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Original Research

# Fungal Laccase-Mediated Enhancement of the Bioactivity of Green Algae Extracts

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**Abstract**

Biomass derived from green marine macroalgae *Ulva* sp. is considered a rich source of bioactive compounds. In the present study, we demonstrate the increased bioactivity of a phenolic-rich extract derived from green marine macroalgae *Ulva intestinalis* after its

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treatment with fungal laccases from *Trametes versicolor* and *Agaricus bisporus*. The phenolic composition of the extract, before and after its enzymatic treatment, was determined through several analytical methods. Furthermore, the antioxidant, enzyme-inhibitory, antimicrobial and cytotoxic activity of enzymatically modified and non-modified extracts was comparatively investigated. Depending on the laccase used, the enzyme-modified extracts exhibited different phenolic content and enhanced bioactivities compared to the non-treated ones. The enzymatically modified extracts presented enhanced antimicrobial, anti-lipoxygenase and anti-collagenase activities, and mild cytotoxicity. These results indicate that the proposed biocatalytic process could be applied to produce green macroalgal natural extracts with enhanced bioactivities paving the way for their implementation in the food, pharmaceutical, and cosmeceutical industries.

**Keywords**

*Ulva intestinalis*; macroalgae; fungal laccases; phenolic compounds; biological activities

## 1. Introduction

Marine organisms, especially macroalgae, constitute a renowned source of bioactive compounds [1]. Among marine macroalgae, *Ulva* species, green seaweeds belonging to the order Ulvales (Chlorophyta), have recently attracted scientific interest [2, 3]. Specifically, *Ulva intestinalis*, a bright grass-green seaweed, rich in polysaccharides, proteins, minerals, and other bioactive substances, such as phenolics has gained increasing attention over the last years [4, 5]. These species produce a wide variety of secondary metabolites with a broad array of bioactivities, including antitumoral, antiviral, antifungal, antibacterial, antiaging, cytotoxic and antiproliferative activity [6, 7]. For example, a protein fraction from *Ulva intestinalis* induced a remarkable increase in collagen and hyaluronic acid production per cell and reduced cell proliferation without increasing cell mortality [8]. Although these species are yet much underexploited, the remarkable potential arising from their exploitation approves their compelling scientific interest, especially in the pharmaceutical and food industries [7, 9].

Green marine algae as well as their naturally derived extracts, exhibit numerous health benefits such as antioxidant, antimicrobial, anti-inflammatory, cytotoxic, neuroprotective, immunomodulatory and cardioprotective, principally attributed to their content in various bioactive components, including polyphenols and non-polyphenolic compounds, such as carotenoids, terpenoids, and alkaloids [10]. Different classes of polyphenols at varying concentrations have been detected in all green algae such as hydroxybenzoic acid and hydroxycinnamic acid derivatives, flavonoids, and bromophenols [11-13]. Thus, a profile of phenolic acids, flavonoids, and phlorotannins (compounds consisting of highly polymerized phloroglucinol units) is typical for *Ulva* sp. Species [14, 15]. A strong correlation between the phenolic and flavonoid content and the antibacterial [16], antioxidant [17], anti-inflammatory and anticancer activity [18] has already been reported for many *Ulva* species. Despite the structural diversity and the variability of the phenolic compounds in these species [19], their potential biological properties establish them as an attractive group for their implementation in medicine [20], as dietary supplements [21], nutraceuticals [22],

or functional foods [23], or in cosmetics.

Phenolic compounds typically comprise one (phenolic acids) or more (polyphenols) aromatic rings with attached hydroxyl groups in their structures. Their biological actions are strongly attributed to their chemical structure [24]. For example, their antioxidant capacities have been related to their hydroxyl groups and phenolic rings [25]. Thus, their chemical structure modifications could alter their bioactivity, either increasing or reducing [26]. A common approach to induce specific changes in the chemical structure of certain compounds, e.g., the proximity of their aromatic rings, relies on enzymatic catalysis [27]. Biocatalysis has emerged as a promising strategy for synthesizing novel bioactive compounds, including flavonoids, hydroxyl cinnamic acid derivatives and phenolics through the targeted modification of natural compounds. Until today, a wide variety of biologically active ingredients, presenting biological activities usually superior to their precursors has been obtained through attractive biotechnological processes [28, 29]. Various classes of enzymes have been applied for the efficient semi-synthesis of diverse biologically active derivatives [30-33] such as the oxidation of naturally occurring phenols by fungal laccases to synthesize fine organic chemicals [33].

Laccase-catalyzed processes constitute a tempting green approach regarding sustainable chemistry due to their incredible potential to act as robust catalysts under mild reaction conditions [34, 35]. Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are copper atom-containing oxidoreductases that catalyze the one single electron oxidation of different phenolic structure- or aniline structure- containing compounds with simultaneous reduction of oxygen to water [36, 37]. Thus, phenolic compounds constitute typical substrates of laccases for the biosynthesis of polyphenols [34]. Laccase-catalyzed oxidation of phenols renders phenoxyl free radicals that in following step lead to coupling-based polymerization or radical rearrangement per se, yielding dead-end products. Laccases have also been exploited in the oxidation of non-laccase substrates acting as mediators and in the production of heteromolecular products with non-laccase substrates [38, 39].

Furthermore, the laccase-catalyzed oxidation of naturally occurring phenolics (including rutin, catechin, ferulic acid and tannic acid), has been proven to produce oligomer products with superior bioactivities (such as antioxidant, antimicrobial, antiaging, anti-inflammatory) compared to their precursor compounds [29, 33]. Additionally, polyphenol-enriched extracts from natural sources (e.g., propolis extracts) have been enzymatically modified with laccases, typically leading to enhanced biological activities [40].

It is worth noting that there are various studies, concerning the enzymatic treatment of extracts derived from green, brown, or red macroalgae such *as Ulva lactuca, Ulva pertusa, Turbinaria ornate, Porphyra dioica* and *Pterocladia capillacea* aiming at the enhancement of their biological activities. The reported studies are often relevant to exploiting hydrolases and lyases for the degradation of polysaccharide- or protein-rich macroalgal extracts to lead to low molecular products with potential enhanced biological activities such as antioxidant, antitumor or antivirus [41-45]. However, there is no previous study regarding the usage of laccases for treating macroalgal phenolic-rich extracts.

The aim of the present study, was to investigate the effect of fungal laccases from *Trametes versicolor* (TvL) and *Agaricus bisporus* (AbL) on both the phenolic content and the bioactivity of phenolic-enriched extracts from green macroalgae *Ulva intestinalis*. Spectrophotometric and chromatographic methods were applied to confirm the successful enzymatic oxidation of the phenolic compounds found in the extracts. The efficiency and specificity of both laccases for the

oxidation of specific phenolic compounds were investigated. Finally, the starting and the enzymatically modified extracts were evaluated for their biological activities, such as antioxidant activity, inhibitory activity against enzymes related to skin aging, antibacterial activity, and cytotoxicity against fibroblast cell line.

## 2. Materials and Methods

### *2.1 Materials*

Ethanol (70% v/v) for extraction, laccase from *Trametes versicolor* (TvL) (0.7 U $mg^{-1}$), laccase from *Agaricus bisporus* (AbL) (>4 U $mg^{-1}$), elastase from porcine pancreas Type I (>4 U $mg^{-1}$), collagenase from *Clostridium histolyticum* Type IA (>125 CDU $mg^{-1}$), lipase from *Candida rugosa* Type VII (724 U $mg^{-1}$), *N*-[3-(2-furyl) acryloyl]-Leu-Gly-Pro-Ala (FALGPA), *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide, *p*-nitrophenyl butyrate (*p*-NPB), caffeic acid (3,4-dihydroxycinnamic acid), N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA + 1% TMCS, analytical standard) and Dulbecco's modified eagle medium (DMEM) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) was purchased from Superlco (Pennsylvania, USA). Iron trichloride hexahydrate ($FeCl_3 \bullet 6H_2O$) was purchased from Thermo Fisher Scientific (Waltham, USA). Lipoxygenase from *Glycine max* (soybean) Type I-B (205716 U $mg^{-1}$) and linoleic acid were obtained from Merck (KGaA Darmstadt, Germany). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate ($Na_2CO_3$) and dimethyl sulfoxide (DMSO) were obtained from Riedel-de Haën (Seelze, Germany). Folin–Ciocalteu and phenol reagent were obtained from Fluka (Switzerland). Luria Bertani Broth (LB Broth) (Lennox) was purchased from Lab M Ltd (Heywood, UK). L-glutamine and penicillin/streptomycin were obtained from Gibco-ThermoFisher (Waltham, USA). Trypsin/EDTA solution and fetal bovine serum (FBS) were purchased from Biochrom AG (Berlin, Germany). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was obtained from TCI (Tokyo, Japan). Thiazolyl blue tetrazolium bromide (MTT) was obtained from Biosynth (Staadt, Switzerland). All the other chemicals and reagents were of analytical grade and procured from reliable sources. Double distilled water was used to prepare all the buffers and solutions.

### *2.2 Methods*

#### 2.2.1 Collection of *Ulva* Seaweed

Fresh seaweeds (*Ulva intestinalis*) were collected from the southeastern tip of Ammoudia beach, Acheron, Preveza, Greece (39° 14'10, 464'' N, 20° 28'38, 172'' E) during the spring of 2019 (March, 2019) (Figure 1) and seaweed identification was performed according to standard taxonomic and literature keys by the Phytobenthos Laboratory at the Institute of Oceanography of the Hellenic Centre for Marine Research [46, 47]. *Ulva* biomass was rinsed thoroughly with tap and deionized water to remove sand, salts and epiphytes and then was air-dried under shade at room temperature for a few days. Finally, dried biomass was ground into flakes and stored in plastic bags, in a dark and dry place, until further use.

**Figure 1** Photographs from the *Ulva* seaweed collection point.

2.2.2 Preparation of the Ethanolic Extract

A 70% (v/v) ethanolic extract was prepared according to Sirbu et al. [48] with slight modifications. Briefly, 5 grams of dry powdered biomass were extracted with 100 mL of 70% ethanol (v/v) overnight (ON) at room temperature under gentle agitation. The suspension was filtered, the resulting filtrate was collected while the algae biomass was re-extracted under the same conditions. Accordingly, the solvent was removed through both rotary evaporation and lyophilization. Finally, the resulting extract was stored at -20°C until further use.

