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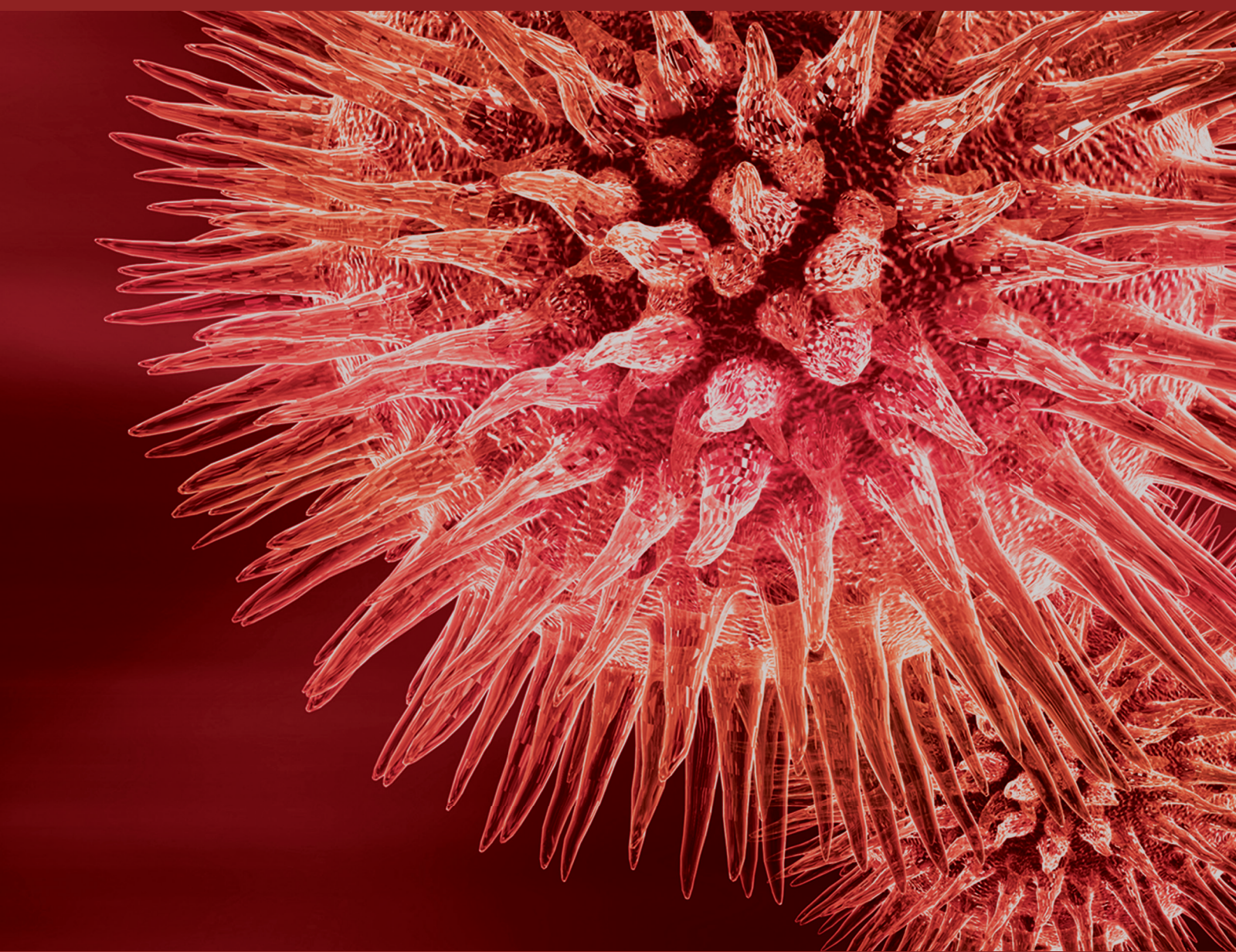

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Contents

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Editorial

Biochemistry: Production of High-Added Value Biomolecules for Industrial Uses

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Natural resources (plant, microorganisms, and algae) constitute a renewable reservoir of high-added value molecules used in various fields such as health, food, pharmaceuticals, and cosmetics. These molecules obtained by extraction or bioconversion are considered natural and have gained an increasing interest at the expense of synthetic products. This is due to the fact that consumers are raising awareness towards the benefits of natural products particularly in food, beverages, and medicines. Moreover, the development of different bioprocesses, particularly techniques for biomolecules extraction and bioconversion (e.g., maceration, supercritical-fluid extraction, fermentation, and enzyme catalysis), allows the discovery of novel bioactive compounds which can be potentially used as drugs or for the fortification of foods.

This special issue contains five papers related to the production of high-added value biomolecules from natural resources and their use in industrial applications. An overview of the research works published in this special issue is given below.

Microorganisms continuously provide new bioactive compounds which are used for the development of novel drugs for the treatment of human, animal, and plant diseases, especially the production of antibiotics more effective against resistant microbes. In this special issue two papers investigate the capacity of microorganisms isolated from extreme conditions to produce active biomolecules. *In the paper* entitled "Antagonistic Properties of Some Halophilic Thermoactinomycetes Isolated from Superficial Sediment of a Solar Saltern and Production of Cyclic Antimicrobial Peptides by the Novel Isolate *Paludifilum halophilum,*" D. F. Dammak et al. have isolated halophilic actinomycetes from a concentrator and crystallizer solar saltern ponds and explored their potential to produce drugs against agricultural and human pathogens. *In the paper* entitled "The Potential of a Brown Microalga Cultivated in High Salt Medium for the Production of High-Value Compounds," S. Boukhris et al. investigated the physicochemical properties of bioactive compounds produced from *Amphora* sp. (Bacillariophyceae) cultivated in a hypersaline medium. The fatty acids profile and biological activities (antioxidant and antibacterial) of the ethanolic extract of *Amorpha* sp. were also determined.

Phytochemicals extracted from plants are a rich source of bioactive molecules including phenolics, vitamins, and flavonoids. These molecules have been recognized as the most promising compounds for the development of medicines used in several pharmacological activities (e.g., anti-inflammation, antimicrobial, antihypertension). This is the subject of the following three papers published in this special issue. *In the paper* entitled "Kinetics of Tyrosinase Inhibitory Activity Using *Vitis vinifera* Leaf Extracts," Y.-S. Lin et al. studied the tyrosinase inhibitory activity of red vine leaf extract (RVLE) containing gallic acid, chlorogenic acid, epicatechin, rutin, and resveratrol, which are effective compounds for skin hyperpigmentation. The authors reported that RVLE had

an effective tyrosinase inhibitory activity and can be used as a whitening agent for cosmetic formulations in the future. *In the paper* entitled "Nutritional Composition and Phytochemical, Antioxidative, and Antifungal Activities of *Pergularia tomentosa* L.", I. Lahmar et al. evaluated the antioxidant properties of extracts from four different organs (roots, stems, leaves, and fruits) of a medicinal Tunisian plant, *Pergularia tomentosa* L. In addition, this work showed that stem and fruit extracts exhibit an antifungal activity against *Fusarium oxysporum* f. sp. *lycopersici,* which could become an alternative to synthetic fungicide. *In the paper* entitled "*Citrus limon* from Tunisia: Phytochemical and Physicochemical Properties and Biological Activities" M. Makni et al. realized quantitative and qualitative characterizations of the zest and the flesh of lemon *(Citrus limon)*. In order to valorize the pharmacological uses of lemon, the authors evaluated its biological activities (antioxidant, antibacterial, antifungal, and antiproliferative activities).

We hope that this special issue provides to the readers with valuable and useful knowledge contributing to the scientific research progress in the biology field.

