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Protective Effect of Tunisian Red Seaweed (*Corallina officinalis*) Against Bleomycin-Induced Pulmonary Fibrosis and Oxidative Stress in Rats

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Abstract

Idiopathic pulmonary fibrosis is a chronic and progressive respiratory disease whose diagnosis and physiopathogenesis are still poorly understood and for which, until recently, there were no effective treatments. Over the past few decades, many studies have demonstrated that marine macroalgae such as red seaweeds are potential alternative sources of useful bioactive compounds possessing various physiological and biological activities. The present study was aimed to investigate the effect of *Corallina officinalis* aqueous extract (COAE) against bleomycin (BLM)-induced lung fibrosis in rat. Thus, Wistar rats were divided into 4 groups of 10 each: control, BLM (2 mg/kg), BLM/COAE-150 mg/kg and BLM/COAE-300 mg/kg once a day for 21 days. Obtained results showed that COAE is rich in phenolic compounds and exhibited relatively high antioxidant activity. COAE might significantly reduce the damage caused by BLM by rewarding the decline in weight and pulmonary index in rats given only BLM. Moreover, lungs, liver and kidneys lipid peroxidation, and sulfhydryl group levels were reversed significantly in a dose-dependent manner in the COAE-treated groups. BLM decreased superoxide dismutase (SOD) and catalase (CAT) activities, while COAE administration increased the antioxidant enzyme activities. Histopathologically, COAE attenuates the severity of the inflammatory lungs state caused by instillation of BLM in rats. These findings suggest that COAE can be a potential therapeutic candidate against BLM-induced lung fibrosis.

Keywords

seaweed, *Coralinas officinalis*, bleomycin, pulmonary fibrosis, antioxidant

Introduction

Pulmonary fibrosis is a chronic progressive lung disease that steadily leads to lung architecture disruption and respiratory failure.[1] Generally, the development of pulmonary pathology is often preceded by acute lung inflammation, caused by a wide variety of etiological factors such as viral and bacterial infections, ionizing radiation, chemotherapy, air irritants and pollutants,[2,3] which were not resolved in time and resulted in the deposition in the lungs and respiratory dysfunction.[4]

Currently, different models of pulmonary fibrosis have been developed including radiation damage, instillation of bleomycin (BLM), silica or asbestos, and transgenic mice or gene transfer employing fibrogenic cytokines.[5] So far, the

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BLM animal model is the most useful experimental tool that is commonly used to investigate treatment options for respiratory disorders. BLM is a chemotherapeutic antibiotic that has been identified as a pro-fibrotic agent when lymphoma patients developed pulmonary fibrosis after intravenous administration of BLM. It has been used in multiple species including mice, rats, guinea pigs, hamsters, dogs, and primates; yet, mice are most common.[6]

The physiopathology of pulmonary fibrosis is still poorly elucidated, and the current treatment based on a combination of corticosteroids (anti-inflammatory) and azathioprine (anti-inflammatory and immunosuppressive) is partially effective.[7,8] Unfortunately, until today, lung transplant is often the only viable treatment option and no therapy has been proven to prolong survival.[9] Therefore, new therapeutic molecules with same properties as the marketed drugs, better efficacy and tolerability and less side effects for the pulmonary fibrosis were needed.

In this context, effects of several natural bioactive compounds, especially plant extracts, on attenuation of BLM-induced pulmonary fibrosis have been reported in the literature, such as *Ginkgo biloba* (100 mg/kg),[10] *Trigonellafoenum graceum* (5-40 mg/kg),[11] *Houttuynia cordata* (1 g/kg),[12] and *Juglans regia* (150 mg/kg).[13] Some of these studies claim that the phenolic compounds like gallic acid and quercetin present in plant extracts might play a major role in protecting or preventing the development of this respiratory pathology.[14] So far, no information regarding benefic effect of marine seaweeds against BLM-induced fibrosis in rat has been documented.

In the last decades, seaweeds are considered a huge reservoir of bioactive compounds such as bioactive peptides, lectins, carotenoids, polysaccharides, fatty acids, flavonoids, proteins, tocopherols, and phytosterols, with different beneficial properties and potential application in food, cosmetic and pharmacological industries.[15,16] Due to these biocompounds, macroalgae present a wide variety of bioactivities such as antioxidant, anti-viral, anti-fungal, antibacterial, antiproliferative, anti-inflammatory, neuroprotective, adipogenesis, and antidiabetic.[17] The red seaweed, *Corallina officinalis*, is a well-known edible seaweed in China and many other countries, and also documented as a drug in traditional Chinese medicine for over 100 years. Several molecules derived from this red species have demonstrated important antimicrobial anti-inflammatory and analgesic bioactivities.[18–20] Thus, the aim of this study was to investigate the protective effect of red seaweed extract COAE against BLM-induced pulmonary fibrosis and oxidative stress in rat.

Material and Methods

Red Seaweed (C Officinalis) Collection

The *C officinalis* biomass was collected in January 2020 from Menzel Abderrahmane city, which is in the north-west of Tunisia (37° 13′ 48″ N and 9° 51′ 36″ E). The collected samples were packed in polyethylene bags and transported to the laboratory within 2 h. Upon arrival, seaweeds were washed with tap water to remove epiphytes, sand and potential contaminants. Fresh seaweeds were subsequently dried in a dark room at ambient temperature for a few weeks. Then, the dried samples were pulverized into a fine powder using a blender (Knife Mill Grindomix GM 200, Retsch, Haan, Germany) and stored in airtight glass jars in the refrigerator for further analysis.

Enzyme-Assisted Extraction

Enzymatic extraction was performed in a small bioreactor in which 1 g of seaweed powder and 100 mL of water were mixed. The reaction vessel was connected to a water bath maintained at optimal temperature needed for the enzyme action. When the optimal temperature has reached, the commercial Alcalase® 2.4 L (5% weight/dry weight, w/dw) were added to the mixture and allowed to react during 24 h at pH 8.0 and 50°C under constant gentle stirring (150 rpm). After the required digestion time, the reaction was stopped by heating the solution at 85°C during 15 min to inactivate the enzyme. After extraction, the samples were filtered, on a Buchner system using a synthetic cloth, and then centrifuged at 10000×g for 20 min at 10°C to separate insoluble and soluble fractions. Finally, the soluble phase was freeze dried, weighed for calculating yield (10.56 ± .02%) and stored at −80°C freezer until further use. The lyophlized powder obtained referred to as *C officinalis* aqueous extract (COAE).

