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Ulva lactuca a natural occurring source of minerals to promote bone health and sustain athlete performance.

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Abstract

The purpose of this study was to identify if the components extracted from Ulva lactuca might have some positive effect in maintaining and improve osteoblast function. The fresh Ulva was lyophilized, and the extract was characterized before testing. The osteoblasts were cultured in standard conditions and the lyophilized extract was tested at 1mg/ml for up to 48 hrs. s markers of osteoblast function and proliferation BMP-6, RUNX-2 and Osteocalcin were analyzed on the mRNA extracted from the osteoblast. The results showed a significant increase, especially after 48 hrs in the production of osteocalcin mRNA which suggest a role for this extract in maintaining bone health. Although

preliminary these data indicate that the extract from Ulva can be used as supplements for human use especially in those conditions where minerals and antioxidants are more needed.

Keywords: ulva lactuca; bone health; BMP-6, RUNX-2, Osteocalcin

Introduction

Ulva lactuca is a green macroalgae involved in the devastating green tides observed worldwide. These green tides or blooms are a consequence of human activities. Ulva blooms primarily occur in shallow waters, and the decomposition of this algae can produce hazardous vapors. Ulva lactuca is a species that typically resembles lettuce, but genetic analyses have shown that other green algae with tubular phenotypes were clades of U. lactuca, although previously described as different species or even genera. The ability of U. lactuca to adopt different phenotypes may be due to environmental parameters, such as the water's salinity or symbiosis with bacteria. Ulva contains commercially valuable components, such as bioactive compounds, food, or biofuels (1). The biomass from this algae, collected annually on beaches, is beginning to be exploited to produce valuable compounds.

The Ganzirri Lake is part of the Capo Peloro lagoon, within the Capo Peloro Nature Reserve, and is a place of significant interest for its morphological, biological, and scenic characteristics. It consists of a complex ecosystem with a rich and varied flora and fauna, thanks to the continuous influx of seawater. In fact, two channels allow exchange with seawater and can be opened or closed to maintain oxygenation and prevent eutrophication, a degenerative process caused by an excessive increase in nutrients. A third channel connects Lake Ganzirri to Lake Faro, which, in turn, is connected to the Tyrrhenian Sea and the Ionian Sea. This geographical arrangement not only allows a constant circulation of seawater but has also led to the formation of a unique micro-ecosystem that enables certain plant and animal species to live exclusively here. Indeed, there is a rich vegetation, including spontaneous growth of many species of plants and algae (2-3). Among the most prevalent algae, we find Ulva lactuca, also known as "Sea Lettuce" due to its shape and texture: it is bright green, with lobed and ruffled leaves resembling lettuce but can reach up to 50 centimeters in length. It is a common green algae in the Mediterranean Sea, growing in brackish water rich in phosphates, which Ulva feeds on. It attaches to rocks or floats freely in shallow waters and research has shown that Ulva lactuca can help mitigate eutrophication by absorbing excess nutrients from the water (4). It is one of the most versatile algae in the fields of food, agriculture, pharmacology, and medicine. This algae contains micro and macronutrients and numerous bioactive compounds that can be extracted and utilized for the production of pharmaceuticals, food products, and cosmetics. It is rich, for example,

in vitamin B12, which plays a crucial role in the functions of the nervous system and the formation of blood cells. It is also rich in sodium, potassium, iodine, aluminum, manganese, and nickel and contains vitamin A, vitamin B1, vitamin C, soluble nitrogen, and phosphorus. It is characterized, above all, by the presence of calcium (3g/100g) and magnesium (2.8g/100g); it has a high percentage of proteins, fibers, sugar, and starch and is a source of carotenoids, chlorophyll, vitamins, essential and non-essential amino acids, at levels comparable to those found in foods such as eggs and legumes (5-6). Thanks to all these bioactive compounds, Ulva is an excellent supplement for vegetarian and vegan diet. Ulva lactuca is increasingly sought after for pharmaceutical and food applications, and there is a growing interest in research related to the extraction, purification, characterization, and food and biomedical application of Ulva lactuca components. For example, its polysaccharides can hydrate and provide elasticity to tissues, promoting research into Ulva lactuca-based hydrogel formulations for the treatment of burns or tissue regeneration (7-8).