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Maha Karra-Chaabouni
Mohamed Trigui
María M. Yust
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Research Article

Citrus limon from Tunisia: Phytochemical and Physicochemical Properties and Biological Activities

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Natural plant extracts contain a variety of phenolic compounds which are assigned various biological activities. Our work aims to make a quantitative and qualitative characterization of the Zest (ZL) and the Flesh (FL) of lemon *(Citrus limon)*, to valorize the pharmacological uses of lemon, by evaluating *in vitro* activities (DPPH, free radical scavenging and reducing power). The antibacterial, antifungal, and antiproliferative activities were sought in the ability of *Citrus limon* extracts to protect DNA and protein. We found that the ZL contains high amounts of phenolics responsible for the important antioxidant properties of the extract. However, the FL is richer in flavonoids than the ZL. The FL extract was also found to be more effective than the ZL in protecting plasmid DNA against the strand breakage induced by hydroxyl radicals. We also concluded that the FL extract exhibited potent antibacterial activity unlike ZL. Analysis by LC/MS-MS identified 6 compounds (Caffeoyl N-Tryptophan, Hydroxycinnamoyl-Oglucoside acid, Vicenin 2, Eriocitrin, Kaempferol-3-O- rutinoside, and Quercetin-3-rutinoside). These preliminary results showed that *Citrus limon* has antibacterial and antioxidant activity *in vitro*. It would be interesting to conduct further studies to evaluate the *in vivo* potential in an animal model.

1. Introduction

In recent years, many diseases have appeared and are mainly due to "oxidative stress" which is the result of an imbalance between the formations or not of prooxidants [1]. Indeed, reactive oxygen species (ROS) are reactive molecules due to the presence of unpaired electrons such as superoxide anion radicals ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), hydroperoxyl radicals ($HOO^{\bullet}$), peroxyl ($ROO^{\bullet}$), and also nonradical species such as hydrogen peroxide (H_2O_2), ozone (O_3), and singlet oxygen ($^1O^{\bullet}_2$) [2].

Oxidative stress is caused by the presence of free radicals that upset stability by electronic pairing with several biological macromolecules such as proteins, lipids, and DNA and cause significant damage to the basic structures of the body (proteins, lipid, and DNA) [1, 3]. Evidences that ROS accumulation in biological systems causes oxidative tissue damage and affects cellular integrity and function are tangible. Oxidative damage caused by ROS has often been the origin of the pathogenesis of several diseases such as aging, arthritis, cancer, inflammation, and heart disease [4].

Lemon is among the most important crops in the world, with an annual production of about 123 million tons in 2010. Lemon (*Citrus limon* L.) occupies the third most important *Citrus* species after orange and mandarin world production by 4.200.000 metric tons [5].

Lemon (*Citrus limon* L.) is a main element of the Tunisian economy. In fact, lemon and lime production reached nearly 27.000 tons in 2005 [6]. The genus *Citrus* includes several important species worldwide, oranges by 56%; tangerines and clementines: 17%; lemons and limes: 11%; and finally grapefruit: 6% of the total [7].

Lemon is very rich in important natural compounds, including citric acid, ascorbic acid, minerals, flavonoids, and essential oils. Therefore, although the new *Citrus* cultivars have been mainly developed for fresh consumption, the

particular characteristics such as their phenolic compound and in particular the flavonoids contents have led to their use in new fields such as pharmacology and food technology [8].

Citrus fruits are mainly used in food industries for the production of fresh juices. Thanks to their important composition in bioactive molecules (natural antioxidants, phenolic acids, and flavonoids), peels, the main fraction of *Citrus* waste which represent approximately half of the mass of the fruit, have been widely studied [2].

Therefore, it is of great interest to screen these plants in order to validate their use in food and medicine and to reveal the active ingredient by characterizing their constituents. The aim of this study was to investigate the *in vitro* antioxidant activities of extracts from the peel (ZL) and the Flesh (FL) of *Citrus limon*. Studies included DPPH free radical scavenging and reducing power. In addition, a determination of the antibacterial and antiproliferative assay was sought. Thus, we made tests of DNA damage and protein to assess the protection ability of extracts.

2. Materials and Methods

2.1. Samples. In his study, *Citrus* fruits *(Citrus limon)* were collected and harvested in mature period: stage yellow color in April 2013 from Sfax, Tunisia. Fruits of lemon cultivar Beldi were yellow-colored and oblate spheroids. The investigation was carried out at the mature stage. Citrus fruits were divided into two parts: the Zest of lemon (ZL) and the Flesh of lemon (FL). Zest is the outer colored portion of the *Citrus* peel and the Flesh is the fruit peels including flavedo (epicarp) and albedo (mesocarp) layers.

2.2. Preparation of the Hydroethanol Extracts. The two extracts ZL and FL were prepared. In brief, 100 g of each part of the plant (ZL and FL) was extracted by 300 ml of ethanol-water (7 : 3, v/v) with shaking for 24 h at a rotational speed of 200 rpm. After 24 h, the ethanol-soluble fraction was filtered and concentrated under reduced pressure at 45°C using a rotary evaporator. Finally, the extract was lyophilized and kept in the dark at 4°C. Extraction yields of ZL and FL were 10.64% and 14.33%, respectively.

2.3. Determination of Phenolic Compound

2.3.1. Total Phenolic Content (TPC). The Folin-Ciocalteu assay, adapted from Zou et al. (2011) [9] with minor modifications was used for the determination of total phenolics present in the *Citrus* fruit extracts. Briefly, 10 μl of appropriately diluted extracts or standard gallic acid solutions was mixed with 20 μl of a Folin-Ciocalteu reagent solution in a 96-well plate and mixed gently. After five minutes, 30 μl of freshly prepared 20% sodium carbonate was added followed by 158 μl of distilled water. The reaction mixture was kept in dark for 2 h and the absorbance of the blue coloration formed was measured at 765 nm against the blank solution, which was prepared by the same procedure described above except the extract solution was substituted by 10 μl of ethanol, using the microplate reader. The TPC was expressed as mg gallic acid equivalent (mg GAE/g).

2.3.2. Determination of Total Flavonoid Content (TFC). Total flavonoids in the extracts were determined using a slightly modified colorimetric method described previously by Zou et al. (2011) [9]. A 30 μl aliquot of appropriately diluted sample solution was mixed with 180 μl of distilled water in a 96 well plate, and subsequently 10 μl of a 5% aqueous $NaNO_2$ solution was added. After six minutes, 20 μl of a 10% of aluminum chloride solution was added and allowed to stand for six minutes; then 60 μl of 4% NaOH solution was added to the mixture and stood for another 15 min. Absorbance of the mixture was determined at 510 nm versus a prepared water blank using a Multiskan Spectrum microplate reader. Total flavonoids were calculated with respect to quercetin standard compound (12.5, 25, 50, 75, and 100 μg/ml). All values were expressed as milligrams of quercetin equivalents per 1 g sample (mg QEeq/g sample).

2.3.3. Determination of Flavonol Content. The flavonol content was measured using a colorimetric assay adapted from Yermakov et al. (1987) [10] with slight modifications. The rutin calibration curve was prepared in a well of 96-well plate by mixing 40 μl of various concentrations of ethanolic solutions of rutin with 40 μl (20 mg/ml) aluminum trichloride and 120 μl (50 mg/ml) sodium acetate. The absorbance at 440 nm was read after 2.5 h. The same procedure was used for 40 μl of plant extract instead of rutin solution. All determinations were carried out in triplicate. The flavonol content was calculated using a standard curve obtained from various concentrations of rutin (0–50 μg/ml). All values were expressed as milligrams of rutin equivalents per 1 g sample (mg REeq/g sample).

2.3.4. Determination of Condensed Tannin Content (CTC). The CTC in the extracts and its fractions was determined using the modified vanillin assay [9]. Ten μl of appropriately diluted sample solution was mixed with 120 μl of 4% vanillin solution (in methanol) in a well of 96-well plate, and then 60 μl of concentrate HCl was added and mixed. After 15 min, the absorbance of the mixture was determined at 500 nm against a blank solution, which was prepared by the same procedure described above except the extract solution was substituted by 10 μl of water. Different concentrations of catechin ranging from 25 to 300 μg/ml were used as standard compound for the quantification of total condensed tannins. All values were expressed as milligrams of catechin equivalents per 1 g sample (mg CEeq/g) [9].