Quantitative Polyphenol Analysis

Total phenolic content of COAE measured with a colorimetric assay using Folin-Ciocalteu's phenol reagent.[21] Briefly, 50 µL of diluted sample was added to 120 µL of Folin-Ciocalteau reagent and 2 mL of distilled water and mixed thoroughly for 5 min. Then, 375 µL of 10% (w/v) sodium carbonate was added and the mixture was allowed to stand for 2 hours at room temperature. The absorbance was measured at 765 nm using UV-VIS spectrophotometer. Gallic acid was used as a phenolic standard to quantify the total phenolic content. The total phenolic content of COAE was expressed as mg gallic acid equivalents (GAE)/g dry weight (DW). The test was carried out in triplicate.

Total flavonoid content of COAE was determined using aluminum chloride colorimetric method.[22] Briefly, 400 µL of diluted sample was mixed with 120 mL of 5% (w/v) $NaNO_2$ and 120 mL of 10% (w/v) AlCl3 was added. At 6 min, 800 µL of NaOH (1 M) was added. The absorbance of the mixture was measured at 510 nm using UV-VIS spectrophotometer. Catechin was used as a condensed flavonoid standard to quantify the total flavonoid content. The total flavonoid content of COAE was expressed as mg quercetin equivalents (QE)/g DW. The test was carried out in triplicate.

Total condensed tannin content in COAE was determined with the acid vanillin assay.[23] Briefly, 50 µL of extract was mixed with 1500 µL of 4% (w/v) vanillin (prepared with absolute methanol) and then 750 µL of hydrochloric acid (36%) was added. The mixture was left to stand at room temperature for 20 min. The absorbance of the mixture was measured at 510 nm. Catechin was used as a condensed tannin standard to quantify the total condensed tannin content. The total condensed tannin content of COAE was expressed as mg catechin equivalents (CE)/g DW. The test was carried out in triplicate.

In vitro *Antioxidant Activities Assays*

The enzymatic COAE at different concentrations were evaluated for antioxidant activity by using three *in vitro* tests, viz, DPPH radical scavenging, iron chelating property, and reducing power. All the analysis was performed in triplicate unless otherwise specified.

DPPH Radical Scavenging Capacity. The scavenging capacity of COAE for DPPH (1,1-diphenyl-2-picrylhydrazyl) radical were examined according to Bersuder et al.[24] Briefly, 500 µL of test sample was mixed with 375 µL of 100% ethanol and 125 µL of .02 mM DPPH ethanol solution. The mixture was vortexed then left for 60 min in the dark at room temperature. The absorbance was read at 517 nm and percentage of DPPH radical scavenging activity was calculated using the following equation

$$\text{DPPH radical} - \text{scavenging}\,(\%) = \left[1 - \frac{A_{517}\ \text{sample}}{A_{517}\ \text{control}}\right] \times 100$$

The control was conducted in the same manner, except that distilled water was used instead of sample. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a standard.

Reducing Power Assay. The ability of COAE to reduce iron (III) was determined according to the method of Yildirim et al.[25] An aliquot of .5 mL sample at different concentrations was mixed with 1.25 mL of .2 M phosphate buffer (pH 6.6) and 1.25 mL of 1% potassium ferricyanide solution. The mixtures were incubated at 50°C for 30 min, followed by addition of 1.25 mL of 10% (w/v) trichloroacetic acid. The mixture was then centrifuged at 3000×g for 10 min. Finally, 1.25 mL of the supernatant was mixed with 1.25 mL of distilled water and 25 mL of .1% (w/v) ferric chloride. After 10 min reaction, the absorbance of the resulting solution was measured at 700 nm. BHA was used as positive control.

Ferrous ion-chelating Ability Assay. The chelating capacity of COAE for Fe^{2+} was measured according to the method described by Dinis et al.[26] To .5 mL of COAE, at different concentrations, 1.6 mL of distilled water and .05 mL 2 mM of $FeCl_2$ was added, followed by the addition of .1 mL of 5 mM ferrozine after 15 min. After a 10 min reaction time at room temperature, the absorbance of the Fe^{2+}–ferrozine complex, with red or violet color, was measured at 562 nm. The chelating antioxidant activity for Fe^{2+} was calculated according to the following formula

$$\text{Chelating activity}\,(\%) = \left[1 - \frac{A_{562}\ \text{of sample}}{A_{562}\ \text{of control}}\right] \times 100$$

The control was conducted in the same manner, except that distilled water was used instead of the sample. EDTA was used as standard.

Animal Model of Bleomycin-Induced Lung Fibrosis

Male Wistar rats, weighing between 180 and 220 g, were obtained from the Central Society of Pharmaceutical Industries of Tunisia (SIPHAT, Ben-Arous, Tunisia). The animals were separated into different groups and acclimatized for 15 days with a standard pellet diet (standard pellet diet Badr-Utique-TN) and water *ad libitum* (22 ± 2°C; 12 h dark/light cycle). All experiments were performed according to the recommendations of the local Ethics Committee on Animal Welfare in strict conformity with NIH guideline for the care and use of laboratory animals.[27]

Bleomycin rat model of lung fibrosis as reported earlier by Abidi et al,[28] was used in this study. All rats underwent anesthesia by intraperitoneal injection of 75 mg/kg body weight (bw) of pentobarbital sodium solution (Sandoz Laboratory, Paris, France). Each anaesthetized rat was immediately suspended from gallows. Induction of fibrosis was done by an intra-tracheal instillation of 2 mg/kg bw of bleomycin (Bleomycin®, Laboratories Aventis, France) in 200 µL saline.[28]

Experimental Design

A total of forty rats were randomly divided into four experimental groups of ten animals each (n = 10). The first (G1) served as a normal control group that received any treatment, only .9% saline (orally 2 mL/kg bw); the second group (G2) served as a positive control (BLM model group) that received a single intratracheal instillation of bleomycin (2 mg/kg bw), 3 days later they received by gavage .9% saline (2 mL/kg bw) once daily for 21 days; the third group (G3) received BLM solution intratracheally (2 mg/kg bw), 3 days later they were treated with COAE (150 mg/kg) weekly for 21 days (BLM/COAE-150 group), and the fourth group (G4) received BLM solution intratracheally (2 mg/kg bw), 3 days later they were treated with COAE (300 mg/kg) weekly for 21 days (BLM/COAE-300 group). For all studied groups, daily gastric gavage was performed each morning at 9 a.m. In the course of the experiment, the body weight of rats in each group, the food

consumption and volume of drunken water were measured on days 3, 4, 7, 14, and 21.