2.2.3 Determination of Total Phenolic Content

The total phenolic content of the extract was determined according to Folin-Ciocalteu as described by Chatzikonstantinou et al. [49] with slight modifications. Briefly, 60 μL of an initial extract stock solution, diluted in DMSO, were mixed with 790 μL of water (at a final concentration of 300 μg $mL^{-1}$), followed by the addition of 50 μL of Folin-Ciocalteu reagent. Then, the mixtures were incubated for three minutes at room temperature and 100 μL of 20% (w/v) sodium carbonate ($Na_2CO_3$) solution were added. The final mixtures were placed in the dark for 1 h at room temperature. Blank samples were also prepared using 60 μL of DMSO instead of the extract. After 1 h, the absorbance of the samples was measured at 725 nm. The results were expressed as milligrams of caffeic acid equivalents per gram of dry extract according to a calibration curve of caffeic acid, prepared under the same conditions in the concentration range of 0-10 μg $mL^{-1}$. All experiments were performed in duplicate.

2.2.4 Enzymatic Modification of the Organic Extract

The enzymatic modification of the ethanolic extract with TvL and AbL was performed in acetate buffer, 15 mM, pH 5. The final concentrations of the extract and the enzyme were 5 mg $mL^{-1}$ and 0.2 U $mL^{-1}$, respectively. Then, the mixtures were incubated overnight at 30°C under shaking at 750 rpm. After the overnight incubation, the samples were lyophilized and stored at -20°C until further

use. One Unit of laccase activity was defined as the amount of the enzyme required to convert one μmole of catechol per minute at pH 5 and 30°C.

### 2.2.5 Characterization of the Starting and the Modified Extracts

The laccase-treated extracts were assessed for their polyphenolic content through the Folin-Ciocalteu assay described in Section 2.2.3.

Ultraviolet-Visible (UV-Vis) Spectroscopy. The changes in the UV-Vis spectra before and after the enzymatic treatment of the extract with both laccases were monitored through Ultraviolet-Visible (UV-Vis) spectroscopy. The UV–Vis spectra were obtained in the range of 200–800 nm on an Agilent Cary 60 UV–Vis spectrophotometer (Agilent, Santa Clara, CA, USA) in a quartz cell with a path length of 1 cm. The concentration of the extract was 0.5 mg $mL^{-1}$.

High Performance Liquid Chromatography (HPLC). According to previous work, both starting and laccase-treated extracts were analyzed through HPLC [50]. Specifically, 10 mg of each sample were diluted in 80-20% (v/v) water-acetonitrile solution and filtered with 0.22 μm filters. The samples were analyzed through HPLC (Shimadzu, Tokyo, Japan) using a Kinetex Evo C18 column, particle size 5 μm, length 250 mm, diameter 4.6 mm, and a diode array UV detector. The mobile phase consisted of acetonitrile (A) and 0.1% (v/v) acetic acid in water (B) with a gradient elution of 10% for solvent A and 90% for solvent B at 0–5 min, 16% for solvent A and 84% for solvent B at 5–18 min, 18% for solvent A and 82% for solvent B at 18–26 min, 28% for solvent A and 72% for solvent B at 26–31 min, 40% for solvent A and 60% for solvent B at 31–32 min, 40% for solvent A and 60% for solvent B at 32–40 min and 10% for solvent A and 90% for solvent B at 40–43 min. The elution conditions were performed at 30°C with a flow rate of 1 mL $min^{-1}$ and the injection volume was 20 μL. HPLC chromatograms were recorded at 280 and 330 nm. External standards were used for the identification of the phenolic compounds contained in the extracts.

Liquid Chromatography-Mass Spectroscopy (LC-MS). Before and after its enzymatic treatment, the extract was analyzed through LC-MS [15]. Specifically, the samples were diluted in 70-30% (v/v) ethanol-water at a final concentration of 7 mg $mL^{-1}$ and filtered with 0.45 μm filters. Analyses were performed on a Liquid Chromatograph-Mass Spectrometer (LC-MS) Advion equipment using a SVEA C18 column, particle size 5 μm, length 150 mm, diameter 4.6 mm from Nanologica with a single quadrupole mass analyzer. The mobile phase consisted of 0.1% (v/v) acetic acid in acetonitrile (A) and 0.1% (v/v) acetic acid in water with a gradient elution of 95% for solvent A and 5% for solvent B at 0-1 min, 100% for solvent B at 1-11 min, 100% for solvent B at 11-15 min, 95% for solvent A and 5% for solvent B at 15-17 min and 95% for solvent A and 5% for solvent B at 17-30 min with a flow rate of 0.3 mL $min^{-1}$. The injection volume was 20 μL. A positive ion source was employed, ESI ( + ), and the parameters were set as follows: capillary voltage at 200°C and 180 V, source voltage offset at 25 V, source voltage span at 20 V, ESI gas temperature at 350°C, ESI voltage at 3500 V, corona discharge at 5 μA. Different external standards were exploited to detect the phenolic compounds contained in the extracts. All the spectra were recorded in the range of 100-1000 m/z.

Gas Chromatography-Mass Spectroscopy (GC-MS). For the GC-MS analysis, both starting and modified extracts (10 mg) were treated with 100 μL of BSTFA + 1% TMCS. The samples were

analyzed according to Wezgowiec et al. [51] with modifications. More specifically, the extracts were incubated at 75°C for 4 h with the derivatization agent. The derivatized samples were diluted with n-hexane before the analysis (1:2 v/v). A volume of 1 µL was used for GC/MS injection. Analysis was performed on a GC/MS system (GCMS-QP2010 SE, Shimadzu, Tokyo, Japan), equipped with a split/splitless injector and a simple quadrupole mass spectrometer operating in electronic ionization (EI) mode (70 eV). A MEGA-5 MS capillary column (30 m length × 0.25 mm internal diameter × 0.25 µm film thickness; MEGA, Legnano, Italy) was used. The oven was programmed from 100°C (hold 1 min) to 190°C with a heating rate of 2°C $min^{-1}$, and to a final temperature of 290°C with a heating rate of 5°C $min^{-1}$ (hold 5 min). The carrier gas was helium set to flow at 0.92 mL $min^{-1}$ and a linear velocity of 35.7 cm $sec^{-1}$. The injector was operated in split mode (1:10) at 280°C. The mass spectrometer was set at the following conditions: ion source temperature at 200°C and interface (transfer-line temperature) at 250°C. The mass spectrometer was operated in full scan mode (m/z 40-1050). The identification of specific compounds was based on the NIST library (NIST 17) and the retention times of standard compounds.

#### 2.2.6 Enzymatic Modification of Individual Phenolic Compounds Detected in Starting Extract

Enzymatic Modification of Individual Phenolic Compounds for UV-Vis Analysis. The enzymatic modification of the individual phenolic compounds with TvL and AbL was performed in acetate buffer, 15 mM, pH 5. The final concentrations of the compound and enzyme in the reaction mixture were 0.2 mM and 0.02 U $mL^{-1}$, respectively. The enzymatic modification of apigenin and luteolin was performed in 10-30% (v/v) methanol reaction solution. The mixtures were incubated at 30°C under shaking at 750 rpm and the UV-Vis spectra of the reactions were monitored periodically. One Unit of laccase activity was defined as the amount of the enzyme required to convert one µmole of catechol per minute at pH 5 and 30°C.

Enzymatic Modification of Individual Phenolic Compounds for HPLC Analysis. Enzymatic modification of individual phenolic compounds with TvL and AbL was performed in acetate buffer, 15 mM, pH 5 and at a final compound and enzyme concentration at 5 mM and 0.2 U $mL^{-1}$, correspondingly. In the case of apigenin and luteolin, the reaction consisted of 50% (v/v) methanol. The mixtures were incubated under shaking at 750 rpm and 30°C and sampling was performed periodically. One Unit of laccase activity was defined as the amount of the enzyme required to convert one µmole of catechol per minute at pH 5 and 30°C.

#### 2.2.7 Characterization of the Individual Phenolic Compounds and Their Laccase-Derived Products

Ultraviolet–Visible (UV-Vis) Spectroscopy. The monitoring of the changes in the UV-Vis spectra during the enzymatic treatment of the individual phenolic compounds with both laccases was performed with Ultraviolet-Visible (UV-Vis) spectroscopy. For this purpose, UV–Vis spectra were obtained in the range of 200–800 nm on an Agilent Cary 60 UV–Vis spectrophotometer (Agilent, Santa Clara, CA, USA) in a quartz cell with a path length of 1 cm at regular time intervals. The final concentration of the non-modified or modified compounds was 0.2 mM.

High Performance Liquid Chromatography (HPLC). The aliquots of the reaction mixtures of the individual phenolic compounds treated with laccases were further analyzed through HPLC according

to Zwane et al. [52] with modifications. More specifically, 125 µL of reaction mixtures were diluted with acetonitrile (1:1), and centrifuged at 4000 rpm for 1 min and the supernatants were collected and filtered with 0.45 µm filters. The samples were analyzed through HPLC (Shimadzu, Tokyo, Japan) using a Bondapack C18 column, particle size 10 µm, length 300 mm, diameter 3.9 mm, and a diode array UV detector. The mobile phase consisted of acetonitrile (A) and 0.1% (v/v) acetic acid in water (B) with a gradient elution of 5% for solvent A and 95% for solvent B at 0–2 min, 80% for solvent A and 20% for solvent B at 2–25 min, 100% for solvent A at 25–30 min and 5% for solvent A and 95% for solvent B at 30–40 min. The elution conditions were performed at 30°C with a flow rate of 1 mL $min^{-1}$ and an injection volume of 20 µL. All the reactions were monitored at different wavelengths ranging from 230 to 350 nm.

2.2.8 Assessment of the Biological Activity of the Extracts

Determination of antioxidant activity. Two methods determined the antioxidant activity of the extracts; namely, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power assays (FRAP), following Chatzikonstantinou et al. [49] and Sharma and Pal Vig, [53] respectively, with slight modifications. More specifically, for the determination of DPPH radical scavenging activity of starting and laccase-treated *Ulva* extracts, 200 µL from initial extract stock solutions, diluted in methanol, were mixed with 100 µL of 1 mM methanolic DPPH solution in a total volume of 1 mL (the final concentration of the extract was 1 mg $mL^{-1}$). Blank samples were also prepared by adding methanol instead of DPPH and a control sample consisting of methanol instead of the sample. The mixtures were left at room temperature for 30 min. After 30 min, the absorbance of each sample was measured at 517 nm. Also, a calibration curve was prepared under the same conditions using Trolox as a standard compound in the concentration range of 0-7.5 µg $mL^{-1}$. The results were expressed as milligrams of Trolox equivalents per gram of dry extract. All experiments were performed in duplicate.