2.4. Liquid Chromatographic and Spectrophotometric Mass Analysis. LC-MS/MS analyses were performed on the apparatus consisting of elements following Thermo LTQ HPLC System, LC system equipped with a quaternary pump, autosampler, and a UV diode array detector and mass spectrometer Agilent Triple Quadrupole Ion Trap XCT MSD: spectrometer mass fitted with an electrospray ionization interface, controlled by software Analyst (version 1.3.1).

The extracts were injected onto a HPLC column Zorbax C-18 300 Å (2.1 × 150 mm). The separation was conducted at ambient temperature with a mobile phase consisting of two water 0.1% formic acid solvent (A) and acetonitrile (B) in the

following conditions: 5% B for 35 min, followed by a 11 min linear gradient from 5 to 100% B, then 100% B for 4 min, and, finally, back to initial conditions (5% B) in two minutes to balance the column before reinjection. For all analyses, the solvents used were HPLC grade; the speed was set at 200 μl/min. The injection volume was 5 μl.

2.5. Antioxidant Capacity Assays

2.5.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging Activity Assay. The antioxidant activity of the extracts was firstly evaluated by monitoring its ability in quenching the stable free radical DPPH. The radical scavenging activity of the extracts and fractions against DPPH free radicals was measured using the method of Clarke et al. (2013) [11] slightly modified as follows: 20 μl of appropriately diluted samples or Vitamin C solutions (10, 50, 100, 500, and 1000 μg/ml) was added to 190 μl of DPPH solution (100 μM) in a well of 96-well plate. The mixture was shaken vigorously and allowed to reach a steady state at room temperature for 30 min. Discoloration of DPPH was determined by measuring the absorbance at 517 nm with a Beckman spectrophotometer. All determination was carried out in triplicate. Ascorbic acid was used as a positive control. The DPPH radical scavenging activity was calculated according to the following equation:

$$\text{Scavenging rate} = \left[1 - \frac{(A_1 - A_2)}{A_0}\right] \times 100\%, \quad (1)$$

where A_0 was the absorbance of the control (blank, without extract), A_1 the absorbance in the presence of the extract, and A_2 the absorbance without DPPH.

2.5.2. Reducing Power Assay. The Fe^{3+} reducing power of the extracts was determined by the method of Verma and Banerjee (2010) [12] with slight modifications. The ethanolic extracts, ascorbic acid, were used at different concentrations (7.8, 15.6, 31.25, 62.5, 125, 250, and 500 μg/ml). One milliliter of each sample was mixed with phosphate buffer (2.5 mL, 0.2 mol·L^{-1}, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 30 mmol·L^{-1}) followed by incubating at 50°C in a water bath for 20 min. The reaction was stopped by adding 2.5 ml of trichloroacetic acid (TCA) solution (10%) and then centrifuged at 3000 r/min for 10 min. The supernatant (100 μl) was mixed with distilled water (100 μl) and $FeCl_3$ (20 μl, 0.1%), in a well of 96-well plate, and the absorbance was measured at 700 nm as the reducing power in a spectrophotometer. Higher absorbance of the reaction mixture indicated greater reducing power.

2.6. Determination of the Antibacterial Activity

2.6.1. Microorganisms for Study. A total of nineteen pathogenic microbial cultures including ATCC strains of bacterial and fungal origin were taken for this study. Eleven of the bacteria and eight fungal strains were isolated from clinical specimen obtained from patient samples and identified by standard laboratory protocol.

Gram Positive *Streptococcus agalactiae B, Streptococcus D, Enterococcus, Staphylococcus aureus*, Gram Negative *Escherichia coli, Citrobacter koseri, Acinetobacter baumannii, Proteus mirabilis, Klebsiella pneumoniae, Salmonella enterica, Pseudomonas aeruginosa,* and Fungal *Aspergillus niger, Penicillium* spp., *Microsporum canis, Trichophyton violaceum, Cryptococcus neoformans, Candida albicans, Candida tropicalis, and Candida glabrata* were considered.

2.6.2. Antimicrobial Activity. The plant extracts were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/mL and tested for antibacterial activity by the agar well diffusion assay. The bacterial culture in Muller Hinton broth was adjusted to the final inoculum density of 1×10^7 CFU/mL (by 0.5 McFarland standards) on molten Muller Hinton agar (MHA) plates. After solidification, wells (diameter 9 mm) were made with a sterile borer in the inoculated MHA plates. About 100 μL solution containing 1 mg of each extract was dispensed in the wells, while DMSO was also tested as the vehicle control. Penicillin G, streptomycin, and gentamicin were the standard drugs used as positive controls in this assay. Antibacterial activity was expressed as the diameters of inhibition zones produced around each well by the plant extracts and antibiotics and was measured after 24 h of incubation at 37°C. Each test was conducted in triplicate to confirm the reproducibility of the observed data [13].

2.6.3. Antifungal Activity. The crude plant extracts as described above were screened for antifungal activity. Fungal culture in Sabouraud dextrose broth containing an inoculum density of 0.5 McFarland (1×10^8 CFU/mL) was diluted at 1 : 10 ratio in SDA plate to obtain the final inoculum concentration of 1×10^7 CFU/mL. Wells (diameter 6 mm) were punched on solidified SDA plates and 100 μL solution containing 1 mg of each extract was dispensed in the wells. Amphotericin-B was used as a standard drug for antifungal assay, and DMSO was tested as the vehicle control. The diameter of the inhibition zone was measured after 24 h of incubation at 35°C. Antifungal activity was expressed as diameters of inhibition zones produced by the plant extracts and antifungal agent. Each test was conducted in triplicate and the reproducibility of the observations was confirmed [13].

2.7. Determination of the Antiproliferative Activity

2.7.1. Cell Line: Strain B95-8 (ATCC: VR-1492). This is a lymphoid line producing virions Epstein-Barr transformants. It was obtained from lymphocytes B of marmoset and irradiated lines from patients with infectious mononucleosis. A fraction of 1–3% of B95-8 cells enters spontaneously in a viral lytic cycle [14]. Original laboratory is laboratory of cell culture, Habib Thameur Hospital of Tunis.

2.7.2. Culture Medium. The culture medium RPMI 1640 (Rosewell Park Memorial Institute) (Gibco) was used for the culture of lymphoblastoid cell line: B95-8. The medium was supplemented with 2 g/l sodium bicarbonate (HCO_3 Na). After adjusting the pH to 7.2 with 1 N HCl, the mixture was filtered through a filter of 0.22 microns and then supplemented

with 10% fetal bovine serum (FBS) (Gibco), gentamycin 1%, and L-glutamine 2 mM.

2.7.3. Cell Culture

(i) Maintenance Culture Cells. All cell lines were cultured in culture flasks (Iwaki) of 25 or 75 cm^2. Transplanting cells was carried out every 3–5 days.

Cells that have reached the saturation concentration were centrifuged for 10 min at 1000 rpm and then suspended in 2 ml of RPMI medium supplemented with 10% fetal bovine serum (FBS). After counting in the presence of trypan blue, the cells were placed in culture at a concentration of $2 \cdot 10^5$ cells/ml [15].

(ii) Trypan Blue Exclusion Test (Cell Count). The test of trypan blue exclusion (Sigma) is based on the evaluation of the integrity cell membrane. It is a specific technique for cell counts and assessment of cell death. It consists of an optical microscope to count the number of cells present in a given volume of cell suspension. Counting was performed on a Malassez cell. 20 μl of cell suspension was diluted with 20 μl of trypan blue. After mixing, a small volume was set in the cell count for Malassez. The concentration of the number of cells per ml was given by the following formula:

$$N = n \times 10 \times \text{dilution factor} \times 1000 \quad (2)$$

where N is number of cells per ml.

(iii) Cellular Cytotoxicity Test (MTT Assay). The MTT (3 Bromide (4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide) (Sigma, Germany) is initially yellow and the substrate is a mitochondrial enzyme succinate dehydrogenase. The latter is capable of cleaving certain covalent bonds of MTT, which transforms it into formazan salt (purple salt), insoluble in aqueous media. This reaction can be monitored quantitatively by spectrophotometry. The DO at 570 nm reflects the activity of mitochondrial cytochromes. This activity can be considered as an index of cell proliferation [16].