Sacrifice, Organ Sampling and Bronchoalveolar Lavage Fluid Recovery

At the end of the treatment, rats were anesthetized with the same procedure described during the induction of fibrosis and then were euthanized by injection of a lethal dose (200 mg/kg bw) of sodium pentobarbital. Blood samples were collected by cardiac puncture and centrifuged at 5000 rpm for 10 min at 4°C. The serum was stored at −80°C for subsequent assays.

Following the sacrifice, the diaphragm and the anterior thorax were cut to extract the heart-lung block. Before extraction, the bronchoalveolar lavage fluid (Balf) was collected from some lobes of the right lung by intratracheal injections of saline (4 × 5 mL) via a catheter and re-aspiration of the liquid between two fractions. The lobes of left lungs were fixed by intratracheal injection of a 10% formalin solution (6 to 8 mL) and immersed in formalin for 48 h before histological examination. Then, the remaining lungs were rapidly excised, trimmed of extraneous tissue, rinsed, and weighed for the lung index determination. The ratio of net lung weight (mg) to the body weight (g) for each rat was used as the lung index. In addition, liver, and kidney tissues were removed and used for a biochemical determination of protein, malondialdehyde (MDA) levels, as well as antioxidant enzyme activities.

Bronchoalveolar Lavage

The Balf sample was centrifuged at 3000 rpm for 5 min at 4°C, and the supernatant was discarded, and the cell pellet was resuspended with 50 µL saline solution. 10 µL of the cell suspension was pipetted and the total number of cells was counted with a hemocytometer.

Then, 30 µL of cell suspension was pipetted, and cell smears were prepared and stained with Wright's-Giemsa to distinguish different types of cells under a light microscope.[29]

Determination of Oxidative Stress biomarkers

Oxidative stress status of lung, liver and kidney was assessed by estimation of malondialdehyde (MDA), thiol group, and protein contents, as well as by the measurement of antioxidant enzymes activities: catalase (CAT) and superoxide dismutase (SOD).

Protein levels were estimated using the Bradford assay method, using bovine serum albumin as standard.[30]

Lipid peroxidation was evaluated by measuring MDA using the double heating method.[31] Briefly, tissues homogenates were combined with a butylated hydroxytoluene (BHT)–trichloroacetic acid (TCA) solution containing 1% BHT (w/v) and 20% (w/v) TCA. The mixture was centrifuged at 1000×g for 5 min at 4°C. The obtained supernatants were blended with .5 N HCl and 120 mM of thiobarbituric acid (TBA) prepared in 26 mM Tris buffer and then heated in a laboratory water bath at 80°C for 10 min. After rapid cooling, an absorbance reading was obtained with a UV-vis spectrophotometer at 532 nm. MDA levels were calculated using an extinction coefficient for the MDA–TBA complex of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

CAT activity was assessed by measuring the rate of H_2O_2 disappearance at 240 nm.[32] Briefly, the assay mixture contained 33 mM H_2O_2, 50 mM phosphate buffer (pH 7) and 30 µL tissue extract. CAT activity was calculated in terms of µmole of H_2O_2 consumed/minute/mg of protein using the extinction coefficient of 40 mM/cm for H_2O_2.

SOD activity was determined as described previously by Misra and Fridovich.[33] It is based on the inhibition of the autooxidation of epinephrine to adenochrome at pH = 10.2. Briefly, the assay mixture included 10 mL bovine catalase (.4 U/mL), 62.5 mM sodium carbonate-sodium bicarbonate buffer and 20 mL epinephrine (5 mg/mL). One unit of SOD is defined as the enzyme required to inhibit 50% of adenochrome generation. The absorbance was recorded at 480 nm.

Sulfhydryl groups (–SH) contents were performed according to Ellman's method.[34]

Histological Analysis

For histological study, rat lungs were fixed in 10% formalin solution for 24 h, dehydrated in a graded series of ethanol, embedded in paraffin, cut into 5 mm thick serial sections, and stained with hematoxylin and eosin to identify inflammatory cells and Masson's trichrome to identify collagen deposition in different sections. The entire lung section was observed at ×100 magnification under light microscope.

Statistical Analysis

All experimental data were analyzed by the professional edition of the Statistical Package for the Social Sciences (SPSS) version 18.0 software package (SPSS Inc. Chicago, IL, USA) and expressed as means ± standard deviation (SD). The data are representative of six to eight distinct observations. Statistical differences between the groups were analyzed with one-way analysis of variance (one-way ANOVA) followed by Bonferroni's post hoc (multiple comparison) test. Differences were considered statistically significant at $P < .05$.

Results

Total Phenolic, Condensed Tannin, and Total Flavonoid Contents of COAE

The results of phytochemical analysis of COAE indicate that the mean polyphenols, flavonoids, and condensed tannins contents were equivalent to 4.56 ± .42 mg GAE/g DW, 3.18 ±

Table 1. Phytochemical composition of COAE.

Total Polyphenol Content (mg GAE/G DW)	4.56 ± .42
Total flavonoid content (mg QE/g DW)	3.18 ± .30
Total condensed tannin content (mg CE/g DW)	.057 ± .02

.3 mg QE/g DW, and .057 ± .02 mg CE/g DW, respectively (Table 1).

In vitro *Antioxidant Activity of COAE*

The antioxidant activity of COAE was evaluated by various antioxidant assays, including 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, reducing power and ferrous ion-chelating ability assay (Figure 1).

DPPH Radical Scavenging Activity. DPPH method is widely used for investigating the free radical scavenging activities of compounds. DPPH is a stable free radical that shows maximum absorbance at 517 nm. When DPPH radicals encounter a proton-donating substrate, such as an antioxidant, the radicals are scavenged and the absorbance is reduced. Figure 1A shows the DPPH free radical-scavenging capacities of COAE and Trolox, used as positive control, at various concentrations. The results clearly indicated that COAE had significantly ($P < .05$) higher ability scavenging activity with a dose-effect relationship (Figure 1A). The maximum scavenging capacity of 96.16 ± .28% was obtained at a relatively high concentration range (6 mg/mL). However, COAE showed lower radical scavenging activity than Trolox at the same concentrations. The concentrations of the studied red seaweed extract and Trolox required to scavenge 50% of the DPPH radicals (IC_{50}) were also determined in this study. The IC_{50} value for the COAE was 1.39 ± .03 mg/mL. On the other hand, the IC_{50} value of the standard (Trolox) was 2.78 ± .14 µg/mL.

