.*, 2014)

25 healthy mice were divided into five groups, group one is control group subjected to free water intake. From group two to group five, mice were orally administered with the water fraction of the methanolic extract of *Ulva lactuca* at concentrations 10, 100, 500, 1000 mg / kg body weight for 15 days daily. Then mice were scarified and the blood sample for each mouse was collected in EDTA tubes to get plasma. Liver function tests, kidney function tests, cholesterol level, triglycerides level and TNF-α cytokine level were measured at plasma samples.

Determination of DPPH (2, 2-Diphenyl-1-picryl hydrazyl) scavenging activity

The assay was done according to (Blois, 1958)

Table 1: Bioscreening of different concentrations of *Ulva lactuca* crude extract and its fractions: 2, 2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity (antioxidant activity), nitric oxide (NO) radical scavenging activity (antiinflammatory activity) and red blood cells (RBCs) detoxicity (antihemolytic

Determination of NO (Nitric oxide) radical scavenging activity

The assay was done according to (Garrat, 1964; Marcocci *et al.*, 1994)

Cytotoxicity study on Human Red Blood Corpuscles (HRBCs) membrane stabilizing

The assay was done according to (Oyedapo *et al.*, 2010)

Routine blood analyses: Liver function tests, kidney function tests, cholesterol level and triglycerides level were measured using commercial kits.

Determination of TNF- α by enzyme linked immunosorbent assay (ELISA): Plasma TNF-α content was measured by using Duo set enzyme-linked immunoassay (ELISA) kits specific for rat TNF-α according to the manufacture's instructions (Bender, Vienna, Austria).

Semi-Quantitative analysis of TNF-α using reverse transcriptase polymerase chain reaction (PCR) technique

TNF-α RNA was isolated from white blood cells (WBCs) and the assay was done according to (Bustin, 2000); where TNF-α sense primer 5-CAGACCCTCACTCAGATCATCTT-3 and antisense primer 5-CAGAGCAATGACTCCAAAGTAGACCT-3 (~150 bp product).

Experimental design and statistical analyses

All data expressed as the mean ± standard deviation (SD). The differences were considered to be statistically significant at $p < 0.05$. Statistical analysis was performed using the unpaired Student's *t*-test and one-way analysis of variance (ANOVA) using primer of Biostatistics (Version 5) software program.

RESULTS AND DISCUSSION

Fraction	Concentration (mg/ml)	DPPH scavenging activity %	NO scavenging activity %	Antihemolytic effect %
Crude extract	1	12.357	6.548	0.001
	2	60.214	33.033	19.002
Water fraction	1	34.096	26.871	0.1
	2	43.992	37.744	14.795

Table 2: HPLC data for *Ulva lactuca* extract

Test	Crude extract	Water fraction of the crude extract
Chlorogenic acid (mg/l)	64.355	5.93e-1
Caffeic acid (mg/l)	50.52	3.86e-1
3,4-Dicaffeoyl quinic acid (mg/l)	34.75	ND
3,5-Dicaffeoyl quinic acid (mg/l)	110.12	ND
4,5-Dicaffeoylquinic acid (mg/l)	42.922	1.064
Gallic acid (mg/ml)	13.354	1.299
Rutin (mg/l)	3.557	ND
Tanic acid (mg/ml)	4.63	ND
Quercetin (mg/l)	1.1	9.25e-3
Retinol (µg/l)	258.623	315.84

Table 3: Effect of the orally administration of different *Ulva* water fraction doses on liver function parameters in mice sera of different experimental animal groups

Groups / Tests	AST	ALT	Total Protein	Albumin
Control	8.612 ± 0.583	29.98 ± 1.361	5.102 ± 0.346	1.105 ± 0.017
10mg/kg	7.89 ± 0.907	29.45 ± 4.477	10.44 ± 0.473 *	0.825 ± 0.092 *
100mg/kg	8.536 ± 0.52	22.03 ± 3.875 *	12.35 ± 0.625 *	1.024 ± 0.177
500mg/kg	8.044 ± 0.515	28.7 ± 1.65	10.73 ± 0.253 *	0.838 ± 0.097 *
1000mg/kg	8.104 ± 0.63	25.16 ± 3.937	10.7 ± 0.6 *	0.984 ± 0.189

* There was a significant difference with control group.