2.8. Protein Damage Protection Assay. The effects of the sample on protein oxidation were carried out according the method of Hu et al. (2012) [17] with minor modifications. BSA was oxidized by a H_2O_2/Fe^{3+}/ascorbic acid system. The reaction mixture (1.0 ml), containing 0.2 ml of sample, 0.2 ml of phosphate buffer (100 mM, pH 7.4), 0.2 ml of BSA (5 mg/ml), 0.2 ml of $FeCl_3$ (250 μM), 0.1 ml of H_2O_2 (20 mM), and 0.1 ml of ascorbic acid (1 mM), was incubated for 6 h at 37°C. After incubation, the reaction mixture was analyzed by electrophoresis in 10% SDS polyacrylamide gel. The gel was stained with a brilliant blue R staining solution for 2 h, destained, and digitally photographed.

2.9. Plasmid DNA Damage Assay. DNA damage and DNA protecting activities of Citrus extracts were prospected on pBR322 plasmid DNA. The plasmid DNA was oxidized with H_2O_2 + UV treatment in the presence or absence of extracts of Citrus according to protocols of Jagtap et al., 2011 [18]. In brief, the experiments were performed in a volume of 15 μl in an Eppendorf tube containing 200 ng of pBR322 plasmid DNA. H_2O_2 was added to final concentration of 100 mM with or without 10 μl of Citrus extracts. The reaction mixture was exposed to UV irradiation and continued at ambient temperature for 5 min on the surface of UV mini transilluminator. After irradiation, the mixture was incubated at room temperature for 15 min. To the mixture, gel loading dye was added and the fragments were separated by electrophoresis. Untreated plasmid DNA was used as a control in each run of gel electrophoresis along with UV and H_2O_2 treatments.

2.10. Statistical Analysis. Experimental results are expressed as means ± SD. All measurements were replicated three times. The data were analyzed by an analysis of variance ($P < 0.05$) and the means separated by Duncan's multiple range test. The IC50 values were calculated from linear regression analysis.

3. Results and Discussion

3.1. Total Phenolic, Flavonoid, Flavonol, and Tannin Contents. Total phenol compounds, as determined by Folin-Ciocalteu method, are reported as gallic acid equivalents with reference to standard curve ($y = 0.003x$, $R^2 = 0.999$). The total phenolic contents were usually significantly higher in Zest ($P \leq 0.001$) with the range of 204.4 ± 9.62 than Flesh with the range of 105.55 ± 4.71 mg gallic acid equivalent/g of extract (Table 1). Polyphenols are the major plant compounds with significant antioxidant activity. The antioxidant activity of these compounds is mainly due to their redox properties [19, 20]. Our results on polyphenol contents were higher than those measured in similar varieties from Iran and Portugal (131 and 87 mg EAG/g extract, resp.) [21, 22]. Indeed, these results indicate that the polyphenol content may be influenced by various factors such as genotypic differences, geographic and climatic conditions, time of harvest, and even cultural practices [23].

de Lourdes Mata Bilbao et al. (2007) [24] showed a rate of polyphenols in Zest of lemon about 3524 mg EAG/100 g of extract, while Guimarães et al. (2010) [22] showed a rate of polyphenols of 87.77 mg EAG/g of extract. This difference probably resulted from the fact that the determination by the Folin-Ciocalteu reagent is not specific to polyphenols, but thousands of compounds may react with the reagent, giving a higher apparent phenolic rate [25, 26]. The phenol content of a plant depends on a number of intrinsic and extrinsic factors [27].

The total flavonoid contents were significantly higher in FC ($P \leq 0.01$) with the range of 56.16 ± 14.14 with respect to ZC with the range of 27.5 ± 6.88 mg QEeq/g of extract powder with reference to standard curve ($y = 0.003x$, $R^2 = 0.981$) (Table 1). In recent years, particular attention has been given to a specific class of phytochemical antioxidants which are flavonoids. Flavonoids are polyphenolic substances naturally present in almost all plant materials and are prominently ubiquitous in cereals, vegetables, fruit, nuts, wine, tea, beer, and cocoa [28]. These flavonoid compounds have a broad spectrum of chemical and biological activities. Indeed, they are compounds which possess strong antioxidant properties.

Table 1: Total phenolic (mg Eq Gallic Acid/g dry weight), flavonoids (mg Eq Quercetin/g dry weight), flavonols (mg Eq Rutin/g dry weight), and condensed tannins (mg Eq Catechin/g dry weight) of ZL and FL extracts of *Citrus Limon*.

	ZL	FL
Total phenol (mg Gallic acid Eq/g)	204.40 ± 09.62***	105.55 ± 04.71
Total flavonoid (mg Quercetin Eq/g)	27.50 ± 06.88	56.16 ± 14.14++
Flavonols (mg Rutin Eq/g)	26.66 ± 07.07***	09.16 ± 03.53
Condensed tannins (mg Catechin Eq/g)	138.33 ± 35.36***	26.66 ± 18.92

++Correlation between FL and ZL difference was statistically significant ($P < 0.01$). ***Correlation between ZL and FL difference was statistically significant ($P < 0.001$). The values are the mean of three determinations ± SD.

Their potential ability to capture and chelate metals and ROS depends on chemical structures and the number and position of hydroxyl groups. Flavonoids such as tea catechins show a high activity of the ferrous iron chelate [4]. Comparative studies by Wang et al., (2014) [29] and Guimarães et al. (2010) [30] proved that the extract of lemon has flavonoid contents of the order of 32.7 and 15.96 mg QEeq/g extract, respectively. Flavonoids, one of the most widespread and diverse groups of natural compounds, are probably the most important natural phenolic compounds. Several biological effects *in vitro* and *in vivo* due to the consumption of foods containing flavonoids were demonstrated. Epidemiological studies showed that increased consumption of flavonoids reduces the risk of cardiovascular disease and certain types of cancer [31].

Flavonols are reported as rutin equivalents with reference to standard curve ($y = 0.002x$, $R^2 = 0.997$). The content of flavonols was significantly higher in Zest ($P \leq 0.001$) with the range of 26.66 ± 7.07 and of the range of 9.16 ± 3.53 mg REeq/g of extract powder (Table 1). We note that the majority of flavonoids for Zest of lemon consist of flavonols. The study of Wang et al. (2014) [29] showed that the majority of flavonols Zest of lemon are quercetin and rutin. In fact, their concentrations are about 0.573 and 0.060 mg mg REeq/g, respectively.

The content of condensed tannin was significantly higher in Zest ($P \leq 0.001$) with the range of 138.33 ± 35.35 than the Flesh with the range of 26.66 ± 18.92 mg catechin equivalent/g of extract powder with reference to standard curve ($y = 0.002x$, $R^2 = 0.994$) (Table 1). The tannins are secondary compounds of various chemical structures, widely produced in the plant kingdom and generally divided into hydrolysable and condensed tannins. Condensed tannins are found primarily in the walls of seeds and play an important role in the defense system of seeds that are exposed to oxidative damage by many environmental factors such as light, oxygen, free radicals, and metal ions [32].

Following the results of the quantitative characterization, lemon is a promising source of beneficial bioactive compounds for human health through its constituent polyphenols and flavonoids.

3.2. Identification of the Phenolic Composition of ZL Extract. The analysis of the ZL extract of *Citrus limon* in liquid chromatography high performance coupled with mass spectrometry (LC-MS/MS) identified compounds which are greater in number of 6 phenolic products (Caffeoyl N-Tryptophan, Hydroxycinnamoyl-Oglucoside acid, Vicenin 2, Eriocitrin, Kaempferol-3-O- rutinoside, and Quercetin-3-rutinoside) as described in Table 2 in order of elution.

The presence of Citrus flavonoids is manifested chiefly in glycoside or aglycone forms [33]. In fact, flavonoids are more abundant in Zest than seeds [34]. Lemon seeds are richer in eriocitrin but poorer in naringin. Meanwhile, the Zest contains important contents of neoeriocitrin, neohesperidin, and naringin and is poor in narirutin [35, 36].