Reducing Power. The reducing power assay is often used to evaluate the ability of natural antioxidant to donate an electron or hydrogen. The determination of the ferric reducing/antioxidant was based on the reduction of Fe^{3+}/ferricyanide complex to the ferrous form in presence of reductants (antioxidants) in the tested samples. The Fe^{2+} was then monitored by measuring the formation of Perl's Prussian blue at 700 nm. In fact, it is widely accepted that higher absorbance at 700 nm is correlated to power reducing. As shown in Figure 1B, the reducing capacity of COAE increased in a concentration-dependant manner. However, the reducing power of COAE remained significantly lower than that of BHA. At 6 mg/mL, the reducing power of COAE reached a maximum value of 2.53 whereas that of BHA was 3.0.

Metal-chelating activity. In the present study, ferrous chelating activities of the COAE, determined at different concentrations (1–6 mg/mL), are presented in Figure 1C. The results revealed that COAE presented a significative higher ($P < .05$) ferrous chelating capacity. It was clear that the effect of the red seaweed extract on the chelating capacity was dose dependent upon concentrations (Figure 1C). COAE was also noted to exhibit excellent ferrous chelating capacity, with the highest level (74.88%) being observed at a concentration of 6 mg/mL. Indeed, the metal chelating activity of EDTA (98.85%) was only 1.3 times higher than COAE activity at the same concentration under the same experimental condition (Figure 1C). The IC_{50} values for the ferrous chelating capacity of COAE and EDTA were 1.01 ± .12 mg/mL and .08 ± .01 mg/mL, respectively.

Effect of COAE on Rat Body Weight, Food Consumption and Water Intake

The change in rats body weight of the different groups between days 0 and 21 was determined and the results are given in Table 2. Bleomycin caused a significant ($P < .05$) body weight loss in exposed rats compared to the control group throughout the experiment period. However, control and COAE groups showed a significant ($P < .05$) and progressive increase in body weight gain, starting on the seventh day (Table 2). Interestedly, treatment with COAE (150 and 300 mg/kg) had a tendency to prevent the body weight loss two weeks after drug administration, but it was not statistically significant ($P > .05$).

Differences in food consumption (Table 3) and water intake (Table 4) were recorded between all groups throughout the treatment period. As shown in Tables 3 and 4, BLM causes a remarkable diminution in these parameters in treated rats compared to normal rats. However, the treatment with COAE at different doses (150 and 300 mg/kg) considerably increases access to water and food consumption in a dose-dependent response in comparison to BLM group. No significant difference ($P > .05$) was detected in the food and water intake between the negative control and the rats treated with 300 mg/kg of COAE.

It is still important to note that throughout the experiment, no deaths were recorded in all groups of rats studied. Also, a decrease in the morbidity of the rats was observed following the induction of fibrosis with BLM. In addition, no toxicity or adverse side effects were noted following gastric gavage treatment of COAE.

Effect of COAE on Lung Index and Bronchoalveolar Fluid Cells

For further investigate the protective effect of COAE on BLM-induced pulmonary fibrosis in rat, the lung index and bronchoalveolar lavage cells (Balf) count were determined among groups and the results are presented in Figure 2. In contrast with body weight loss, the lung index of the BLM-treated rats

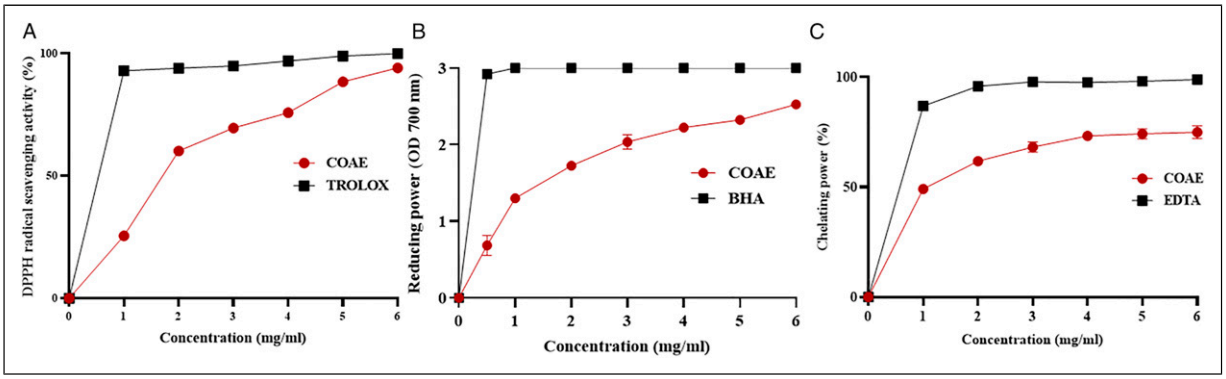

Figure 1. Antioxidant activities of the COAE; (A) DPPH radical scavenging activity, (B) reducing power assay; (C). Metal-chelating activity. COAE, *Coralina officinalis* aqueous extract.

Table 2. Effect of bleomycin instillation and/or COAE treatment on rat body weight variation.

	Body Weight (g)				
	Day 0	Day 3	Day 7	Day 14	Day 21
Control	302.33 ± 11.23	309.33 ± 8.08	308.33 ± 12.22	311.66 ± 10.40	323.33 ± 35.11
BLM	296.66 ± 15.27[a]	281.66 ± 12.58[a]	303.00 ± 8.00[a]	309.33 ± 10.01	316.00 ± 10.14[a]
BLM/COAE-150	290.50 ± 10.53[a,b]	270.25 ± 4.78[a,b]	295.75 ± 11.02[a,b]	310.25 ± 9.32	328.50 ± 6.85[a,b]
BLM/COAE-300	306.25 ± 18.87[a,b]	287.00 ± 11.46[a,b]	297.75 ± 18.98[a,b]	325.25 ± 18.26[a,b]	346.00 ± 23.07[a,b]

Number of rats: n = 10/group. Control: Negative control group, BLM: Intratracheal instilled bleomycin group, BLM/COAE: *C. officinalis* aqueous extract at different doses (150 and 300 mg/kg) after BLM instillation. Values are the mean ± SD;
[a]$P < .05$ compared to control group and
[b]$P < .05$ compared to BLM group (ANOVA test).

Table 3. Effect of bleomycin instillation and/or COAE treatment on food intake of rats.