Table 4: Effect of the orally administration of different *Ulva* water fraction doses on kidney function parameters in mice sera of different experimental animal groups

Groups / Tests	Urea	Creatinine	Uric Acid
Control	81 ± 9.263	0.633 ± 0.082	6.124 ± 0.096
10mg/kg	81.11 ± 7.842	0.717 ± 0.041	6.005 ± 0.12
100mg/kg	65.85 ± 4.26 *	0.717 ± 0.117	5.98 ± 0.143
500mg/kg	55.7 ± 6.455 *	0.862 ± 0.133 *	6.225 ± 0.262
1000mg/kg	56.34 ± 7.991 *	0.9 ± 0.089 *	6.106 ± 0.526

* There was a significant difference with control group.

Table 5: Effect of the orally administration of different *Ulva* water fraction doses on lipids level in mice sera of different experimental animal groups

Groups / Tests	Total Cholesterol	Triglycerides
Control	75.83 ± 3.898	310.4 ± 3.289
10mg/kg	76.46 ± 5.632	305.1 ± 5.567
100mg/kg	78.12 ± 1.576	312.1 ± 10.87
500mg/kg	83.4 ± 3.627 *	317 ± 12.7
1000mg/kg	81.6 ± 4.341	366.1 ± 46.35 *

* There was a significant difference with control group.

Table 6: Tumor necrosis factor (TNF-α) level and gene expression fold

Groups / Tests	TNF-α (pg/ml)	TNF-α gene expression fold
Control	12830.8 ± 104.301	1.142 ± 0.009
10mg/kg	11201 ± 202.416 *	1.121 ± 0.003 *
100mg/kg	9201 ± 164.256 *	0.922 ± 0.004 *
500mg/kg	9000 ± 180.412 *	0.91 ± 0.001 *
1000mg/kg	13830.8 ± 150.341 *	1.2 ± 0.005 *

* There was a significant difference with control group.

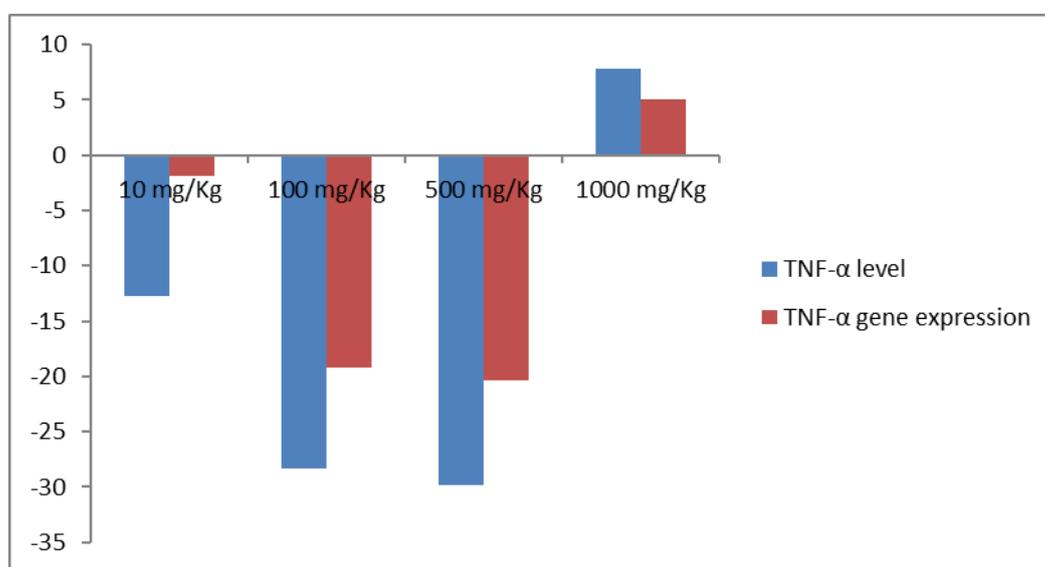


Fig. 1: The percent of increasing or decreasing of TNF-α level and gene expression in different treated groups