Miyake et al. [37] performed the isolation of two C-glucosyl flavones from lemon fruit: diosmetin 6, 8-di-C-glucoside and diosmetin 6-C-d-glucoside. Moreover, such flavones are found in limes, rather than other kinds of Citrus fruit [34, 38]. Lemon juices were less rich in vicenin-2, and diosmin [39–41]. However, three most abundant flavones were found in lemon Zest: diosmetin 6,8-di-C-glucoside [37], vicenin-2, and diosmin [35].

Rutin and myricetin were most identified in lemon juice [42, 43], but quercetin and kaempferol existed in Zest and juice as well [36, 41, 42]. Hydroxycinnamic acids were also detected in very low concentrations (caffeic, chlorogenic, ferulic, sinapic, and p-coumaric acids) [40, 41, 44, 45].

3.3. Antioxidant Activity of Citrus limon Extracts. The antioxidant activity cannot be evaluated by only a single method due to the complex nature of phytochemicals. Also, the antioxidant activity determination is reaction-mechanism dependent. Therefore, it is important to employ multiple assays to evaluate the antioxidant activity of plant extract or phytochemicals [9].

3.3.1. The Scavenging Activity for DPPH Radicals. DPPH is a stable organic free radical with a strongest adsorption at 517 nm, the color of which turns from purple to yellow followed by the formation of DPPH upon absorption of hydrogen from an antioxidant [46].

DPPH molecules that contain a stable free radical have widely been used to evaluate the radical scavenging ability of antioxidants. The free radical scavenging activities of the two extracts, ZL and FL, were assayed by using DPPH. As shown in Figure 1, both ZL and FL reacted directly with and quenched DPPH radicals to different degrees with increased activities at higher concentrations. At all of the concentrations tested, ZL showed significantly stronger activities than FL. However, at similar concentrations, the scavenging effect of FL was only 20.3% ± 3.9. The IC50 of ZL was about

Table 2: Identification and analysis of the phenolic composition of ZL extracts using liquid chromatography high performance coupled with mass spectrometry (LC-MS/MS).

Pic	TR (min)	UV (nm)	$[M-H]^-$	MS^2	Structure
(1)	5.78	326	365.1446	263, 125, 142, 221, 302, 320	Caffeoyl N-Tryptophan
(2)	6.60	300 sh, 330	355.0666	147, 191, 209, 337	Fer-glc (acid Hydroxycinnamoyl-Oglucoside)
(3)	7.58	268, 338	593.1503	473, 353, 383, 503, 575	Vicenin 2
(4)	9.35	284, 334 sh	595.1659	287	Eriocitrin
(5)	10.02	256, 266, 350	593.1504	285, 151, 175, 199, 216, 241, 257	Kaempferol-3-O- rutinoside
(6)	11.09	263, 298 sh, 356	609.1819	301, 151, 178, 255, 271	Quercetin-3-rutinoside (Rutin)

434.50 μg/ml ± 5.9. To obtain the same IC50 scavenging activity, the concentration needed for FL was 1126990.76 mg/ml ± 9.2, almost 2596 times; although both ZL and FL showed DPPH scavenging activity, ZL was a considerably better DPPH radicals scavenger. The antioxidant potential of extracts was different may be due to the difference in chemical structures of their phenolic compounds, as suggested by previous work as regards the relationship between the chemical structure and antioxidant potential of phenolic compounds by means of the DPPH method [46]. The antioxidant capacity is worth evaluating in three structural groups [47], the first of which is the B-ring *ortho*dihydroxy (catechol) structure. This structure favors the stability to aroxyl radicals, possibly thanks to hydrogen bonding. It also leads to electron dislocation. The 2, 3-double bond conjugated with a 4-oxo function is the second structure responsible for B-ring electron dislocation. Finally, we mention hydroxyl groups. Evidently, a combination of these chemical and structural elements is responsible for the flavonoid antioxidant capacity. An example is the presence or absence of glycosides or aglycones and the amount and position of eventually esterified hydroxyls [48, 49].

At position 3 in flavanones and flavones, the lack of a hydroxyl group affects their antioxidant ability. However, at 2 and 3 the double bond increases the structure reactivity. Thus, apigenin is denoted as a moderate antioxidant compound, while naringenin is not active against the superoxide ion.

3.3.2. The Reducing Power. The reducing power has widely been used as a significant marker of the antioxidant activity. In this assay, the yellow color of the solution acquired various green and blue shades due to the reducing power of compounds. Antioxidants lead to the Fe^{3+} reduction in the presence of a ferricyanide complex to the ferrous (Fe^{2+}) form through a one electron donation [50].

As shown in Figure 2, we obtained a significant value of reducing power in both ZL and FL extracts. Furthermore, the data indicated a concentration-dependent mode for the reducing powers of both extracts. In addition, the latter also increased in parallel with concentrations. This is due to their richness in bioactive molecules that act as antioxidants. The considered extracts' relatively strong reducing power was noticeable. However, the ZL extract was found to have slightly higher reducing activity than FL. The hydrogen- or electron-donating capacity of these extracts could be the cause behind

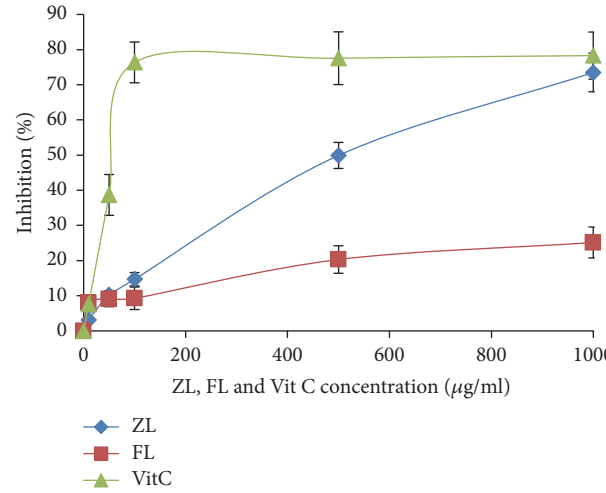

Figure 1: Radical scavenging effect (%) on DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals of ZL and FL extracts of *Citrus limon*. The values are the mean of three determinations ± SD.

this phenomenon [51]. Accordingly, relatively higher amounts of reductones could be found in both extracts. Possibly, these reductones could react with free radicals to stabilize and block radical chain reactions.

3.4. Antimicrobial Activity

3.4.1. Antibacterial Activity. We evaluated the antimicrobial activity of extracts of *Citrus limon* by the method of diffusion in a solid medium. The activity was revealed on 11 bacterial strains Gram (+) and Gram (−). Then for each disk, we measured the diameters of zones of growth inhibition of bacterial cultures. The results of antibacterial screening extracts are shown in Table 3.

No zone of inhibition was observed in goshawks discs of lemon Zest after the end of incubation for most of the bacterial cultures listed above. These strains have a very high resistance against the action of this extract. For standard antibiotic (OFX), zones of inhibition ranged from 10 mm in *Proteus mirabilis* to 42 mm in *E. coli*. That antibiotic resistance was therefore seen in *E. coli, Citrobacter koseri, Streptococcus Group B, and Group D enterococci,* while other strains were sensitive to this antibiotic. *Pseudomonas aeruginosa* was resistant to rifampicin with an inhibition diameter of 22 mm.

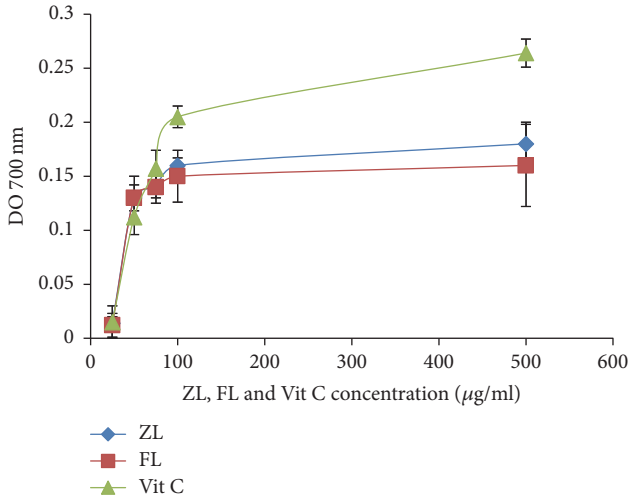

Figure 2: Reducing power of ZL and FL extracts of *Citrus limon*, as measured by changes in DO at 700 nm. The values are the mean of three determinations ± SD.