Concerning the FRAP assay, a fresh FRAP solution consisting of 10-1-1 of 300 mM acetate buffer pH 3.6, 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3$ $6H_2O$ solution, respectively, was prepared. 1.14 mL FRAP solution and 60 µL from different extract solutions, diluted in methanol, (at a final concentration of 133.3 µg $mL^{-1}$), were mixed and incubated for 10 min at 37°C. A control solution was also prepared to contain methanol instead of the sample and a blank solution consisting of 1.14 mL acetate buffer and 60 µL of the sample. After 10 min, the absorbances were measured at 593 nm. The results were expressed as milligrams of Trolox equivalents per gram of dry extract according to a calibration curve of Trolox, prepared under the same conditions in the concentration range of 0-7 µg $mL^{-1}$. All experiments were performed in duplicate.

Determination of antimicrobial activity. The antibacterial activity of the starting and enzymatically modified *Ulva* extracts was estimated according to Chatzikonstantinou et al. [49] with slight adaptations. More specifically, *Ulva* extracts were tested against *Escherichia coli* BL21 (*E.coli*) and *Corynebacterium glutamicum* ATCC 21253 *(C. glutamicum*). Initially, bacterial cells were grown overnight in LB broth medium, under shaking at 37°C. After overnight incubation, 25 µL of exponential phase cells (~$10^7$ CFU $mL^{-1}$) and 25 µL of different *Ulva* solutions diluted in 0.9% w/v NaCl, in the concentration range of 2.5-50 mg $mL^{-1}$, were transferred to 96-well microwell plates, containing 200 µL fresh LB broth medium. A control was also prepared by adding 25 µL of 0.9% w/v

NaCl instead of the sample. Then, the plates were incubated at 37°C for eight hours under shaking and their absorbance was measured at 600 nm at one-hour intervals. Finally, a growth curve of the bacterial population was prepared according to the $OD_{600}$ values, and the results were expressed as $IC_{50}$ (fitting a non-linear dose-dependent four-parameter curve) which is defined as the concentration required to reduce the growth of the bacterial population about 50%, without pre-incubation of the microorganisms with potential inhibitors. All measurements were performed in triplicate.

Determination of lipoxygenase inhibitory activity. Lipoxygenase inhibitory activity was determined according to Papadopoulou et al. [54] with adaptations. Briefly, the reaction mixture consisted of phosphate buffer 15 mM pH 6.8 with 1% (v/v) Tween 20, 100 μg $mL^{-1}$ lipoxygenase, 0.09 mM linoleic acid and 0.5 mg $mL^{-1}$ extract in a final total volume of 200 μL. The lipoxygenase inhibitory activity of the starting and modified extracts was determined based on the following protocol. Briefly, 20 μL of lipoxygenase and 20 μL of each sample were mixed with buffer and pre-incubated for 10 min at 37°C in a 96-well microwell plate. After pre-incubation, 18 μL of the substrate were added and the absorbance at 234 nm was measured instantly at one-minute intervals for 5 min in a microplate spectrophotometer (Multiskan spectrum, Thermo Fisher Scientific, Waltham, USA) to determine the initial velocity of enzyme in the presence of the sample ($V_{sample}$). A negative control was also prepared to contain buffer instead of the extract ($V_{negative\ control}$) and blank solutions containing extract with enzyme or substrate solution. Hydroxytyrosol was used as a positive control. All experiments were performed in duplicate. Inhibitory activity was calculated according to the following formula (*):

$$\text{Inhibitory activity (\%)} = (1 - V_{sample}/V_{negative\ control}) * 100 \quad (*)$$

Determination of collagenase inhibitory activity. Collagenase inhibitory activity was measured according to Thring et al. [55] with adaptations. Briefly, the reaction mixture consisted of tricine buffer 50 mM pH 7.5 with 400 mM NaCl and 10 mM $CaCl_2$, 40 μg $mL^{-1}$ collagenase, 0.25 mM *N*-[3-(2-furyl) acryloyl]-Leu-Gly-Pro-Ala (FALGPA) and 1 mg $mL^{-1}$ extract in a final total volume of 200 μL. To determine collagenase inhibitory activity of the starting and modified extracts, 16 μL of collagenase and 40 μL of each sample were mixed with buffer and pre-incubated at 37°C for 10 min in a 96-well microwell plate. After pre-incubation, 20 μL of the substrate were added and the absorbance was measured at 340 nm instantly at thirty-second intervals for 5 min in a microplate spectrophotometer (Multiskan spectrum, Thermo Fisher Scientific, Waltham, USA) to determine the initial velocity of enzyme in the presence of the sample ($V_{sample}$). A negative control was also prepared by adding buffer instead of extract ($V_{negative\ control}$) and blank solutions containing extract with enzyme or substrate. Catechin was used as a positive control. All experiments were performed in duplicate and inhibitory activity was calculated.

Determination of elastase inhibitory activity. Elastase inhibitory activity was determined according to Thring et al. [55] with adaptations. Briefly, the reaction consisted of tris-HCl buffer 25 mM pH 8, 3.25 μg $mL^{-1}$ elastase, 0.75 mM *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide and 1 mg $mL^{-1}$ extract in a total volume of 200 μL. To determine the elastase inhibitory activity of the starting and modified extracts, 15 μL of elastase and 40 μL of each sample were mixed with buffer and pre-incubated for 10 min at 37°C in a 96-well microwell plate. After a pre-incubation period, 60 μL of the substrate

were added and the absorbance at 405 nm was measured immediately and at one-minute intervals for 5 min in a microplate spectrophotometer (Multiskan spectrum, Thermo Fisher Scientific, Waltham, USA) to determine the initial velocity of enzyme in the presence of the sample ($V_{sample}$). A negative control was also prepared by adding buffer instead of extract ($V_{negative\ control}$) and blanks containing the extract solution with enzyme or substrate solution. Quercetin was used as a positive control. All experiments were performed in duplicate and inhibitory activity was calculated.

Determination of lipase inhibitory activity. Lipase inhibitory activity was measured according to Gatto et al. [56] with adaptations. Briefly, the reaction mixture consisted of phosphate buffer 15 mM pH 7, 3.75 μg $mL^{-1}$ lipase, 0.25 mM *p*-NPB and 1 mg $mL^{-1}$ extract in a total volume of 200 μL. To determine the lipase inhibitory activity of the starting and modified extracts, 30 μL of lipase and 40 μL of each sample were mixed with buffer and pre-incubated for 10 min at 25°C in a 96-well microwell plate. After pre-incubation, 20 μL of the substrate (diluted in DMSO) were added and the absorbance was measured instantly at 405 nm at one-minute intervals for 5 min in a microplate spectrophotometer (Multiskan spectrum, Thermo Fisher Scientific, Waltham, USA) to determine the initial velocity of enzyme in the presence of the sample ($V_{sample}$). A negative control was also prepared by adding buffer instead of extract ($V_{negative\ control}$) and blank solutions containing extract with enzyme or substrate. All experiments were performed in duplicate. Orlistat was used as a positive control and inhibitory activity was calculated.

Determination of cell viability. Albino Swiss mouse embryo fibroblasts (NIH/3T3, ATCC CRL-1658) were used in this study. Cells were grown in high glucose (4.5 mg $L^{-1}$) Dulbecco's Modified Eagle Medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, and 100 units $mL^{-1}$ of penicillin and 100 μg $mL^{-1}$ streptomycin in a humidified incubator (5% CO2, 95% air) at 37°C. Cells were plated for 24 h in 96-well plates at a density of $5\times10^3$ cells per well before the treatment for 24 h and 48 h with various concentrations (20–500 μg $mL^{-1}$) of the extracts. 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) assay (stock solution of 3 mg $mL^{-1}$) was added to each well for 3 h. Absorbance was determined at 570 nm (background measurements at 690 nm were subtracted) using a microplate spectrophotometer (Multiskan spectrum, Thermo Fisher Scientific, Waltham, USA). All experiments were carried out in triplicate.

## 3. Results

### *3.1 Determination of Phenolic Profile of the Ulva Intestinalis Extract*

Various green marine macroalgae possess an appreciable content and an interesting profile of phenolic compounds which exert significant bioactivities rendering them useful for various applications [14, 48]. To investigate the phenolic profile of *Ulva intestinalis*, the phenolic compounds were extracted with 70% (v/v) ethanol as described in section 2.2.2. In the present extract, 12 milligrams of caffeic acid equivalents per gram of dry extract were recovered as similarly reported in other studies [14, 17, 57].

A combination of HPLC, LC-MS and GC-MS analytical methods was employed to characterize the main phenolic compounds in *Ulva*'s starting extract. Additionally, the UV-Vis spectrum of the ethanolic extract was recorded. In the case of HPLC analysis, the characteristic phenolic peaks were identified by comparing their retention times and UV spectra with those of external phenolic

standards. In the case of GC-MS analysis, the chemical profile was analyzed according to the NIST library and external phenolic standards. In contrast, LC-MS analysis was based on external phenolic standards.

Interestingly, ten compounds were identified in the ethanolic extract (Table 1), which are classified under three representative classes of phenolic compounds including simple phenols (tyrosol), phenolic acids (caffeic acid, gallic acid, ferulic acid, syringic acid, *p*-coumaric acid and *p*-hydroxybenzoic acid) and flavonoids (catechin, apigenin and luteolin), accordingly to previous studies [14, 15, 48]. It is worth to be mentioned that the detected phenolic compounds have been previously reported to be included in extracts of *Ulva* species as well as in extracts derived from other green, red and brown macroalgae such as *Caulerpa taxifolia, Udotea indica, Fucus vesiculosus, Sargassum muticum, Palisada perforata, Botryocladia leptopoda* indicating that they are widely distributed among the macroalgae [48, 58-60]. Additionally, multiple peaks were detected on the UV-Vis spectrum of the extract indicating the presence of compounds of different nature (Figure S2).

**Table 1** Detection of different phenolic compounds in the starting extract through HPLC, GC-MS and LC-MS analyses*. The compounds in GC-MS analysis were identified as Trimethylsilyl (TMS) derivatives.

| | **HPLC** | **GC-MS** | **LC-MS** |
|---|---|---|---|
| **Standard compound** | Rt (min) | Rt (min) and major fragments (m/z) | Rt (min) and m/z |
| Caffeic acid[a,b,c] | 11.21 | 50.291/[73,219,396] | 15.96/[181.2] |
| Gallic acid[a,b] | 3.30 | 43.050/[73,281,458] | 16.70/[171.1] |
| Ferulic acid[a,b,c] | 20.72 | 48.880/[338,323,308] | 8.85/[195.2] |
| *p*-Coumaric acid[a,b] | 17.84 | 42.107/[73,293,219] | - |
| Syringic acid[a,b,c] | 11.74 | 40.401/[327,312,297] | 8.70/[199.2] |
| *p*-Hydroxybenzoic acid[a,b] | 8.74 | 7.975/[267,223,73] | - |
| Catechin[a,b,c] | 8.43 | 64.203/[73,368,369] | 13.39/[291.3] |
| Apigenin[a,c] | 36.82 | - | 16.87/[271] |
| Luteolin[a,c] | 35.41 | - | 16.47/[287.2] |
| Tyrosol[a,b] | 7.49 | 23.283/[179,73,282] | - |

*a, b and c stand for detection through HPLC, GC-MS and LC-MS, correspondingly, while Rt stands for Retention time.