The antioxidant, antibacterial, anti-inflammatory characteristics of Ulva lactuca are well-established (9-14), and lately, its beneficial properties on the skeletal system are under study. In fact, it is believed that this algae may have a preventive action against osteoporosis.

Specifically, osteoporosis is a skeletal disease characterized by a reduction in bone mineral density and a compromise of bone microarchitecture. This condition makes bones fragile and susceptible to fractures, which can occur even following minor traumas or without apparent cause. It is often considered a "silent disease" as it can progress without obvious symptoms until a fracture occurs. The treatment of osteoporosis is based on a combination of lifestyle modifications, diet, calcium and vitamin D supplementation, physical exercise, and medications.

Therefore, to assess the effectiveness of Ulva lactuca in improving bone health, we used osteoblasts treated with Ulva lactuca extract derivatives. Osteoblast cells, being involved in the formation of new bone tissue, express a series of genes during their activity that are essential for the synthesis and deposition of the bone extracellular matrix. Some of the key regulators of this process are: i) BMP6 (Bone Morphogenic Protein 6), which encodes a protein functioning as an essential growth factor for bone formation, repair, and regeneration; ii) RUNX2 (Runt-related transcription factor 2), which induces the formation of a protein crucial for osteoblast differentiation, collagen synthesis, mineral deposition, and the formation of an organized bone matrix; and iii) BGLAP (Osteocalcin), which promotes the synthesis of a protein involved in bone mineralization. Assessing the expression of genes such as BMP-6, RUNX2, and Osteocalcin in treated cells allows for the evaluation of Ulva lactuca extract in preventing osteoporosis.

Materials and Methods

To extract and characterize the compounds in U. Lactuca (Figure 1) several procedures for extraction and physicochemical characterization are required.

Sample collection and analysis of components

The samples of Ulva lactuca were collected from Lake Ganzirri and stored at -40°C, then lyophilized for about 72 hours until completely dehydrated. The lyophilization cycle consisted of four distinct phases:

1) The sample is frozen on refrigerated shelves.
2) The shelves are heated, and the ice is sublimated (primary drying), while the water vapor is condensed.
3) Unfrozen water remaining in the product is removed by a careful temperature increase of the shelves (secondary drying).
4) The condenser is heated to melt and remove the collected ice.

Through this process, 99.694 grams of lyophilized material were obtained, of which approximately 1 gram was treated with 20 mL of a mixture of methanol and water (70:30 v/v) and placed in an ultrasonic bath (50°C, 90 min, 40 kHz), then filtered and dried with a rotary evaporator. This methanolic extraction is a technique used to extract compounds or active principles from natural substances, using methanol as the extracting solvent. The extract, containing the desired compounds, undergoes further purification processes to obtain a concentrated and purified extract. The obtained extract had a yield of 22.8%.

Figure 1. A sample of Ulva Lactuca from Lake Ganzirri before extraction

At this point, the obtained extract was chemically determined:

- Dry matter: Determined according to the Association of Official Analytical Chemists (AOAC, 1990) indications.
- Protein content: Total proteins were determined using the Kjeldahl method and calculated using a nitrogen conversion factor of 6.25 (15). Data were expressed as a percentage of dry weight.
- Fat content: Lipids were extracted following the protocol described in the NF V 03-713 standard (16). 8 g of algal powder samples were hydrolyzed with a mixture of ethanol, formic acid, and 70% hydrochloric acid for 20 minutes at 75°C, followed by oily extraction using chloroform as a solvent. The sample was cooled under agitation, 16 mL of ethanol and 50 mL of chloroform were added, and the mixture was stirred for 20 minutes. The extraction procedure was repeated twice and dried with a rotary evaporator at 50°C (BUCHI R-210, Merck KGaA, Darmstadt, Germany). The result was expressed as a percentage of lipids in the dry matter of algal powder.
- Soluble sugars: Sugars were extracted with ethanol (960 mL/L) at 50°C for 30 minutes. After centrifugation, the supernatant was collected, and the sugar content was analyzed with the phenol/sulfuric acid reagent.
- Dietary fiber: Insoluble and soluble dietary fibers were determined according to the enzymatic-gravimetric method AOAC (17). Samples were gelatinized with heat-stable α-amylase (A-3306, Sigma Chemical Co., St. Louis, MO, USA) for 30 minutes in a boiling water bath. Then, they were enzymatically digested with protease (P-5380, Sigma Chemical Co., St. Louis, MO) (60°C, pH 7.5, 30 min) to solubilize proteins, followed by incubation with amyloglucosidase (A-9268, Sigma Chemical Co., Poole, Dorset, UK) (60°C, pH 4.5, 30 min) to remove starch. Subsequently, they were filtered, washed (with water, 95% ethanol, and acetone), dried, and weighed to determine insoluble fiber. Four volumes of absolute ethanol were added to the filtrate and water washes. Then, the precipitates were filtered and washed twice with 80% ethanol and acetone. Subsequently, the residues (soluble fiber) were dried and weighed. The obtained values were corrected for ash and proteins. Total dietary fiber was determined by summing insoluble and soluble dietary fiber.