	Food Intake (g)				
	Day 0	Day 3	Day 7	Day 14	Day 21
Control	20.65 ± .87	18.97 ± .54	19.50 ± .60	19.25 ± .77	21.07 ± .75
BLM	19.89 ± .66	8.75 ± .75[a]	12.43 ± .33[a]	14.35 ± .43[a]	16.86 ± .66[a]
BLM/COAE-150	18.66 ± .43[a,b]	15.76 ± .61[a,b]	15.71 ± .55[a,b]	16.15 ± .67[a,b]	19.88 ± .65[a,b]
BLM/COAE-300	16.69 ± .38[a,b]	14.8 ± .73[a,b]	15.68 ± .27[a,b]	17.09 ± .37[a,b]	20.88 ± .75[b]

Number of rats: n = 10/group. Control: Negative control group, BLM: Intratracheal instilled bleomycin group, BLM/COAE: *C. officinalis* aqueous extract at different doses (150 and 300 mg/kg) after BLM instillation. Values are the mean ± SD;
[a]$P < .05$ compared to control group and
[b]$P < .05$ compared to BLM group (ANOVA test).

(15.76 mg/g) increased due to lung weight gain after bleomycin injection compared with that of the normal control rats (9.53 mg/g) (Figure 2A). On the other hand, COAE (150 and 300 mg/kg) treatment decreased the lung index compared to the bleomycin group (Figure 2A). Especially, the COAE-300 mg/kg group has a similar lung index, which almost restored to the normal level of control group, in order of 9.31 mg/g.

The effect of COAE on inflammatory cell infiltration in the lung was investigated by counting these later in collected Balf (Figure 2B). The results obtained showed that the total number of cells in the BLM group (35.25 ± 2.73 104/mL) were significantly ($P < .05$) higher than the control group (13.33 ± 1.03 104/mL). In contrast, the COAE-treated groups presented a significant ($P < .05$) decrease in total cells count in a dose-dependent manner compared to the BLM group, which suggested that COAE could effectively reduce the total cells count of Balf.

Furthermore, BLM significantly increased monocytes (11.00 ± 1.78 10^4/mL) and lymphocytes (22.5 ± 1.25 10^4/mL) count in Balf ($P < .05$) (Figure 2B). After treatment with COAE (150 and 300 mg/kg), the number of

Table 4. Effect of bleomycin instillation and/or COAE treatment on water intake in rats.

	Water Intake (mL)				
	Day 0	Day 3	Day 7	Day 14	Day 21
Control	44.16 ± 2.31	43.32 ± 1.29	43.00 ± 1.44	44.05 ± 1.53	44.03 ± 1.72
BLM	44.35 ± 1.27	25.57 ± .45[a]	28.29 ± 1.08[a]	32.30 ± 1.13[a]	37.95 ± .62[a]
BLM/COAE-150	44.55 ± .67	25.36 ± 1.14[a]	25.27 ± .70[a,b]	30.75 ± .80[a]	40.55 ± .61[a,b]
BLM/COAE-300	44.71 ± .82	28.00 ± 1.29[a,b]	30.79 ± 1.14[a,b]	32.33 ± 1.17[a]	44.09 ± .95[b]

Number of rats: n = 10/group. Control: Negative control group, BLM: Intratracheal instilled bleomycin group, BLM/COAE: *C. officinalis* aqueous extract at different doses (150 and 300 mg/kg) after BLM instillation. Values are the mean ± SD;
[a]$P < .05$ compared to control group and
[b]$P < .05$ compared to BLM group (ANOVA test).

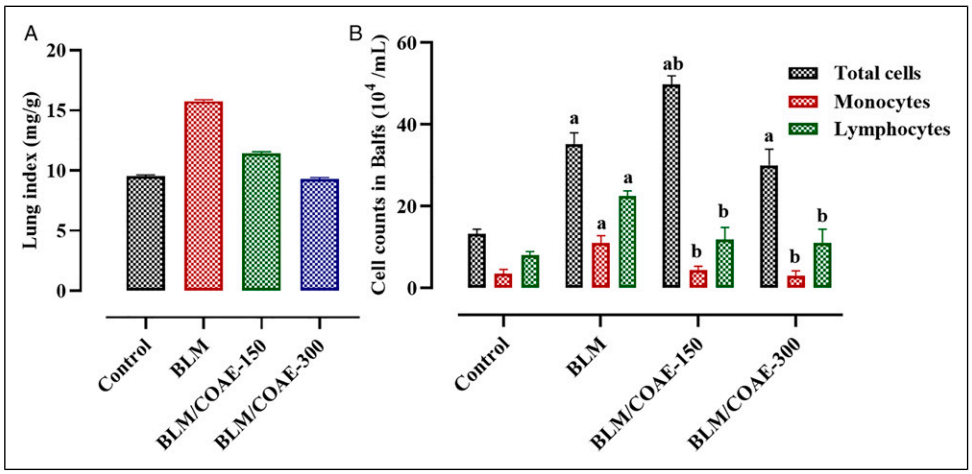

Figure 2. Effects of COAE and/or BLM on lung index of BLM-induced PF in rat (A) Cell counts and classification in Balf (B). Values are the mean ± SD; a: $P < .05$ compared to control group and b: $P < .05$ compared to BLM group (ANOVA test). COAE, *Coralina officinalis* aqueous extract; BLM, Bleomycin; Balf, Bronchoalveolar lavage fluid.

monocytes and lymphocytes decreased significantly ($P < .05$) in a dose-dependent manner compared to the BLM group. The above results demonstrate that the bioactive extract of *C officinalis* could significantly reduce the production of inflammatory cells in Balf, suggesting an anti-inflammatory effect of COAE that leads to a remarkable attenuation of the damage caused by pulmonary fibrosis induced by BLM.