### *3.2 Enzymatic Modification of the Ulva Extract-Determination of Total Phenolic Content and Characterization of the Phenolic Profile of the Enzyme-Treated Extracts*

Various studies have shown that the enzymatic oligomerization of phenolic compounds could positively impact the parent compounds' biological activities [29, 33, 40]. In the present study, the phenolic-rich extract was treated with two microbial laccases aiming to alter its phenolic profile and thus its overall bioactivity. The laccase-treated phenolic-rich extracts were assessed with Folin-Ciocalteu assay and analyzed by HPLC, GC-MS and LC-MS chromatography to quantify their phenolic content and characterize the phenolic profile after the enzymatic treatment. The laccase-treated extracts presented lower polyphenolic content (4 ± 1.2 milligrams of caffeic acid equivalents per gram of dry extract for TvL-modified extract and 7 ± 0.3 milligrams of caffeic acid equivalents per

gram of dry extract for AbL-modified extract) than the starting extract. The lower phenolic content of the laccase-treated extracts is in accordance with the one reported for laccase-treated wheat straws [61, 62] and laccase-treated propolis extracts [40]. It could be attributed to the oxidation of phenolic compounds towards forming oligomeric products [40, 63].

The effect of the enzymatic treatment on the phenolic compounds of the starting extract was investigated through various analytical methods and UV-Vis spectroscopy. The obtained UV-Vis spectra concerning the enzymatically modified extracts were much differentiated from the ones of the starting extract (Figure S2) as peak shift and decrease in absorption intensity were observed. The enzymatic treatments of the extract with both laccases resulted in the consumption of the parent phenolic compounds and the appearance of newly synthesized compounds as clearly demonstrated through all the applied analytical methods (Figures S3, S4). Although key information was provided from the analytical methods, no data concerning the reaction products can be extracted, as anticipated, given the complicated nature of the oxidized products and their low content. Other researchers have reported similar challenges in their attempt to characterize the chemical nature of the products deriving from enzymatically modified phenolic compounds [64].

### *3.3 Enzymatic Modification and Characterization of the Main Phenolic Compounds of the Ulva Extract*

In order to gain further insights into the different selectivity and affinity of the fungal laccases used in the present work, the main phenolic compounds in the starting extract of *Ulva*, were separately modified by both laccases and the progress of the corresponding reactions was monitored through UV-Vis spectroscopy and HPLC chromatography.

The conversion yields of the biocatalytic oxidations of the phenolic compounds were calculated by comparing the initial and final peak areas on HPLC chromatograms and are summarized in Table 2. In contrast, the corresponding HPLC chromatograms are presented in the supplementary section (S8-S15). As shown in Table 2, TvL completely oxidized most of the tested phenolic compounds while the ability of AbL to catalyze the above oxidations was reduced, in accordance with that reported elsewhere [65]. Interestingly, the treatment with TvL and AbL led to different chromatographic profiles (S8-S15) indicating these laccases' different ability and selectivity to catalyze the oxidation of the selected phenolic compounds.

**Table 2** Reaction conversion (%) of phenolic compounds after overnight incubation with TvL and AbL.

| **Phenolic compounds** | **% Conversion yields** | |
|---|---|---|
| | **TvL** | **AbL** |
| Caffeic acid | 100 | 90 |
| Gallic acid | 99 | 39 |
| Ferulic acid | 100 | 21 |
| *p*-Coumaric acid | 98 | 32 |
| Syringic acid | 99 | 72 |
| *p*-Hydroxybenzoic acid | 0 | 0 |
| Catechin | 100 | 20 |
| Luteolin | 85 | 35 |

| | | |
|---|---|---|
| Apigenin | 0 | 0 |
| Tyrosol | 100 | 57 |

UV-Vis absorption spectra of the main phenolic compounds before and after the enzymatic treatments were also recorded (Figures S6, S7). In all cases, typical UV absorbance peaks, attributed to the π-system of their aromatic rings were observed in the range of 200-400 nm [66, 67]. The differentiation of peak positions in the UV-Vis spectra among the tested phenolic compounds is a result of their ionization state and the nature of the substituent [67]. Most of the phenolic compounds were successfully oxidized by both laccases used. During the oxidation reactions, the UV-Vis peaks of the standard compounds were decreased, broadened, and shifted to other wavelengths, typical for the conjugation of phenolic compounds with C-C or C-O-C coupling and the resulting $n$-$\pi^*$ transitions.

New peaks in the 400-800 nm range were also observed due to the colored products formed [66]. Both laccases are unable to catalyze the oxidation of *p*-hydroxybenzoic acid and apigenin since the UV-Vis spectra of the reaction mixtures remain unchanged after the enzymatic treatment. Similar results have been previously reported concerning transformations with TvL towards *p*-hydroxybenzoic acid and apigenin and AbL towards *p*-hydroxybenzoic acid, respectively [65, 68]. In the enzymatic treatments described before, both *p*-hydroxybenzoic acid and apigenin were consumed after the action of both laccases. It can be assumed that various phenolic compounds, present in the extract, after their oxidation can act as mediators leading to the oxidation of the rest phenolic compounds found in the extract [69]. It is interesting to note that the observed UV-Vis spectra of the reaction mixtures were significantly different regarding the observed peaks or the intensity of the common peaks (Figures S1, S6, S7) indicating the different ability of TvL and AbL to catalyze the oxidation of these phenolic compounds and thus the different composition of the reaction mixtures at the end of the enzymatic modification.

### *3.4 Antioxidant Activity of the Starting and Enzyme-Treated Ulva Extracts*

The antioxidant activity of the starting and laccase-treated extracts was investigated by DPPH and FRAP assays commonly employed for the in vitro evaluation of the antioxidant activity of algal extracts [70, 71]. The results were expressed as milligrams of equivalents of the strong antioxidant compound Trolox per gram of dry extract and are presented in Figure 2. The starting extract demonstrated significant antioxidant activity with both assays, presenting a greater ability to reduce the $Fe^{+3}$-TPTZ complex than the DPPH radical. This antioxidant activity is higher than the one observed for other *Ulva* extracts [72, 73] which could probably be attributed to the extraction method used or to the different geographic areas and seasonal periods of the macroalgae collection [17, 57]. Concerning the results obtained for the laccases-treated extracts, it can be seen that the antioxidant activity decreased (Figure 2). More specifically, the enzymatic treatment of the extract with TvL, led to a higher reduction of the antioxidant activity than that observed after the treatment with AbL. These results could be correlated with the phenolic content of the enzymatically treated extracts since higher antioxidant activity was observed in extracts with higher phenolic content. This is in accordance with the recent study by Botta et al. [40] reporting the decreased antioxidant activity of propolis extract after its treatment with TvL.

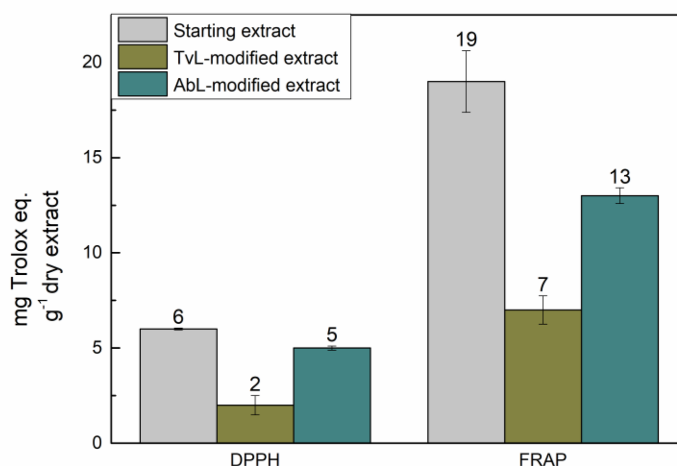


**Figure 2** Estimation of antioxidant activity of starting and laccase-treated extracts through DPPH and FRAP methods. The results are expressed as milligrams of Trolox equivalents per gram of dry extract.

### *3.5 Antimicrobial Activity of Starting and Enzyme-Treated Extracts*

The antimicrobial activity of the starting and laccase-treated extracts was tested against a gram-negative and a gram-positive bacterium, *Escherichia coli* BL21 (*E. coli*) and *Corynebacterium glutamicum* ATCC 21253 (*C. glutamicum*), respectively. The $IC_{50}$ values were determined and are presented in Table 3. The starting extract presented a stronger antimicrobial activity against *C. glutamicum* than *E. coli*. Similar results have been previously reported in the literature concerning organic extracts from *Ulva intestinalis* that proved to be stronger antibacterial agents against gram-positive than gram-negative bacteria, under the same concentrations, probably attributed to the different composition of bacterias' cell wall and, consequently, their permeability [57, 74]. However, different factors determine the biological activity of plant or algal extracts such as the possible interactions between the extract's components that may act synergistically [57].

**Table 3** Antimicrobial activity of starting and laccase-treated extracts expressed as $IC_{50}$ concentration (mg $mL^{-1}$). $IC_{50}$ is defined as the concentration of the tested extract that is able to induce 50% inhibition of bacterial cells' growth.

| **Extracts** | ***E. coli*** | ***C. glutamicum*** |
|---|---|---|
| Starting | 5 ± 0.8 | 2.3 ± 0.2 |
| TvL-modified | 4.2 ± 0.7 | 1.5 ± 0.5 |
| AbL-modified | 4.4± 0.7 | 1.8 ± 0.1 |

On the other hand, the laccase-mediated oxidization of various phenolic compounds has been proposed as a good strategy for enhancing their antimicrobial activity [75, 76]. In the present work, using laccases to treat phenolic-enriched extracts led to an enhancement of their antimicrobial activity, as indicated by $IC_{50}$ values (Table 3). Higher antimicrobial activity was observed after the

treatment with TvL than with AbL. The enhanced antimicrobial activity could be attributed to oxidative products formed due to the enzymatic treatment. It has been proposed that due to the laccase-catalyzed oxidation of phenolic compounds, oligomeric phenolic compounds are formed that present higher antimicrobial activity than the precursor compounds [77, 78]. Even though the exact mechanism of action of the oligomers against the bacteria has not been elucidated; it is attributed to factors such as the higher electron delocalization and the branched nature of the produced oligomers [78].