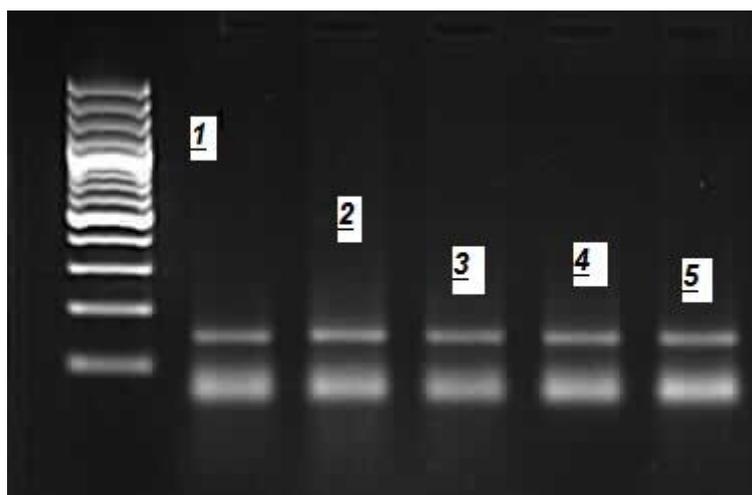


Fig. 2: TNF- α gene expression in different treated groups. 1st lane was the DNA ladder, lane no. 1 was control group, lane no. 2 was 10mg/kg group, lane no. 3 was 100mg/kg group, lane no. 4 was 500mg/kg group and lane no. 5 was 1000mg/kg group

We investigated the bioscreening activities for the water fraction of the methanolic *Ulva lactuca* extract, and our results provide that crude extract and its fractions had antioxidants, anti-inflammatory and anti hemolytic activities. These effects were concentration dependent where 2mg/ml of each one showed the more potent activity. For DPPH scavenging activity, the most powerful one was crude extract followed by water fraction (Table 1). While for NO scavenging activity, butanol was the most potent fraction followed by water one (Table 1). Finally for antihemolytic activity, crude extract was the most effective one followed by water fraction one (Table 1). Overall among tested fractions, water one was the most effective therefore it was chosen as a candidate for *in vivo* study. Since *Ulva* water fraction showed these amazing activities the next step was the determination of safe dose and the possible toxicity.

In LD₅₀ test, there was no mortality detected among mice through 15 days of injection with *Ulva* water fraction at different doses (10-1000 mg/kg). Therefore, we could not calculate the LD₅₀ and accounted it as safe agent as shown in tables 3, 4 and 5. But at dose 500 mg/kg and 1000 mg/kg it was noticed that the total protein concentration (table 3) was dramatically increased which

associated with decreased albumin level that could be due to formation of new protein like globulin (inflammatory protein) due to hepatitis as Thapa and Walia (2007) mentioned. Also urea level (table 4) significantly decreased compared to control due to impairment in urea cycle caused by hepatocyte inflammation. Also creatinine level (table 4) significantly increased compared to control which means that renal injury began to occur by increasing the dose. Also increasing *Ulva* water fraction dose accompanied by hypercholesterolemia and high triglycerides level (table 5). All together the chosen safe dose was 100 mg/Kg body weight.

It was clearly noticed that *Ulva* water fraction had therapeutic antioxidant and antiinflammatory effects since HPLC analysis revealed the presence of phenolic and flavonoid active constituents in high content e.g. gallic acid, quercetin and retinol (table 2) acting in synergism to increase its bioactivity such as antioxidant and antiinflammatory activities. Cook and Samman (1996), Aneta *et al.*, (2007) and Lui *et al.*, (2010) reported that phenolic and flavonoid compounds have high antioxidant and antiinflammatory capacities.

Also *Ulva* water fraction lightened the inflammation through decreasing the

proinflammatory cytokine TNF- α level and down regulation of its gene (table 6 and figure 1 and 2).

The results of this study suggest that *Ulva lactuca* and its water fraction had phenolic and flavonoid active constituents offered a potential use as antioxidant and antiinflammatory candidate to prevent the progression of many disorders. However, further detailed investigations are needed to isolate the phytoconstituents responsible for their and antiinflammatory actions.

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