Effect of COAE on Protein Levels

In order to assess the extent of fibrosis, the lungs, kidneys and liver protein levels were determined (Figure 3). As shown in Figure 3, the protein levels in all tissues sampled from BLM-treated rats was decreased markedly ($P < .05$) compared with that of the normal control rats. At day 21 after bleomycin instillation, the protein levels recorded at lungs, kidneys and liver are 16.19 g/100 g, 15.70 g/100 g, and 23.37 g/100 g, respectively. The decreased protein contents in all homogenates were significantly ($P < .05$) reduced dose dependently with COAE (150 and

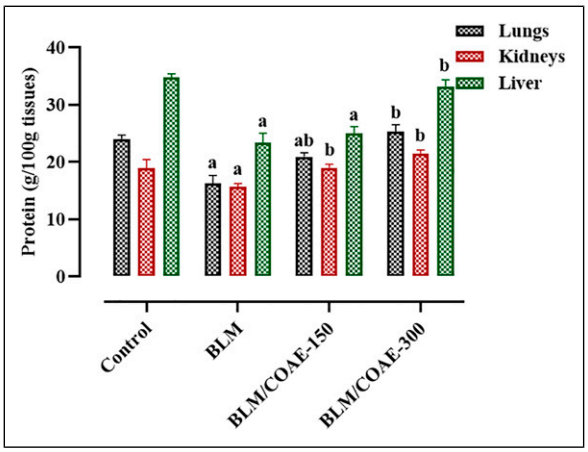

Figure 3. Effects of COAE and/or BLM on lungs, kidneys, and liver Protein levels. Values are the mean ± SD; a: $P < .05$ compared to control group and b: $P < .05$ compared to BLM group (ANOVA test). Number of rats: n = 10). COAE, *Coralina officinalis* aqueous extract; BLM, Bleomycin.

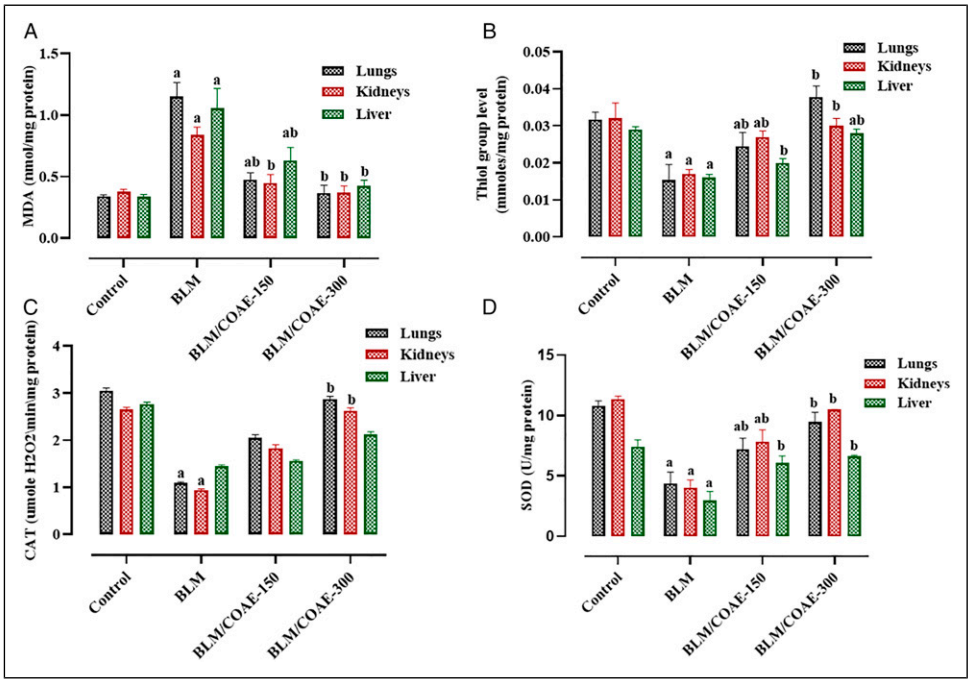

Figure 4. Effects of COAE and/or BLM on lungs, kidneys, and liver MDA levels (A), sulfhydryl groups (B), CAT (C) and SOD (D). Values are the mean ± SD; a: $P < .05$ compared to control group and b: $P < .05$ compared to BLM group (ANOVA test). Number of rats: n = 10. COAE, *Coralina officinalis* aqueous extract; BLM, Bleomycin; MDA, malondialdehyde; CAT, Catalase; SOD, Superoxyde dismutase.

300 mg/kg) administration (Figure 3). Our results showed also a significant increase in lung protein content following BLM/COAE-300 mg/kg treatment to reach a level slightly higher than the normal control rats (25.23 ± 1.28 vs 24.01 ± .66 g/100 g of lung tissue, respectively, $P < .05$).

Effect of COAE on Lipoperoxidation and Antioxidant Enzymes Activities

To investigate whether the effect of COAE on BLM-induced fibrosis was associated or not with antioxidant activity *in vivo*, some oxidative stress markers including MDA, SOD, CAT and sulfhydryl group were further determined (Figure 4). Lipid peroxidation was assessed by MDA determination and the results are shown in Figure 4A. MDA levels in all tissues tested significantly increased in BLM treated rats compared to control group ($P < .05$). Indeed, MDA lung level is significantly higher in the BLM group compared to the control group (1.15 ± .11 vs .34± .10 nmol/g protein, ($P < .05$), respectively. COAE administration at 300 mg/kg to BLM treated rats significantly lowered the MDA levels compared to bleomycin group ($P < .05$); whereas 150 mg/kg caused a moderate change in MDA levels especially in lung (Figure 4A). In addition, instillation with BLM alone dramatically decreased the levels of thiol group in the lungs, liver, and kidneys tissues. However, treatment with COAE counteracted efficiently the BLM-induced oxidation processes within all studied tissues (Figure 4B).

On the other hand, the activities of catalase in lung, liver, and kidneys tissues, as shown in Figure 4C, were significantly reduced by BLM treatment when compared with control (1.08 ± .31 vs 3.05 ± .98 µmol/min/g protein, 1.44 ± .35 vs 2.76 ± .46 µmol/min/g protein, .94 ± .23 vs 2.64 ± .52 µmol/min/g protein, respectively, ($P < .05$)), while in BLM/COAE-150 mg/kg and BLM/COAE-300 mg/kg groups, this activity is significantly restored when compared to the BLM group. Similarly, BLM significantly decreased the activities of SOD in all tissues samples as compared to the normal control rats ($P < .05$) (Figure 4D). Meanwhile, COAE (150 et 300 mg/kg) significantly enhanced the activity of SOD as compared to the BLM group ($P < .05$).