### *3.6 Enzyme Inhibitory Activity of Ulva Starting and Laccase-Modified Extracts Against Enzymes of Pharmaceutical and Cosmeceutical Interest*

In the present work, the effect of the *Ulva* extract, before and after its enzymatic treatment, was investigated on the activity of several enzymes of pharmaceutical and cosmeceutical interest such as lipoxygenase, collagenase, elastase and lipase.

#### 3.6.1 Effect on the Activity of Lipoxygenase from *Glycine max* (Soybean)

Lipoxygenase is implicated in the metabolism of arachidonic acid to produce leukotrienes and plays an essential role in inflammatory and allergic diseases [79]. Additionally, high levels of arachidonic acid in the skin can result from inflammatory skin diseases or aging [80]. Thus, inhibitors of this enzyme could play a key role as promising antiaging agents. The present work investigated the effect of starting and laccase-treated extracts against lipoxygenase from *Glycine max* at the concentration of 0.5 mg $mL^{-1}$. Hydroxytyrosol was used as a positive control since it inhibits lipoxygenase activity [81]. As shown in Figure 3A, the starting ethanolic extract presented a low effect on lipoxygenase activity (approx. 5%) at the tested concentration in contrast with the positive control, in accordance with the study by Shobier et al. [82] for ethanolic extracts from *Ulva fasciata* and *Ulva linza* specimens. On the other hand, the inhibitory activity of the enzymatically modified extracts was up to 4-fold higher than that observed for the initial ethanolic extract, probably attributed to the oxidation products formed after the enzymatic treatment. Similar studies have also reported the inhibitory effect of the oxidative phenolic products formed by chemical oxidation reactions on lipoxygenase activity [81].

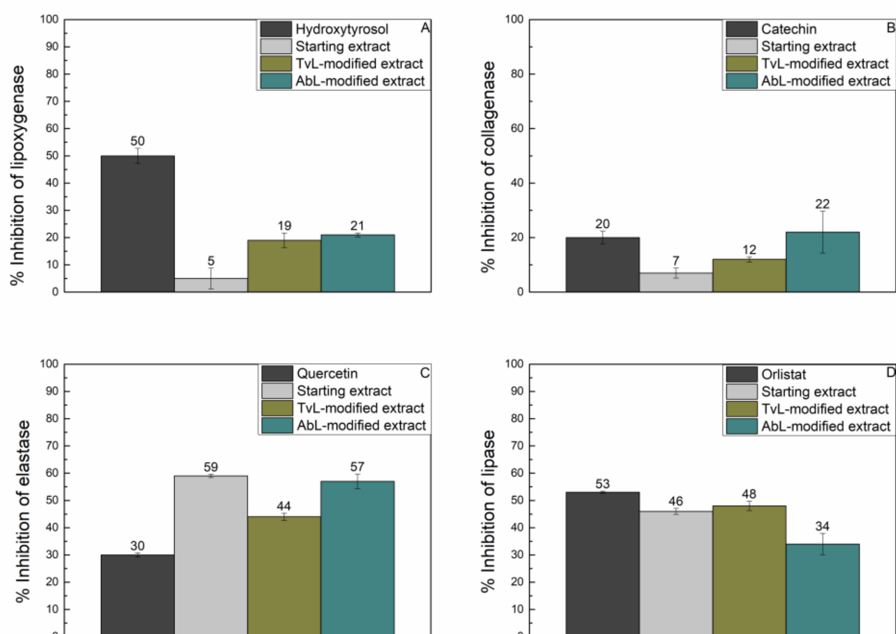


**Figure 3** Inhibitory activity of starting and enzyme-treated extracts at the concentration of **(A)** 0.5 mg $mL^{-1}$ against lipoxygenase from *Glycine max* (hydroxytyrosol was chosen as a positive control at the concentration of 100 μg $mL^{-1}$) **(B)** 1 mg $mL^{-1}$ against collagenase from *Clostridium histolyticum* (catechin was chosen as a positive control at the concentration of 100 μg $mL^{-1}$) **(C)** 1 mg $mL^{-1}$ against elastase from porcine pancreas (quercetin was chosen as a positive control at the concentration of 100 μg $mL^{-1}$) **(D)** 1 mg $mL^{-1}$ against lipase from *Candida rugosa* (orlistat was chosen as a positive control at the concentration of 100 μg $mL^{-1}$).

### 3.6.2 Effect on the Activity of Collagenase from *Clostridium histolyticum*

The collagenase enzyme, acting on collagen, plays a crucial role in tissue remodeling, homeostasis, and wound healing. However, in many cases it is essential the suppression of its over-activation as it is implicated in skin-aging processes affecting the appearance of skin as a result of collagen digestion [83].

One of the main antiaging strategies includes the search for inhibitory agents against this enzyme [83]. In the present work, starting and enzymatically modified extracts of *Ulva* were tested against collagenase from *Clostridium histolyticum* at a final concentration of 1 mg $mL^{-1}$. Catechin was used as a positive control since it can act as an inhibitor of collagenase activity [84]. The inhibitory activity of the starting ethanolic extract was significantly lower than the catechin at the tested concentration (Figure 3B). A previously reported study [85] demonstrated that fractions of phenolic compounds deriving from *Ulva pertusa* exhibited significant inhibitory ability against three different types of collagenases. Notably, the enzymatically modified extracts prepared in this study induced up to 3-fold enhanced inhibitory ability against collagenase, indicating that the enzymatic oxidation of phenolics leads to novel products with enhanced collagenase inhibitory activity than the parent compounds.

3.6.3 Effect on the Activity of Elastase from Porcine Pancreas

Elastase is the responsible enzyme for elastin's breakdown. Its increased activity is associated with several diseases such as psoriasis, dermatitis, premature skin aging, and consequently, wrinkles formation [86]. While there are different studies concerning the inhibitory potential of polyphenols against elastase [84, 87, 88] there is not much research on the elastase inhibitory activities of algae [89]. Herein, both starting and enzymatically modified extracts were tested for elastase inhibitory activity at 1 mg $mL^{-1}$. Quercetin was used as a positive control since it can act as an inhibitor of elastase activity [89]. As it is presented in Figure 3C, the starting ethanolic extract exhibited significant elastase inhibitory activity. This inhibition potential is significantly higher than that reported for similar ethanolic extracts from *Ulva australis* that were not able to inhibit elastase activity even at a concentration of 10 mg $mL^{-1}$ [90]. Regarding the inhibitory activity of the enzymatically modified extracts, relatively reduced inhibitory ability was observed after the enzymatic treatment with both laccases (43.6% for TvL-modified extract and 56.8% for AbL-modified extract) indicating that the newly synthesized products considerably interacted with the enzyme.

3.6.4 Effect on the Activity of Lipase from *Candida rugosa*

*Candida rugosa* lipase (CrL) inhibitors have gained scientific interest, especially in developing treatments against *C. rugosa* strains refractory to antifungal therapy [91, 92]. The ethanolic starting and laccase-treated extracts prepared in the present work were tested for their inhibitory activity against CrL. The well-known lipase inhibitor orlistat was used as a positive control [93]. As shown in Figure 3D, the starting ethanolic extract presented significant CrL inhibitory activity at the tested concentration of 1 mg $mL^{-1}$ which is in contrast to that reported for organic extracts from *Ulva rigida* where no lipase inhibitory activity was observed even at a concentration of 3 mg $mL^{-1}$ [93]. It should be highlighted that the CrL inhibitory activity of the TvL-treated extract was slightly increased. In contrast, the AbL-treated extract showed reduced inhibitory activity, pointing out the different selectivity of the two laccases that led to diverse products, affecting the lipase inhibitory activity of the Ulva extracts differently.

### *3.7 Determination of Cell Viability of NIH/3T3 Cells after Exposure to Starting and Laccase-Treated Extracts*

The cell viability of NJH/3T3 cells after exposure to starting and laccase-treated extracts was investigated. According to the results, cell viability of NIH/3T3 cells remained stable (around 70-80%) across a broad range of concentrations of TvL- and AbL-modified extracts (20–500 μg $mL^{-1}$) indicating that the modified extracts exerted a mild cytotoxic effect. On the other hand, the starting extract did not produce any significant toxic effect after incubation for 24 hours (Figure 4A). Longer exposure (48 h) amplified the starting extract's cytotoxicity resulting in a 35% decrease in the cell population. On the other hand, the TvL-modified extract maintained the same cytotoxic profile as seen in 24 h whereas the AbL- modified extract increased cell death at concentrations higher than 250 μg $mL^{-1}$ (Figure 4B).

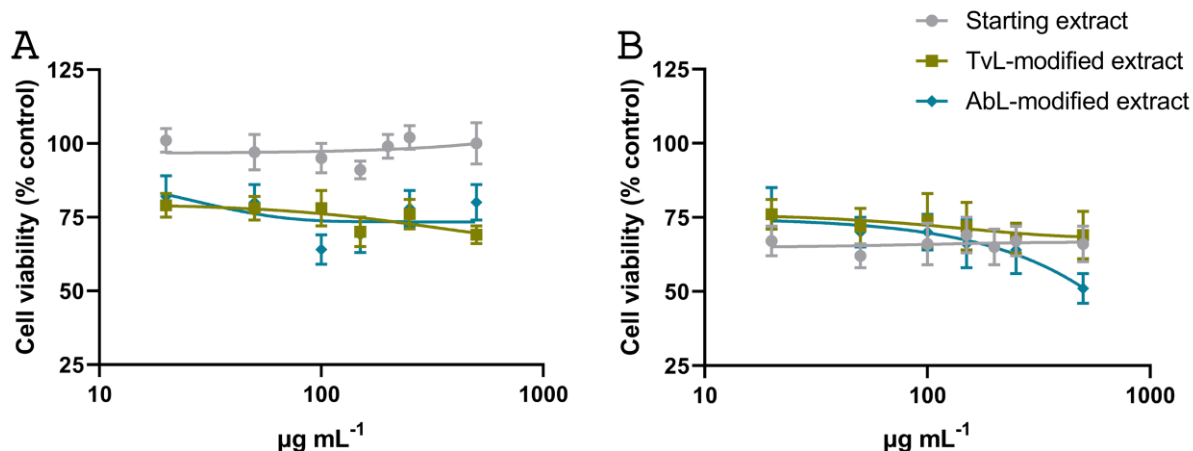


**Figure 4** Viability of NIH/3T3 after exposure to the starting, TvL- and AbL- modified extracts for 24 **(A)** and 48 **(B)** hours.