Table 3: Diameter (mm) of inhibition zones of Microbial strains of *Citrus limon* extracts.

Bacterial strains	Diameter of inhibition (mm)	
	ZL	FL
Escherichia coli	0	$16 \pm 2^{***}$
Staphylococcus aureus	0	$30 \pm 3^{***}$
Acinetobacter baumannii	0	$24 \pm 2^{***}$
Proteus mirabilis	0	$19 \pm 1.5^{***}$
Klebsiella pneumoniae	0	$22 \pm 3.5^{***}$
Citrobacter koseri	0	$21 \pm 1.6^{***}$
Salmonella enterica	0	$32 \pm 1.9^{***}$
Pseudomonas aeruginosa	0	$22 \pm 2.2^{***}$
Streptococcus agalactiae B	32 ± 1.2	$28 \pm 2.6^{**}$
Streptococcus D	21 ± 0.9	$24 \pm 1.9^{*}$
Enterococcus	30 ± 3.1	31 ± 3.3

*Correlation between FL and ZL inhibition was statistically significant ($P < 0.05$). **Correlation between FL and ZL inhibition was statistically significant ($P < 0.01$). ***Correlation between FL and ZL inhibition was statistically significant ($P < 0.001$).

When compared to the ZL extract inhibition, the FL extract presented significant values of inhibition for all bacterial strains. These values ranged between 16 and 32 mm.

The antibacterial test on β hemolytic *Streptococcus* showed growth inhibition for all extracts of both parts of lemon.

According to Massé et al. (2003) [52], sensitivity to Gram + bacteria is due to the inhibitory action on protein silybin synthesis and RNA. Furthermore, Pathak et al. (1991) [53] linked the sensitivity of bacteria to polyphenols to the inhibition of enzymes necessary for the production of energy in the bacterial cell or the change in the permeability of the cell and also to the inhibition of RNA synthesis.

In a study of the polyphenolic relationship, the antimicrobial potency of bacteria causing food spoilage, Lucera et al., (2012) [54] concluded that the sensitivity of microorganisms to polyphenols depends on itself and the structure of the polyphenol. However, knowledge of the action of antibiotics (action on Gram +) mechanisms can explain the sensitivity of the strains to these antibiotics.

3.4.2. Antifungal Activity. The disk diffusion method allowed us to demonstrate the antifungal potency extracts of *Citrus limon* vis-à-vis the tested fungal strains.

The antifungal activity is indicated by the presence or the absence of mycelial growth. It results in a translucent halo around the sterile agar disc [55].

Only Nystatin antifungal drug used as a control at a dose of 100 μg presented a zone of inhibition of growth of the strains, which confirms the validity of the method used.

No zone of inhibition was observed around discs impregnated with different extracts and none of the extracts inhibited the growth of these strains. This could be explained by the lack of substances with antifungal activity such as alkaloids [56]. These results indicate that ZL and FL extracts do not contain antifungal agents.

3.5. Antiproliferative Activity. Cytotoxic effects on the line B95-8 were studied by MTT assay. Our results showed a cytotoxic effect of the extracts of the plant on line B95-8 (Figure 3); a dose-response was observed.

Our results showed that the extracts of *Citrus limon* have an inhibitory effect on the line B95-8 and this position is characterized by a remarkable increase in cytotoxicity as a function of increasing concentrations of the samples tested.

Cell proliferation was assessed by MTT assay using the B95-8 cells treated with varying concentrations of the extracts for 48 h. As shown in Figure 3, each sample inhibits cell proliferation in a dose-dependent manner. The proliferation of B95-8 cells was significantly reduced ($P \leq 0.001$) by 50% after 48 h of exposure with 0.074 g/ml ZL or 0.0087 g/ml FL.

In a concentration of 0.015 g/ml of FL, only 20.11% of viable cells were present, while a concentration of 0.00087 had a low inhibitory power on B95-8 cells with a percentage of 95.44% of viability.

The strongest inhibitor power was observed at a concentration of 0.34 mg/ml for ZL (49.35% cell toxicity). Beyond these concentrations (0.015 and 0.034 mg/ml for ZL and FL, resp.), we observed a significant decrease in the inhibitory potency, and the effect of these extracts on the line B 95-8 was antagonistic.

3.6. Protein Damage Assay. Proteins are major targets for oxidants due to their high abundance in biological systems and high rate constants for the reaction of oxidants [57]. Previous scientific investigation has demonstrated that free radicals induced protein damage which plays a significant role in aging and pathological events. Electron leakage, metal-ion dependent reactions, and autooxidation of lipids and sugars have possibly led to radical-mediated damage to proteins [58]. Electrophoretic patterns of BSA after incubation with the Fe^{3+}/H_2O_2/ascorbic acid system in the presence of samples were assayed with SDS–PAGE (Figure 4). In the

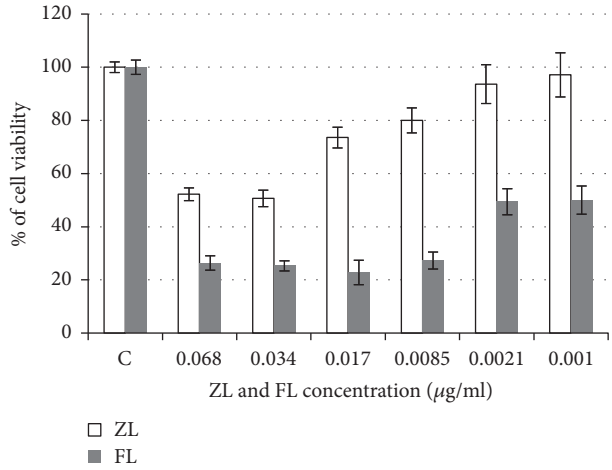

Figure 3: Effect of ZL and FL extracts of *Citrus limon* on proliferative activity of B95-8 cell line.

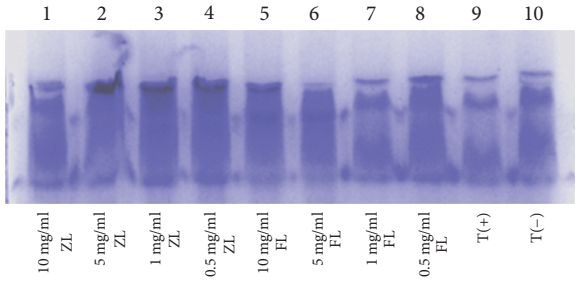

Figure 4: Effect of the ZL and FL *Citrus limon* extracts on protein damage.

current study, analysis of protein bands and quantified gel image showed the protective effect of both ZL and FL extracts against ROS attacks. At 1 mg/ml, extracts protected significantly BSA and remarkably restored the protein band intensity. This protective ability was mainly due the antioxidant activity of extracts. In fact, phenolic compounds are considered as major active components of the plant extracts responsible for the strong antioxidant capacity.

3.7. Inhibitory Effect of the Citrus limon Extracts on the Oxidative DNA Damage Caused by H_2O_2. The inhibitory effects of the Flesh and the peel extracted from lemon on oxidative DNA damage caused by H_2O_2 were investigated through *in vitro* DNA migration assay. According to Figure 5, a gel electrophoretogram of the FL and ZL effect on *in vitro* oxidative damage of plasmid DNA by hydroxyl radicals was generated through Fenton reaction between Fe^{2+} and H_2O_2. The plasmid DNA was mainly of the super-coiled form in the absence of Fe^{2+} and H_2O_2 (control). The addition of Fe^{2+} and H_2O_2 leads to the decrease of the DNA super-coiled form and conversion into the relaxed circular and linear form. The further fragmentation of linear form however decreased in the presence of FL and ZL. DNA migration assay is a sensitive biomarker of DNA damage. At concentrations of 5 mg/ml, we observed a significant dose-dependent decrease in DNA migration.