Neutral and acid detergent fibers (NDF, ADF) and acid detergent lignin (ADL) were determined according to Van Soest, et al. (18).

Mineral elements: Mineral element analysis was performed by initial acid digestion with a microwave mineralizer, followed by inductively coupled plasma mass spectrometry (ICP-MS).

Fatty acid profile: The fatty acid profile was obtained by initial extraction with a Soxhlet apparatus and subsequent analysis with a gas chromatograph (GC) equipped with a split/splitless injector and flame ionization detector (FID).

Photosynthetic pigments: The profile of photosynthetic pigments in Ulva lactuca was determined using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system (Shimadzu, Kyoto, Japan), equipped with an SIL-20AXR autosampler, electrospray ionization (ESI) source, and Agilent Zorbax SB-C18 column (5 microm 4.6 x 250 mm). The multiple reaction monitoring (MRM) mode allowed for both quantitative and qualitative analysis.

Treatment of osteoblastic cells.

Ulva lactuca extract was used at various concentrations (from 0.1 to 10 mg/ml) to assess potential toxicity, and the dose of 1 mg/mL of the extract was chosen for treating the osteoblastic cells. The osteoblastic cells (MC3T3-L1) were cultured in DMEM medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin–streptomycin (Sigma–Aldrich, Milan, Italy) at 37°C in a humidified atmosphere containing 5% CO2. When reaching confluence, were treated in vitro with the extract for a total of 24h and 48h to evaluate the anti-osteoporotic capabilities of Ulva lactuca. At the end of 24h and 48h, the cells were collected, and RNA was extracted.

Total mRNA was extracted from osteoblasts using Trizol LS reagent (Thermo Fisher Scientific, Waltham, MA), as previously described.22 First-strand DNA (1 µL) was added to the BrightGreen qPCR Master Mix (Applied Biological Materials Inc., Richmond, Canada) in a total volume of 20 µL per well to evaluate gene expression of target genes: BMP6 (bone morphogenetic protein 6), RUNX2 (runt-related transcription factor 2), and osteocalcin, using specific mouse primers (Table 1). Samples were loaded in duplicate and GADPH was used as housekeeping gene; the reaction was performed using the two-step thermal protocol suggested by the manufacturer (Applied Biosystems, Foster City, California). Results were quantified using the $2^{\Delta\Delta Ct}$ method and expressed as n-fold increase of gene expression by using untreated osteoblasts as calibrator.

Table 1

GADPH	Primer F CTC ATG ACC ACA GTC CAT GC Primer R TTC AGC TCT GGG ATG ACC TT
Osteocalcin	Primer F AAG CAG GAG GGC AAT AAG GT Primer R TGC CAG AGT TTG GCT TTA GG
RUNX2	Primer F GCC GGG AAT GAT GAC AAC TA Primer R GGA CCG TCC ACT GTC ACT TT
BMP6	Primer F CTC TTC GGG CTT CCT CTA TC Primer R CCA ACA CCG ACA GGA TCT

Data and statistical analysis

All the results are expressed as mean ± SD. The reported values are the result of at least three experiments. All assays were performed in duplicate to ensure reproducibility. The differences between the groups were evaluated by one-way ANOVA with Tukey's post-test. A p-value less than 0.05 was considered significant. Graphs were prepared using GraphPad Prism Version 8.0 for macOS (GraphPad Software Inc., La Jolla, CA, USA).