Histopathological Analysis

Figure 5A displays histopathological findings in lung tissue stained with hematoxylin-eosin. As expected, control lungs exhibited normal pulmonary architecture with normal alveolar spaces and normal thickening of alveolar septa (Figure 5A, G1). Whereas, lungs from rats treated with BLM alone were characterized by lymphocytic inflammatory redesign, disturbance of the cellular architecture with fibrous alterations as well as the presence of small nodules. These animals present also a severe lung inflammation with massive infiltration of inflammatory cells obvious alveolar wall thickening and the presence of inter and intra-alveolar plasma cells (Figure 5A, G2). Treatment with COAE reduced the severity almost all BLM-induced lung damages. In fact, the BLM/COAE-

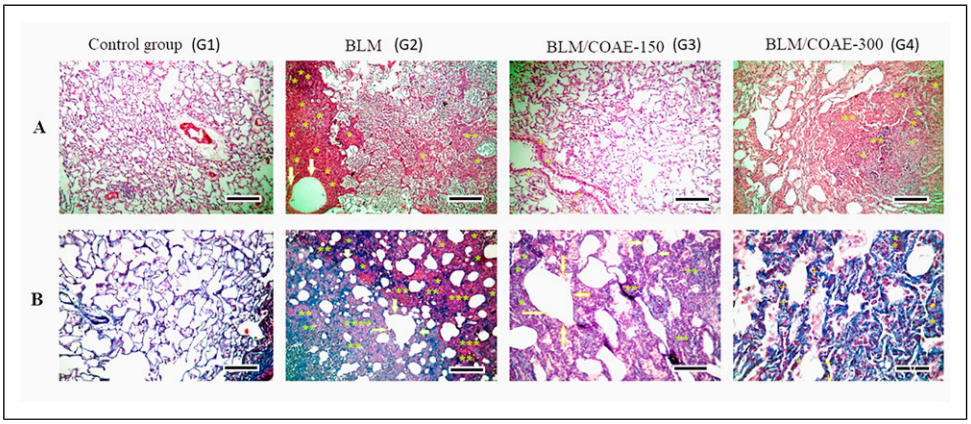

Figure 5. Evolution of histological alteration of lung rat after BLM instillation and/or COAE treatment. (A) Hematoxylin and eosin-stained lung tissue (Magnification, ×100; scale bar, 10 μm), (B) Masson's Trichrome stained lung tissue (Magnification, ×100; scale bar, 10 μm). Histological observation showed Fibrotic infiltrate: Dilatation of cystic bronchi with disappearance of alveoli and presence of necrotic material (*), Small fibroblastic foci (+), and Honeycomb ( ). (G1) A Control group; (G2) a rat receiving BLM (G3) a rat receiving BLM/COAE-150 and (G4) a rat receiving BLM/COAE-300. (Magnification, ×100; scale bar, 10 μm). Number of rats: n = 10. BLM, Bleomycin; COAE, *Coralina officinalis* aqueous extract.

150 mg/kg group showing a minimal thickening of walls and the lack of detectable damage of the lung architecture (Figure 5A, G4).

Figure 5B displays histopathological findings in lung tissue stained with Masson's trichrome. Condensed bundles of collagen recognized in green color are markedly evident in the BLM group (Figure 5B. G2) compared with the control rats (Figure 5B. G1). Treatment with COAE at 150 mg/kg showed moderate decrease in collagen bundles compared to BLM group (Figure 5B. G3). On the other hand, pulmonary lesion was significantly lower in BLM/COAE +300 mg/kg group compared to BLM group. Collagen deposition is moreover increasingly less abundant in these groups with better protection in the group treated with COAE at 300 mg/kg (Figure 5B. G4).

Discussion

Idiopathic pulmonary fibrosis (IPF) is an incurable, progressive, fatal lung disease caused by genetic and environmental factors or associated to other lung disorders. Currently, FDA-approved drugs for IPF include nintedanib and pirfenidone, which show poor efficacy in most patients.[35] Therefore, we need to discover novel drugs with more efficacy and less adverse effects for the treatment of IPF. Recently, several agents, especially from plant resources, with potent antioxidant and anti-inflammatory activities exhibit protective effects against bleomycin-induced lung injury.[36,37] Seaweed and seaweed components have revealed prospective medical values with potent antioxidant and anti-inflammatory effects.[38] To our knowledge, the effect of red seaweed extract on pulmonary fibrosis has never been reported. For the first time in the present study, the possible protective effect of COAE against pulmonary fibrosis and oxidative stress induced by bleomycin was investigated in rats.

In this study, we showed the beneficial and potential therapeutic effects of COAE on the progression of lung fibrosis in BLM-treated rats. Our results confirm by the determination of biochemical markers, enzymatic activities and histological analysis that COAE can attenuate BLM-induced lung fibrosis by normalizing pro-oxidant parameters and by enhancing the activities of antioxidant enzymes. This COAE effect appears to be mediated through inhibition of both inflammation and oxidative stress in BLM-induced pulmonary fibrosis in rats.

Furthermore, the rigidity and structural complexity of the algal cell wall is the major obstacle to efficient extraction of intracellular bioactive constituents. Enzymatic extraction has also been reported to increase the extractability of bioactive compounds from several marine algae.[39,40] In the present study, enzyme-assisted extraction of *C officinalis* was proposed as an eco-friendly method of red seaweed valorization. Commercial Alcalase® used during extraction might have broken down different peptide bonds in the cell wall proteins as well as the proteins inside the cells releasing more soluble bioactive compounds.

Generally, the amount of specific secondary metabolites, with the most important of them being phenolic molecules, dictates the effective bioactive potential of seaweeds. Our results show that COAE has appreciable amounts of total polyphenols and flavonoids. Moreover, COAE has demonstrated high capacities of DPPH free radical scavenging, ferric iron reducing and metal chelating, which reflects the pronounced antioxidant potential of this red seaweed. It has been reported that the presence of phytoconstituents such as flavonoids, tannins and polyphenols prevent a number of diseases through their free radical scavenging activity.[41]

However, the synthesis and diversity of phenolic chemicals are intimately tied to the seaweed taxonomic group and individual species.[42] Furthermore, phenolic acids such as benzoic acid, p-hydroxybenzoic acid, salicylic acid, gentisic acid, protocatechuic acid, vanillic acid, gallic acid, and syringic acid have been found in the genus Gracilaria (Rhodophyta, red alga).[43,44] By contrast, several studies investigating different seaweed species have reported that antioxidant activity is not necessarily correlated with the content of phenolic compounds in the seaweed extracts, suggesting the presence of other substances such as pigments (chlorophyll and carotenoids), small peptides, and sulfated polysaccharides of low molecular weight.[45,46]

In the present study, BLM administration resulted in body weight loss, lung index increase, progressive and significant inflammation, and severe alveolar destruction in rat lungs. In addition, significant reductions in antioxidant enzymes activities and sulfhydryl group levels and elevation of MDA content were detected in the rat lung tissues following BLM exposure. However, our results demonstrated that COAE clearly attenuated fibrotic lesions in the bleomycin group. The body weight loss, lung index, and total and differential cell numbers of Balf, histopathological damages of fibrosis were significantly reversed by COAE in a dose-dependent manner. In addition, COAE reversed the CAT and SOD activity, as well as proteins and MDA levels, near to normal values in the BLM/COAE-treated groups.