The results indicated that the enzymatic modification of the extracts led to the abolishment of the time-dependent cytotoxic effect exerted by the starting extract, especially for the TvL-modified extract. Potentially, the enzymatic modification increased modified phenolic compounds' cellular uptake. According to the literature, the cytotoxicity of *Ulva* extracts varies among the different species, the extraction procedure used, and the different cell lines tested. Acharya et al. [94] showed that the aqueous marine algae *Ulva lactuca* extract was non-toxic against human colorectal cancer cells (HCT-116) to doses up to 1 mg mL$^{-1}$ and exposure for 24 hours. On the contrary, ethanolic extracts of marine algae, *Ulva fasciate* Delile exerted dose- and time-dependent cytotoxicity at the same cell line (HCT-116) after incubation for 24 and 48 hours. Cell cytometry revealed that the extracts induced apoptosis and mitochondrial dysregulation [95].

## 4. Conclusions

In the present work, we comparatively investigated for the first time, the biological activity of both non-treated and enzyme-treated extracts derived from the green macroalgae *Ulva intestinalis*. More specifically, a phenolic-rich extract from *Ulva intestinalis* macroalgae, collected in Epirus (Greece) was prepared and enzymatically modified with two fungal laccases (TvL and AbL) to convert its phenolic compounds into hybrid oligomeric products and thus alter its biological activity to enhance it. The starting and the modified extracts were assessed for their chemical profile through analytical methods and their antioxidant, antimicrobial and enzyme-inhibitory activities. Several phenolic acids, flavonoids and phenols were identified in the starting extract, in accordance with other studies for *Ulva* species extracts. After the enzymatic treatments, analytical methods confirmed the consumption of the phenolic compounds in the starting extract and their transformation into new products.

Additionally, the specific activity and selectivity of the laccases used were studied against a range of phenolic substrates and remarkable differences were observed. As anticipated, the differentiated selectivity and affinity of the tested biocatalysts affected the composition of the enzyme-treated extracts and their biological activity. In most cases, the extract's bioactivity enhancement was observed after the laccase treatments. Thus, the laccase-treated extracts presented enhanced antimicrobial, anti-collagenase and anti-lipoxygenase activity in combination with mild cytotoxicity,

with the observed enhancements to range depending on the laccase used. In conclusion, this study highlights the importance of biocatalytic transformations to alter the bioactivity of natural extracts expanding their potential applications in food, pharmaceutical, and cosmeceutical industries.

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**Author Contributions**

Stamatia Spyrou: Conceptualization, Methodology, Investigation, Writing-Original draft, Alexandra V. Chatzikonstantinou: Conceptualization, Methodology, Investigation, Writing-Original draft-Review and Editing, Archontoula Giannakopoulou: Writing-Original draft-Review and Editing, Renia Fotiadou: Methodology, Investigation, Writing-Original draft, Silvia Priska: Investigation, Methodology, Data curation, Yannis V. Simos: Investigation, Methodology, Data curation, Writing-Original draft, Aliki Tsakni: Investigation, Data curation, Dimitrios Peschos: Writing-Review and Editing, Dimitra Houhoula: Writing-Review and Editing, Epaminondas Voutsas: Writing-Review and Editing, Haralambos Stamatis: Conceptualization, Fund raising, Writing-Review and Editing, Supervision.

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**Competing Interests**

The authors have declared that no competing interests exist.

**Additional Materials**

The following additional materials are uploaded at the page of this paper.

1. Figure S1: The color change of the reaction mixtures of the modification of tyrosol with AbL (yellow mixture) and TvL (pale mixture) after overnight incubation.
2. Figure S2: UV-Vis spectra of the starting and laccase-treated extracts at the concentration of 0.5 mg $mL^{-1}$.
3. Figure S3: HPLC chromatograms of the starting (A) and laccase-treated extracts [TvL-modified (B) and AbL- modified (C)] at 330 nm.
4. Figure S4: HPLC chromatograms of the starting (A) and laccase-treated extracts [TvL-modified (B) and AbL- modified (C)] at 280 nm.

5. Figure S5: GC-MS total ionic chromatogram of the starting extract.
6. Table S1: Phenolic compounds that were detected in the natural extract through GC-MS analysis.
7. Figure S6: UV-Vis spectra of the enzymatic modifications of phenolic compounds that were detected in the natural extract with TvL at various time intervals. ON stands for overnight incubation.
8. Figure S7: UV-Vis spectra of the enzymatic modification of phenolic compounds that were detected in the natural extract with AbL at various time intervals. ON stands for overnight incubation.
9. Figure S8: Chromatogram at 280 nm of (A) standard compound tyrosol (Retention time = 5.867 min) (B) reaction of tyrosol with TvL after overnight incubation (Retention $time_{product1}$ = 14.697 min, Retention $time_{product2}$ = 18.644 min) (C) reaction of tyrosol with AbL after overnight incubation (Retention $time_{product1}$ = 3.194 min, Retention $time_{product2}$ = 4.696 min, Retention $time_{product3}$ = 15.029 min).
10. Figure S9: Chromatogram at 280 nm of (A) standard compound syringic acid (Retention $time_1$ = 10.974 min, Retention $time_2$ = 11.358 min) (B) reaction of syringic acid with TvL after overnight incubation (Retention $time_{product1}$ = 9.027 min, Retention $time_{product2}$ = 15.180 min, Retention $time_{product3}$ = 18.957 min) (C) reaction of syringic acid with AbL after overnight incubation (Retention $time_{product1}$ = 9.102 min, Retention $time_{product2}$ = 15.180 min, Retention $time_{product3}$ = 18.980 min).
11. Figure S10: Chromatogram at 280 nm of (A) standard compound gallic acid (Retention $time_1$ = 3.219 min, Retention $time_2$ = 4.140 min) (B) reaction of gallic acid with TvL after 30 min incubation (C) reaction of gallic acid with TvL after overnight incubation (Retention $time_{product1}$ = 2.130 min, Retention $time_{product2}$ = 2.285 min, Retention $time_{product3}$ = 2.624 min) (D) reaction of gallic acid with AbL after overnight incubation (Retention $time_{product}$ = 2.302 min).
12. Figure S11: Chromatogram at 320 nm of (A) standard compound ferulic acid (Retention time = 13.569 min) (B) reaction of ferulic acid with TvL after 10 min incubation (C) reaction of ferulic acid with TvL after overnight incubation (Retention $time_{product1}$ = 18.127 min, Retention $time_{product2}$ = 22.975 min) (D) reaction of ferulic acid with AbL after overnight incubation (Retention $time_{product1}$ = 16.654 min, Retention $time_{product2}$ = 18.042 min, Retention $time_{product3}$ = 20.352 min).
13. Figure S12: Chromatogram at 310 nm of (A) standard compound *p*-coumaric acid (Retention time = 12.642 min) (B) reaction of *p*-coumaric acid with TvL after 30 min incubation (C) reaction of *p*-coumaric acid with TvL after overnight incubation (Retention $time_{product1}$ = 16.760 min, Retention $time_{product2}$ = 20.070 min, Retention $time_{product3}$ = 20.762 min, Retention $time_{product4 =}$ 21.419 min (D) reaction of *p*-coumaric acid with AbL after overnight incubation.
14. Figure S13: Chromatogram at 280 nm of (A) standard compound catechin (Retention time = 10.398 min) (B) reaction of catechin with TvL after 10 min incubation (C) reaction of catechin with TvL after overnight incubation (D) reaction of catechin with AbL after overnight incubation (Retention $time_{product1}$ = 14.032 min, Retention $time_{product2}$ = 15.304 min).
15. Figure S14: Chromatogram at 320 nm of (A) standard compound caffeic acid (Retention time = 10.994 min) (B) reaction of caffeic acid with TvL after 10 min incubation (C) reaction of caffeic acid with TvL after overnight incubation (D) reaction of caffeic acid with AbL after overnight incubation (Retention $time_{product1}$ = 13.210 min, Retention $time_{product2}$ = 13.813 min, Retention $time_{product3}$ = 14.354 min).
16. Figure S15: Chromatogram at 350 nm of (A) standard compound luteolin (Retention time = 17.499 min) (B) reaction of luteolin with TvL after overnight incubation (C) reaction of luteolin with AbL after overnight incubation.

**References**

1. Biris-Dorhoi ES, Michiu D, Pop CR, Rotar AM, Tofana M, Pop OL, et al. Macroalgae—A sustainable source of chemical compounds with biological activities. Nutrients. 2020; 12: 3085.
2. Wichard T, Charrier B, Mineur F, Bothwell JH, de Clerck OD, Coates JC. The green seaweed Ulva: A model system to study morphogenesis. Front Plant Sci. 2015; 6: 72.
3. Dominguez H, Loret EP. Ulva lactuca, a source of troubles and potential riches. Mar Drugs. 2019; 17: 357.
4. Ghosh S, Greiserman S, Chemodanov A, Slegers PM, Belgorodsky B, Epstein M, et al. Polyhydroxyalkanoates and biochar from green macroalgal Ulva sp. biomass subcritical hydrolysates: Process optimization and a priori economic and greenhouse emissions break-even analysis. Sci Total Environ. 2021; 770: 145281.
5. Kazir M, Abuhassira Y, Robin A, Nahor O, Luo J, Israel A, et al. Extraction of proteins from two marine macroalgae, Ulva sp. and Gracilaria sp., for food application, and evaluating digestibility, amino acid composition and antioxidant properties of the protein concentrates. Food Hydrocoll. 2019; 87: 194-203.
6. Jimenez-Lopez C, Pereira AG, Lourenço-Lopes C, García-Oliveira P, Cassani L, Fraga-Corral M, et al. Main bioactive phenolic compounds in marine algae and their mechanisms of action supporting potential health benefits. Food Chem. 2021; 341: 128262.
7. Ktari L. Pharmacological potential of Ulva species: A valuable resource. J Anal Pharm Res. 2017; 6: 00165.
8. Bodin J, Adrien A, Bodet PE, Dufour D, Baudouin S, Maugard T, et al. Ulva intestinalis protein extracts promote in vitro collagen and hyaluronic acid production by human dermal fibroblasts. Molecules. 2020; 25*:* 2091.
9. Juul L, Stødkilde L, Ingerslev AK, Bruhn A, Jensen SK, Dalsgaard TK. Digestibility of seaweed protein from Ulva sp. and Saccharina latissima in rats. Algal Res. 2022; 63: 102644.
10. Kellogg J, Lila MA. Chemical and in vitro assessment of Alaskan coastal vegetation antioxidant capacity. J Agric Food Chem. 2013; 61: 11025-11032.
11. Zertuche-González JA, Sandoval-Gil JM, Rangel-Mendoza LK, Gálvez-Palazuelos AI, Guzmán-Calderón JM, Yarish C. Seasonal and interannual production of sea lettuce (Ulva sp.) in outdoor cultures based on commercial size ponds. J World Aquac Soc. 2021; 52: 1047-1058.
12. Holdt SL, Kraan S. Bioactive compounds in seaweed: Functional food applications and legislation. J Appl Phycol. 2011; 23: 543-597.
13. Cotas J, Leandro A, Monteiro P, Pacheco D, Figueirinha A, Gonçąlves AMM, et al. Seaweed phenolics: From extraction to applications. Mar Drugs. 2020; 18: 384.
14. Wekre ME, Kåsin K, Underhaug J, Holmelid B, Jordheim M. Quantification of polyphenols in seaweeds: A case study of Ulva intestinalis. Antioxidants. 2019; 8: 612.
15. Mezghani S, Csupor D, Bourguiba I, Hohmann J, Amri M, Bouaziz M. Characterization of phenolic compounds of Ulva rigida (Chlorophycae) and its antioxidant activity. Eur J Medicinal Plants. 2016; 12: 1-9.
16. Jang JY, Shin H, Lim JW, Ahn JH, Jo YH, Lee KY, et al. Comparison of antibacterial activity and phenolic constituents of bark, lignum, leaves and fruit of Rhus verniciflua. PLoS One. 2018; 13: e0200257.
17. Farasat M, Khavari-Nejad RA, Nabavi SMB, Namjooyan F. Antioxidant activity, total phenolics