Results

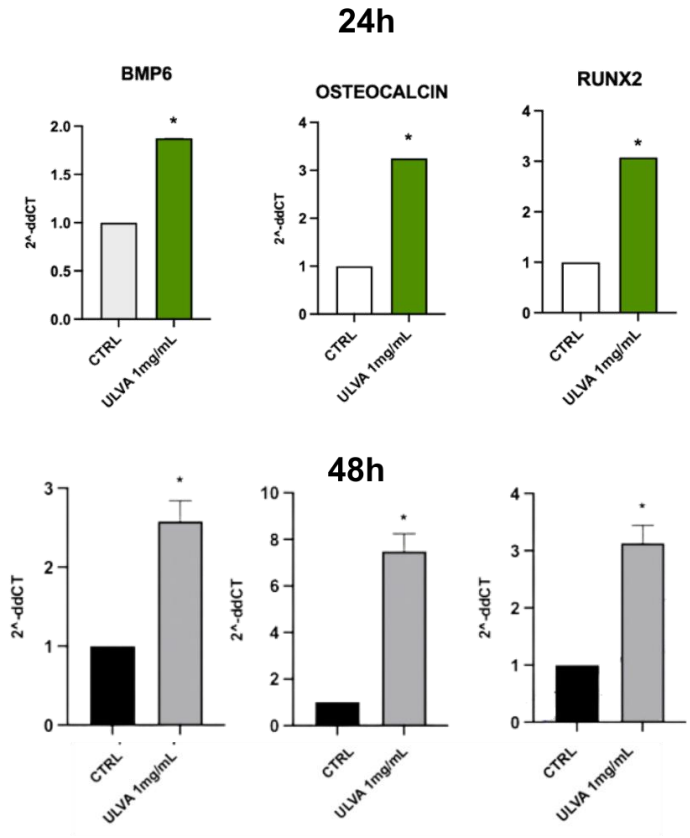
Characterization of the Ulva Lactuca extract

According to the above described methods it was possible to determine that the sample collected from Lake Ganzirri contained:

- Dry matter (85.05 g/100g)
- Total proteins (8.46 g/100g)
- Fats (7.87 g/100g), among which the most abundant fatty acids were oleic (15.93 g/100g) and palmitic (59.40 g/100g)
- Total fibers (54.9 g/100g)
- Mineral elements (magnesium, calcium, and potassium)
- Photosynthetic pigments (chlorophyll a and b, and α and β carotene)

Effects on osteoblast cells

The Ulva lactuca extract tested for 24 hours on osteoblast cells at the dose of 1mg/ml stimulated the expression of BMP6 ($p<0.05$ vs control), osteocalcin and RUNX2 ($p<0.01$ vs control). At 48 hours the same concentration of Ulva extract produced a further increase in the expression of BMP6 ($p<0.01$), osteocalcin and RUNX2 ($p<0.005$ vs control).



Conclusion

This preliminary study was interesting to demonstrate the simplicity and efficiency of the extraction process, the robust activity of the U. lactuca extracts in stimulating osteoblast and its high-value compound content. Given the concentration of proteins, fatty acids, minerals, and anti-oxidants, these extracts could potentially be used as additives or ingredients in cosmetic, pharmaceutical, and food products (19). Among food products those intended as supplements for sport performance could be formulated with the Ulva extract which contains all the important micro and macronutrients needed by athletes. The extract of Ulva obtained from Ganzirri Lake demonstrated a concentration of bioactive molecules similar to what already reported in other papers (20). Considering that the maximum of the effect on osteoblasts was observed on Osteocalcin which binds to calcium and integrates it into the bone matrix, facilitating bone mineralization and ensuring bone strength and density, the effects of this extract can be useful to maintain physical performances.

Ulva lactuca is a remarkable example of nature's bounty, offering numerous health, environmental, and industrial benefits. Its resilience and adaptability make it a valuable resource in various sectors. The growing body of research underscores its potential and highlights the need for sustainable harvesting practices to ensure its availability for future generations.

The potential uses demonstrated in this small scale experiment are of great remark since the re-use of a biomass provides a benefit for the environment and the components derived from the alga are a free and renewable source of nutrients for human use.

Declarations

Conflict of Interest

AB and NI are co-funders of SunNutraPharma srl a spin-off company of the University of Messina

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Author Contributions

GF and AB conceived and wrote the paper
RL and LDM produced data
NI and NC revised and analyzed data

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