Body weight variation is among appropriate indicators for monitoring animal health status during BLM-induced lung injury progression. Treatment with BLM caused a significant weight loss and a significant lung index increase in rats. In the more commonly used model of lung fibrosis, where BLM is administered by intratracheal instillation, mice show rapid, and substantial loss of body weight.[47] COAE administration increased body weight while these treatments decreased organ-body weight.

On the other hand, the oxidative stress plays a crucial role in the pathological development of pulmonary fibrosis and the production of oxygen-reactive species (ROS) has been reported to be implicated, at least, in part in the pathogenesis of fibrosis.[48,49] MDA is one of the most commonly used biomarkers for lipid peroxidation and its increased intracellular level synonymous with oxidative cell membrane damage in BLM lung injury.[50] Our results founded that BLM induced an increase of MDA level in all tissues studied, especially in lung. However, COAE treatment significantly reduced the MDA levels, indicating decreased degree of oxidative stress and subsequent lipid peroxidation. This protective effect of COAE is thought to be connected to its free radical scavenger effect and antioxidant activity, decreasing oxidative stress caused by BLM via generation of ROS.

Endogenous antioxidant enzymes such as SOD and CAT are an important indicator for oxidant and antioxidant status and have the ability to remove toxic insults derived from chemicals, oxidative stress and metabolites from cells.[51] SOD maintain the dynamic balance of free radical generation and scavenging, which can catalyze the dismutation of the highly reactive superoxide anion to O_2 and to the less reactive species H_2O_2.[52] CAT catalyzes the degradation or reduction of H_2O_2 to water and molecular oxygen, consequently completing the detoxification process imitated by SOD.[53] In agreement with previous studies, our results showed that BLM markedly decreased the SOD and CAT activities, as well as the thiol group level, in the different studied tissues including lung.[36,54] Meanwhile, treatment with COAE at 300 mg/kg resulted in a significant decrease in lung MDA with a significant increase in SOD and CAT activities imply that COAE is beneficial in maintaining oxidant-antioxidant balance. This effect may be attributed to the stimulating effect of bioactive substances of red seaweed such as peptides, mycosporine-like amino acids, polysaccharide and polyphenols which have been described to play antioxidant effects.[55,56] In addition, the restoration of the enzymatic activities of SOD and CAT could be due to the possible stimulation of the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway by the pro-oxidant biomolecules contained in COAE. Indeed, Nrf2 is a crucial regulator of the cellular antioxidant response that induces the expression levels of various antioxidant genes, in order to enhance SOD and catalase activities, as well as other cytoprotective enzymes.[57]

In laboratory animals, Balf is commonly used to monitor inflammatory responses, immune mechanisms, and infectious disease processes that occur in the pulmonary airways.[58] It has been demonstrated that macrophages and lymphocytes contributed substantially to BLM-induced pulmonary inflammation and fibrosis.[59,60] After BLM injection, these inflammatory cells were activated, migrating into the inflammatory foci, synthesizing, and secreting various cytokines, inflammatory mediators, proteases, and ROS.[59,60] Also, BLM-induced pulmonary fibrosis is accompanied by chronic inflammation characterized by an increase in the number of inflammatory cells in the Balf during the fibrotic phase.[61] In agreement with previous studies, the present results showed that BLM could induce a significant increase in the number of total cells, monocytes and lymphocytes in the Balf on day 21 after BLM administration.[36,62] However, COAE treatment ameliorated these pathological changes in Balf, suggesting a probable anti-inflammatory effect of COAE able to inhibit inflammatory reactions and maintaining the number of immune agents close to their normal value. However, COAE treatment ameliorated these pathological changes in Balf, suggesting a probable anti-inflammatory effect of COAE capable of inhibiting the progression of inflammatory reactions and maintaining the near-normal number of immune agents.

Histological examination under light microscope of the lung tissue from BLM group animals revealed evident distortion of lung architecture, where marked with severe alveolar structural damage, a large number of inflammatory cell infiltration and fibroblast hyperplasia, a significantly enlarged

alveolar septum, and a large amount of collagen fiber deposition in the alveolar septum. However, treatment with COAE at 150 or 300 mg/kg attenuates the extent and severity of the histopathological characteristics of lung tissues damage, suggesting that COAE protects BLM-induced lung fibrosis in rats. These findings are consistent with previous studies showing that several natural bioactive compounds exert a potent protective effect against BLM-induced lung fibrosis in rats.[36,54]

Conclusion

Our current study demonstrates the protective effect of COAE against BLM-induced lung inflammation and fibrosis in rats as confirmed by biochemical assays and histopathological evaluation. COAE markedly ameliorated lung morphology. COAE reduced lipid peroxidation and increased antioxidant defense enzyme (SOD and CAT) levels. Significant COAE protective effect was observed with doses of 300 mg/kg body weight. This protective effect of COAE may be due to its confirmed antioxidant potential able to preventing the production or removing of oxygen free radicals and also its anti-inflammatory capacities. Further research is needed in order to identify and characterize the chemical components responsible for the protective effect of red seaweed extract. Also, more detailed work is necessary to completely expose the molecular mechanisms behind the protective effect of actives ingredients of COAE against BLM-induced pulmonary injury.

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Author's Contributions

Dhaouafi J ensured the preparation of the experimental protocol and its realization, was involved in all the analyses carried out, wrote the manuscript, and interpreted the results obtained.

Dhaouafi J is identified as the guarantor of the paper, taking responsibility for the integrity of the work as a whole, from inception to published article.

Abidi A ensured the chemical analysis of the seaweed extract, antioxidant activities, the protocol realization, the writing and the revision of the manuscript.

Nedjar N ensured the conceptualization, methodology, supervision, writing-review, and editing. Romdhani M ensured the bronchoalveolar fluid analysis and the protocol realization.

Tounsi H read the H&E and trichrome histological sections and is involved in the interpretation of its results.

Sebai H participated in revision and correction of manuscripts.

Balti R ensured the formal analysis, resources, writing—review and editing, visualization, supervision, project administration, and funding acquisition.

All authors have reviewed and approved the submission of this manuscript version.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethics Approval

Animals were cared for according to the principles of the local Ethics Committee on Animal Welfare (University of Jendouba: UJ2021-03-4022) in accordance with the recommendations of the International Council of Laboratory Animal Science.

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