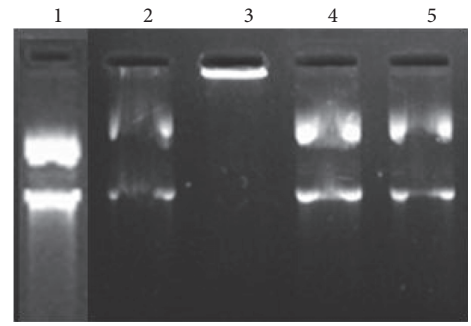

Figure 5: Inhibitory effect of the ZL and FL *Citrus limon* extracts on the oxidative DNA damage caused by H_2O_2. *Line 1*: untreated plasmid; *Line 2*: plasmid treated with 5 mg/ml ZL extract; *Line 3*: plasmid treated with 5 mg/ml of FL extract; *Line 4*: positive control (quercetin (10 mg/ml) + Rf Fenton); *Line 5*: negative control (plasmid + Rf Fenton).

Flavonoids possess an ideal structure for trapping free radicals because they have a number of hydroxyls acting as hydrogen donors depicted as an important antioxidant [59]. This is shown in our results, since the lemon Flesh is richer in flavonoids than lemon Zest, which favors better DNA protection.

Numerous tumors and ROS-mediated signaling and genomic instability are marked by oxidative stress. It obviously contributes to the initiation and progression of cancer. About 80% of the DNA damage resulting in the development of cancer is caused by ROS such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hydroxyl radical (OH). Therefore, avoiding oxidative DNA damage induced by ROS is very important for cancer prevention [60].

4. Conclusion

In conclusion, the results of the present study indicate that the extracts from *Citrus limon* exhibit powerful antioxidant properties, expressed by its capacity to scavenge DPPH radicals and to reduce power, and the extracts reduce H_2O_2-induced DNA via its antioxidant activities. These antioxidant activities and inhibitory effects of the extracts on DNA and cell damage may further prove that *Citrus limon* is useful as a medicinal plant for cancer chemoprevention.

The results obtained show that *Citrus limon* extracts contain high enough levels of phenolic and flavonoid compounds. This is correlated with a remarkable antioxidant activity towards the reduction of iron, and a relatively high power against scavenging free radicals. So, *Citrus limon* extracts could be a promising antioxidant source for the prevention and/or treatment of oxidative stress-related diseases or as food additives.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Research Article

Antagonistic Properties of Some Halophilic Thermoactinomycetes Isolated from Superficial Sediment of a Solar Saltern and Production of Cyclic Antimicrobial Peptides by the Novel Isolate *Paludifilum halophilum*

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This study has focused on the isolation of twenty-three halophilic actinomycetes from two ponds of different salinity and the evaluation of their ability to exert an antimicrobial activity against both their competitors and several other pathogens. From the 23 isolates, 18 strains showed antagonistic activity, while 19 showed activities against one or more of the seven pathogen strains tested. Six strains exhibited consistent antibacterial activity against Gram-negative and Gram-positive pathogens characterized at the physiological and molecular levels. These strains shared only 94-95% 16S rRNA sequence identity with the closely related species of the Thermoactinomycetaceae family. Among them, the potent strain SMBg3 was further characterized and assigned to a new genus in the family for which the name *Paludifilum halophilum* (DSM 102817^T) is proposed. Sequential extraction of the antimicrobial compounds with ethyl acetate revealed that the crude extract from SMBg3 strain had inhibitory effect on the growth of the plant pathogen *Agrobacterium tumefaciens* and the human pathogens *Staphylococcus aureus, Salmonella enterica, Escherichia coli, and Pseudomonas aeruginosa*. Based on the HRESI-MS spectral data, the cyclic lipopeptide Gramicidin S and four cyclic dipeptides (CDPs) named cyclo(L-4-OH-Pro-L-Leu), cyclo(L-Tyr-L-Pro), cyclo(L-Phe-L-Pro), and cyclo(L-Leu-L-Pro) were detected in the fermentation broth of *Paludifilum halophilum*. To our knowledge, this is the first report on the isolation of these compounds from members of the Thermoactinomycetaceae family.

1. Introduction

Actinomycetes are considered as an intermediate group of bacteria and fungi and recognized as prokaryotic organisms. Traditionally, these bacteria have been isolated from terrestrial sources although the first report of mycelium-forming actinomycetes recovered from marine sediments appeared several decades ago [1, 2]. It is only recently that marine-derived actinomycetes have become recognized as a source of novel antibiotics and anticancer agents with unusual structures and properties [3, 4]. However, considering the rising need of new antibiotics to combat the emergence of drug-resistant bacteria, many microbiologists have focused their recent research on actinomycetes from nonconventional environments where particular chemical and physical factors contribute to the selection of species that are best adapted to

that extreme environment. To cope with their environmental stressful factors, these microorganisms have developed a complex stress management for their survival, which is being unrevealed for multiple purposes [5, 6]. Accordingly, groups of acidophilic and alkaliphilic, psychrophilic and thermophilic, halophilic and haloalkaliphilic, and xerophilic actinomycetes have been described [7, 8].

In recent years, novel halophilic and halotolerant actinomycetes of diverse genera from diverse families have been isolated from hypersaline environments [9–11]. On the basis of phenotypic, chemotaxonomic, and phylogenetic analysis, several of these halophilic strains were affiliated to the Thermoactinomycetaceae family of the phylum Firmicutes, which was created for the first time in 2006 by Matsuo et al. [12] and included six genera named *Thermoactinomyces, Laceyella, Thermoflavimicrobium, Seinonella* [13], *Planifilum* [14], and *Mechercharimyces* [12]. Recently, numerous novel genera, such as *Melghirimyces, Salinithrix*, and *Croceifilum*, were added to this family and the number was extended to seventeen [15, 16]. Except some genera having mesophilic growth below 45°C, growth in a thermophilic range is a main feature of the Thermoactinomycetaceae family [14]. In addition, several *species* of the family, such as *Melghirimyces algeriensis* isolated from an Algerian salt lake [17], *Salinithrix halophila* from the soil of hypersaline wetland in the north of Iran [18], and *Paludifilum halophilum* from a superficial sediment of Tunisian solar saltern [16], are halotolerant or halophilic able to support until 20% (w/v) of salinity. Despite the increasing number of halophilic thermoactinomycetes, these microorganisms are still of the least explored ones for novel secondary metabolites. In the field of antimicrobial substances, only some new antibiotics, such as chinikomycin and lajollamycin, are detected in halophilic or halotolerant actinomycete species [4] and several biotechnology companies and academic institutions are currently working on new strategies for the pharmaceutical applications of these new compounds.

Sfax solar saltern, located in the central east of Tunisia, is one of the largest marine salterns in the region. Even though a number of culture-dependent and culture-independent studies were carried out on the biodiversity of eukaryotic [19] and prokaryotic [20, 21] microbial assemblages inhabiting different ponds, there are no reports on any exclusive diversity or biotechnological potential of actinomycetes inhabiting this ecosystem. In a continuous effort to explore the prokaryotic diversity and discover new antimicrobial compounds, we performed a screening procedure to isolate rare halophilic actinomycetes from a concentrator and crystallizer solar saltern ponds and explore their potential to produce drugs against agricultural and human pathogens. The novel isolate *Paludifilum halophilum* strain SMBg3 with significant antimicrobial activity was characterized further and shown to be potential producer of Gramicidin S and four cyclic antimicrobial dipeptides.