and flavonoid contents of some edible green seaweeds from northern coasts of the Persian Gulf. Iran J Pharm Res. 2014; 13: 163-170.

18. Zhang L, Khoo C, Koyyalamudi SR, Pedro ND, Reddy N. Antioxidant, anti-inflammatory and anticancer activities of ethanol soluble organics from water extracts of selected medicinal herbs and their relation with flavonoid and phenolic contents. Pharmacologia. 2017; 8: 59-72.
19. Trigui M, Gasmi L, Zouari I, Tounsi S. Seasonal variation in phenolic composition, antibacterial and antioxidant activities of Ulva rigida (Chlorophyta) and assessment of antiacetylcholinesterase potential. J Appl Phycol. 2013; 25: 319-328.
20. Yu-Qing T, Mahmood K, Shehzadi R, Ashraf MF. Ulva lactuca and its polysaccharides: Food and biomedical aspects. J Biol Agric Healthc. 2016; 6: 140-151.
21. Liao ZH, Ibarra-Arana MJ, Chen JC, Huang HT, Lin YJ, Nan FH. The effects of dietary supplement of Ulva fasciata, fermented soybean and probiotics on the growth of Apostichopus japonicus. Aquac Nutr. 2021; 27: 1363-1369.
22. Taboada C, Millán R, Míguez I. Composition, nutritional aspects and effect on serum parameters of marine algae Ulva rigida. J Sci Food Agric. 2010; 90: 445-449.
23. Udayangani C, Wijesekara I, Wickramasinghe I. Characterization of sea lettuce (Ulva lactuca) from Matara, Sri Lanka and development of nutribars as a functional food. Ruhuna J Sci. 2019; 10: 96-107.
24. Uyama H. Artificial polymeric flavonoids: Synthesis and applications. Macromol Biosci. 2007; 7: 410-422.
25. Chen J, Yang J, Ma L, Li J, Shahzad N, Kim CK. Structure-antioxidant activity relationship of methoxy, phenolic hydroxyl, and carboxylic acid groups of phenolic acids. Sci Rep. 2020; 10: 2611.
26. Kim J, Pälijärvi M, Karonen M, Salminen JP. Oxidatively active plant phenolics detected by UHPLC-DAD-MS after enzymatic and alkaline oxidation. J Chem Ecol. 2018; 44: 483-496.
27. Groussin AL, Antoniotti S. Valuable chemicals by the enzymatic modification of molecules of natural origin: Terpenoids, steroids, phenolics and related compounds. Bioresour Technol. 2012; 115: 237-243.
28. Gkantzou E, Chatzikonstantinou AV, Fotiadou R, Giannakopoulou A, Patila M, Stamatis H. Trends in the development of innovative nanobiocatalysts and their application in biocatalytic transformations. Biotechnol Adv. 2021; 51 :107738.
29. Antonopoulou I, Varriale S, Topakas E, Rova U, Christakopoulos P, Faraco V. Enzymatic synthesis of bioactive compounds with high potential for cosmeceutical application. Appl Microbiol Biotechnol. 2016; 100: 6519-6543.
30. Tibrewal N, Tang Y. Biocatalysts for natural product biosynthesis. Annu Rev Chem Biomol Eng. 2014; 5: 347-366.
31. Cardullo N, Muccilli V, Tringali C. Laccase-mediated synthesis of bioactive natural products and their analogues. RSC Chem Biol. 2022; 3: 614-647.
32. Navarra C, Goodwin C, Burton S, Danieli B, Riva S. Laccase-mediated oxidation of phenolic derivatives. J Mol Catal B. 2010; 65: 52-57.
33. Jeon JR, Baldrian P, Murugesan K, Chang YS. Laccase-catalysed oxidations of naturally ocurring phenols: From in vivo biosynthetic pathways to green synthetic applications. Microb Biotechnol. 2012; 5: 318-332.
34. Su J, Fu J, Wang Q, Silva C, Cavaco-Paulo A. Laccase: A green catalyst for the biosynthesis of

poly-phenols. Crit Rev Biotechnol. 2018; 38: 294-307.
35. Potdar MK, Kelso GF, Schwarz L, Zhang C, Hearn MTW. Recent developments in chemical synthesis with biocatalysts in ionic liquids. Molecules. 2015; 20: 16788-16816.
36. Yang J, Li W, Ng TB, Deng X, Lin J, Ye X. Laccases: Production, expression regulation, and applications in pharmaceutical biodegradation. Front Microbiol. 2017; 8: 832.
37. Shekher R, Sehgal S, Kamthania M, Kumar A. Laccase: Microbial sources, production, purification, and potential biotechnological applications. Enzyme Res. 2011; 2011: 217861.
38. Chauhan PS, Goradia B, Saxena A. Bacterial laccase: Recent update on production, properties and industrial applications. 3 Biotech. 2017; 7: 323.
39. Jones SM, Solomon EI. Electron transfer and reaction mechanism of laccases. Cell Mol Life Sci. 2015; 72: 869-883.
40. Botta L, Brunori F, Tulimieri A, Piccinino D, Meschini R, Saladino R. Laccase-mediated enhancement of the antioxidant activity of propolis and poplar bud exudates. ACS Omega. 2017; 2: 2515-2523.
41. Abou El Azm N, Fleita D, Rifaat D, Mpingirika EZ, Amleh A, El-Sayed MMH. Production of bioactive compounds from the sulfated polysaccharides extracts of Ulva lactuca: Post-extraction enzymatic hydrolysis followed by ion-exchange chromatographic fractionation. Molecules. 2019; 24: 2132.
42. Chi Y, Zhang M, Wang X, Fu X, Guan H, Wang P. Ulvan lyase assisted structural characterization of ulvan from Ulva pertusa and its antiviral activity against vesicular stomatitis virus. Int J Biol Macromol. 2020; 157: 75-82.
43. Ermakova SP, Menshova RV, Anastyuk SD, Vishchuk OSM, Zakharenko AM, Thinh PD, et al. Structure, chemical and enzymatic modification, and anticancer activity of polysaccharides from the brown alga Turbinaria ornata. J Appl Phycol. 2016; 28: 2495-2505.
44. Pimentel FB, Cermeño M, Kleekayai T, Harnedy-Rothwell PA, Fernandes E, Alves RC, et al. Enzymatic modification of Porphyra dioica-derived proteins to improve their antioxidant potential. Molecules. 2020; 25: 2838.
45. Fleita D, El-Sayed M, Rifaat D. Evaluation of the antioxidant activity of enzymatically-hydrolyzed sulfated polysaccharides extracted from red algae; Pterocladia capillacea. LWT. 2015; 63: 1236-1244.
46. Lauret M, Oheix J, Derolez V, Laugier T. Guide de reconnaissance et de suivi des macrophytes des lagunes du Languedoc-Roussillon. Ifremer, Cépralmar, Agence de l'Eau RM&C, Région Languedoc-Roussillon: Réseau de Suivi Lagunaire; 2011. Available from: https://www.semanticscholar.org/paper/Guide-de-reconnaissance-et-de-suivi-des-macrophytes-Lauret-Oheix/ca21c0d5de18e2b551a980eebc9164ded64ee802.
47. Rodríguez-Prieto C, Ballesteros E, Boisset F, Afonso-Carrillo J. Guía de las macroalgas y fanerógamas marinas del Mediterráneo ocidental Omega (Algae and Phanerogams of the Mediterranean). SA Barcelona; 2013. Available from: https://www.researchgate.net/publication/357795728_Guia_de_las_macroalgas_y_fanerogamas_marinas_del_Mediterraneo_Occidental.
48. Sirbu R, Stanciu G, Tomescu A, Ionescu AM, Cadar E. Evaluation of antioxidant and antimicrobial activity in relation to total phenolic content of green algae from Black Sea. Rev Chim. 2019; 70: 1197-1203.
49. Chatzikonstantinou AV, Giannakopoulou A, Spyrou S, Simos YV, Kontogianni VG, Peschos D, et

al. Production of hydroxytyrosol rich extract from Olea europaea leaf with enhanced biological activity using immobilized enzyme reactors. Environ Sci Pollut Res Int. 2022; 29: 29624-29637.

50. Pappou S, Dardavila MM, Savvidou MG, Louli V, Magoulas K, Voutsas E. Extraction of bioactive compounds from Ulva lactuca. Appl Sci. 2022; 12: 2117.
51. Wezgowiec J, Wieczynska A, Wieckiewicz W, Kulbacka J, Saczko J, Pachura N, et al. Polish propolis-Chemical composition and biological effects in tongue cancer cells and macrophages. Molecules. 2020; 25: 2426.
52. Zwane RE, Parker A, Kudanga T, Davids LM, Burton SG. Novel, biocatalytically produced hydroxytyrosol dimer protects against ultraviolet-induced cell death in human immortalized keratinocytes. J Agric Food Chem. 2012; 60: 11509-11517.
53. Sharma S, Vig AP. Preliminary phytochemical screening and in vitro antioxidant activities of Parkinsonia aculeata Linn. BioMed Res Int. 2014; 2014: 756184.
54. Papadopoulou AA, Katsoura MH, Chatzikonstantinou A, Kyriakou E, Polydera AC, Tzakos AG, et al. Enzymatic hybridization of α-lipoic acid with bioactive compounds in ionic solvents. Bioresour Technol. 2013; 136: 41-48.
55. Thring TSA, Hili P, Naughton DP. Anti-collagenase, anti-elastase and anti-oxidant activities of extracts from 21 plants. BMC Complement Altern Med. 2009; 9: 27.
56. Gatto MT, Falcocchio S, Grippa E, Mazzanti G, Battinelli L, Nicolosi G, et al. Antimicrobial and anti-lipase activity of quercetin and its C2-C16 3-O-acyl-esters. Bioorg Med Chem. 2002; 10: 269-272.
57. Kosanić M, Ranković B, Stanojković T. Biological activities of two macroalgae from Adriatic coast of Montenegro. Saudi J Biol Sci. 2015; 22: 390-397.
58. Kalasariya HS, Pereira L. Dermo-cosmetic benefits of marine macroalgae-derived phenolic compounds. Appl Sci. 2022; 12: 11954.
59. Mekinić IG, Skroza D, Šimat V, Hamed I, Čagalj M, Perković ZP. Phenolic content of brown algae (Pheophyceae) species: Extraction, identification, and quantification. Biomolecules. 2019; 9: 244.
60. Li GL, Guo WJ, Wang GB, Wang RR, Hou YX, Liu K, et al. Sterols from the green alga Ulva australis. Mar Drugs. 2017; 15: 299.
61. Oliva-Taravilla A, Moreno AD, Demuez M, Ibarra D, Tomás-Pejó E, González-Fernández C, et al. Unraveling the effects of laccase treatment on enzymatic hydrolysis of steam-exploded wheat straw. Bioresour Technol. 2015; 175: 209-215.
62. Kolb M, Sieber V, Amann M, Faulstich M, Schieder D. Removal of monomer delignification products by laccase from Trametes versicolor. Bioresour Technol. 2012; 104: 298-304.
63. Muñiz-Mouro A, Oliveira IM, Gullón B, Lú-Chau TA, Moreira MT, Lema JM, et al. Comprehensive investigation of the enzymatic oligomerization of esculin by laccase in ethanol: Water mixtures. RSC Adv. 2017; 7: 38424-38433.
64. Adelakun OE, Kudanga T, Parker A, Green IR, le Roes-Hill M, Burton SG. Laccase-catalyzed dimerization of ferulic acid amplifies antioxidant activity. J Mol Catal B. 2012; 74: 29-35.
65. Teymennet-Ramírez KV, Martínez-Morales F, Muñoz-Garay C, Bertrand B, Morales-Guzmán D, Trejo-Hernández MR. Laccase treatment of phenolic compounds for bioethanol production and the impact of these compounds on yeast physiology. Biocatal Biotransformation. 2022; 40: 38-49.
66. Cusola O, Valls C, Vidal T, Roncero MB. Using electrochemical methods to study the kinetics of

laccase-catalyzed oxidation of phenols. Ind Eng Chem Res. 2018; 57: 2434-2439.
67. Božiĉ M, Gorgieva S, Kokol V. Laccase-mediated functionalization of chitosan by caffeic and gallic acids for modulating antioxidant and antimicrobial properties. Carbohydr Polym. 2012; 87: 2388-2398.
68. Yan Q, Tang X, Zhang B, Wang C, Deng S, Ma X, et al. Biocatalytic oxidation of flavone analogues mediated by general biocatalysts: Horseradish peroxidase and laccase. RSC Adv. 2019; 9: 13325-13331.
69. Kaczmarek MB, Kwiatos N, Szczesna-Antczak M, Bielecki S. Laccases – enzymes with an unlimited potential. Biotechnol Food Sci. 2017; 81: 41-70.
70. Matsukawa R, Dubinsky Z, Kishimoto E, Masaki K, Masuda Y, Takeuchi T, et al. A comparison of screening methods for antioxidant activity in seaweeds. J Appl Phycol. 1997; 9: 29-35.
71. Silva A, Rodrigues C, Garcia-Oliveira P, Lourenço-Lopes C, Silva SA, Garcia-Perez P, et al. Screening of bioactive properties in brown algae from the Northwest Iberian Peninsula. Foods. 2021; 10: 1915.
72. Álvarez-Gomez F, Korbee N, Figueroa FL. Analysis of antioxidant capacity and bioactive compounds in marine macroalgal and lichenic extracts using different solvents and evaluation methods. Cienc Mar. 2016; 42: 271-288.
73. García V, Uribe E, Vega Gálvez A, Delporte Vergara C, Valenzuela Barra G, López J, et al. Health-promoting activities of edible seaweed extracts from Chilean coasts: Assessment of antioxidant, anti-diabetic, anti-inflammatory and antimicrobial potential. Rev Chil Nutr. 2020; 47: 792-800.
74. Srikong W, Bovornreungroj N, Mittraparparthorn P, Bovornreungroj P. Antibacterial and antioxidant activities of differential solvent extractions from the green seaweed Ulva intestinalis. ScienceAsia. 2017; 43: 88-95.
75. Panda L, Duarte-Sierra A. Recent advancements in enhancing antimicrobial activity of plant-derived polyphenols by biochemical means. Horticulturae. 2022; 8: 401.
76. Yu C, Liu X, Pei J, Wang Y. Grafting of laccase-catalysed oxidation of butyl paraben and p-coumaric acid onto chitosan to improve its antioxidant and antibacterial activities. React Funct Polym. 2020; 149: 104511.
77. Chakroun H, Bouaziz M, Yangui T, Blibech I, Dhouib A, Sayadi S. Enzymatic transformation of tyrosol by Trametes trogii laccases: Identification of the product and study of its biological activities. J Mol Catal B. 2013; 87: 11-17.
78. Elegir G, Kindl A, Sadocco P, Orlandi M. Development of antimicrobial cellulose packaging through laccase-mediated grafting of phenolic compounds. Enzyme Microb Technol. 2008; 43: 84-92.
79. Onar HC, Yusufoglu A, Turker G, Yanardag R. Elastase, tyrosinase and lipoxygenase inhibition and antioxidant activity of an aqueous extract from Epilobium angustifolium L. leaves. J Medicinal Plants Res. 2012; 6: 716-726.
80. Rinnerthaler M, Bischof J, Streubel MK, Trost A, Richter K. Oxidative stress in aging human skin. Biomolecules. 2015; 5: 545-589.
81. Shingai Y, Fujimoto A, Nakamura M, Masuda T. Structure and function of the oxidation products of polyphenols and identification of potent lipoxygenase inhibitors from Fe-catalyzed oxidation of resveratrol. J Agric Food Chem. 2011; 59: 8180-8186.
82. Shobier AH, Ismail MM, Hassan SWM. Variation in anti-inflammatory, anti-arthritic, and antimicrobial activities of different extracts of common Egyptian seaweeds with an emphasis

on their phytochemical and heavy metal contents. Biol Trace Elem Res. 2022. doi: 10.1007/s12011-022-03297-1.

83. Hartmann A, Gostner J, Fuchs JE, Chaita E, Aligiannis N, Skaltsounis L, et al. Inhibition of collagenase by mycosporine-like amino acids from marine sources. Planta Med. 2015; 81: 813-820.
84. Wittenauer J, MäcKle S, Sußmann D, Schweiggert-Weisz U, Carle R. Inhibitory effects of polyphenols from grape pomace extract on collagenase and elastase activity. Fitoterapia. 2015; 101: 179-187.
85. Choi JS, Ha YM, Joo CU, Cho KK, Kim SJ, Choi IS. Inhibition of oral pathogens and collagenase activity by seaweed extracts. J Environ Biol. 2012; 33: 115-121.
86. Era B, Floris S, Sogos V, Porcedda C, Piras A, Medda R, et al. Anti-aging potential of extracts from Washingtonia filifera seeds. Plants. 2021; 10: 151.
87. Brás NF, Gonçalves R, Mateus N, Fernandes PA, Ramos MJ, de Freitas V. Inhibition of pancreatic elastase by polyphenolic compounds. J Agric Food Chem. 2010; 58: 10668-10676.
88. Jakimiuk K, Gesek J, Atanasov AG, Tomczyk M. Flavonoids as inhibitors of human neutrophil elastase. J Enzyme Inhib Med Chem. 2021; 36: 1016-1028.
89. Castejón N, Thorarinsdottir KA, Einarsdóttir R, Kristbergsson K, Marteinsdóttir G. Exploring the potential of Icelandic seaweeds extracts produced by aqueous pulsed electric fields-assisted extraction for cosmetic applications. Mar Drugs. 2021; 19: 662.
90. Trentin R, Custódio L, Rodrigues MJ, Moschin E, Sciuto K, da Silva JP, et al. Exploring Ulva australis Areschoug for possible biotechnological applications: In vitro antioxidant and enzymatic inhibitory properties, and fatty acids contents. Algal Res. 2020; 50: 101980.
91. Grippa E, Valla R, Battinelli L, Mazzanti G, Saso L, Silvestrini B. Inhibition of Candida rugosa lipase by berberine and structurally related alkaloids, evaluated by high-performance liquid chromatography. Biosci Biotechnol Biochem. 1999; 63: 1557-1562.
92. Ruiz C, Falcocchio S, Xoxi E, Villo L, Nicolosi G, Pastor FIJ, et al. Inhibition of Candida rugosa lipase by saponins, flavonoids and alkaloids. J Mol Catal B. 2006; 40: 138-143.
93. Neto RT, Marçal C, Queirós AS, Abreu H, Silva AMS, Cardoso SM. Screening of Ulva rigida, Gracilaria sp., Fucus vesiculosus and Saccharina latissima as functional ingredients. Int J Mol Sci. 2018; 19: 2987.
94. Acharya D, Satapathy S, Yadav KK, Somu P, Mishra G. Systemic evaluation of mechanism of cytotoxicity in human colon cancer HCT-116 cells of silver nanoparticles synthesized using marine algae Ulva lactuca extract. J Inorg Organomet Polym Mater. 2022; 32: 596-605.
95. Ryu MJ, Kim AD, Kang KA, Chung HS, Kim HS, Suh IS, et al. The green algae Ulva fasciata Delile extract induces apoptotic cell death in human colon cancer cells. In Vitro Cell Dev Biol Animal. 2013; 49: 74-81.