2. Materials and Methods

2.1. Study Site and Samples Collection. The study was conducted in the solar saltern of Sfax located in the central eastern coast of Tunisia (34°39′N and 10°43′E). It is an artificial ecosystem consisting of a series of interconnected ponds extending over an area of 1500 ha along 12 km of coastline (Figure 1). These ponds are shallow (20–70 cm deep), with a salinity of between 4 and 43% (w/v). The process begins by storing seawater in 17 primary ponds to increase water salinity by evaporation. When the salt concentration reaches the 40–75 g/L range, the seawater is moved to an internal section of five parallel water ponds where it is kept until the salt concentration reaches 130 g/L. After this stage, the seawater is distributed into the six precrystallization ponds to attain a salt concentration of 300 g/L. At the final stage (crystallizer ponds), where the salt precipitates, the brines reach a very high salt concentration (400 to 430 g/L).

Superficial sediment and water samples were taken in December 2012 and February and Mars 2013, from two different salinity ponds, the concentrator pond M1 (salinity 20% (w/v)) and the crystallizer pond TS18 (salinity 38% (w/v)), and immediately stored cold until processing in laboratory within 2 hours of collection. Salinity of the water samples above the sediment was determined at the site with a hand refractometer (ZUZI 5032020), while pH and temperature were measured in situ using, respectively, a digital pH-meter (ISTEK NeoMet pH-220L) and a mercury glass thermometer (Nahita 72075150). Samples for dissolved organic carbon (DOC) were filtered through a 0.22 μm pore size membrane and the concentrations were measured as CO_2 generated by catalytic combustion using a Shimadzu TOC-V carbon analyzer.

2.2. Isolation of Halophilic Actinomycetes. An aliquot of 1 ml of the water sample or 1 g of the superficial sediment (the 0–2 cm fraction) treated with double sonication (Ultrasonic Homogenizers Sonopuls HD 2070) was dispersed in 9 mL of filter sterilized (pore size 0.22 μm) saline water with 15% NaCl. Additional series of dilution were also made and 0.1 mL of the proper dilution was spread on the surface of different selective media, namely, Glucose-Tryptone-Yeast (GTY) [22], Starch Casein Agar (SCA) [23], Bennett medium [24], complex medium (CA) [24], ISP2, and Bergey's Streptomyces medium [25]. Each medium was supplemented with 0.2 μm pore size filtered cycloheximide (25 μg/mL) and nalidixic acid (25 μg/mL) and 15% (w/v) NaCl.

The aerobic development and growth characteristics of halophilic actinomycetes were followed daily at 37°C on plates and colonies were recognized by their characteristic chalky leather appearance and their severe and dry appearance. After four weeks of growth, colonies were counted and twenty-three, with diverse morphologies, pigmentation, and sizes, were randomly selected from the different media, subcultured several times on their isolation media to obtain pure cultures, and stored at −80°C in 20% (v/v) glycerol. A code of three letters and one number was assigned for each strain: the first letter of S and W refers, respectively, to the sediment or water origin of the strain; the second letter corresponds to the isolation pond: T (for TS18 pond) and M (for M1 pond); the third letter designates the isolation medium: B (for Bennett medium), G (for GTY medium), Bg (Bergey's medium), C (for CM medium), S (for SCA

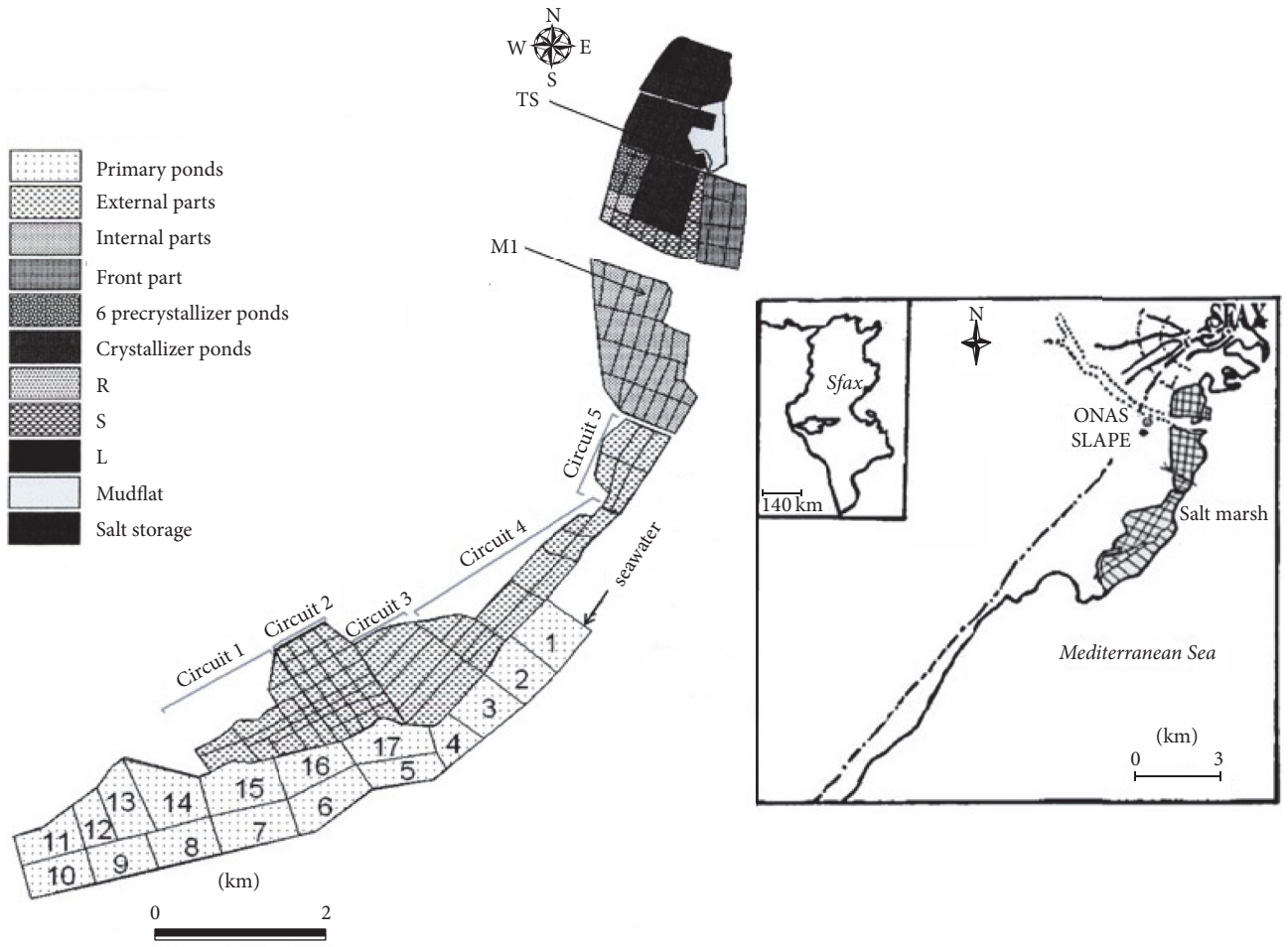


Figure 1: Map of the location of the two sampling ponds (TS18 and M1) of the Sfax solar saltern.

medium), and I (for ISP2 medium). The final number refers to the number of the isolates.

2.3. In Vitro Antimicrobial Activity. In order to study the antagonistic interaction between environmental actinomycetes, the isolates were grown on Bennett agar plates (10% NaCl) for 21 days at 37°C. Agar cylinders (6 mm in diameter) were then taken with hollow punch and deposited on the surface of the Bennett agar plate which had previously been seeded with one ml of 4-day cultured target-actinomycete strain. Plates were kept at 4°C for 2 h and then incubated at 37°C for 7–14 days. The inhibition zones were measured after incubation and expressed in mm.

The antimicrobial activities of the isolates were also tested against Gram − (*Escherichia coli* BW25113, *Agrobacterium tumefaciens, Salmonella enterica* ATCC43972, and *Pseudomonas aeruginosa* ATCC49189) and Gram+ (*Micrococcus luteus* LB 14110, *Staphylococcus aureus* ATCC6538, and *Listeria ivanovii* BUG 496) bacterial pathogens. The search for antibacterial activity was carried out by the method of disc agar [26], where actinomycete isolates were grown on Bennett agar medium for 14 days at 37°C and agar cylinders (6 mm in diameter) were then taken and deposited on the surface of the Mueller–Hinton agar plates previously seeded with the test microorganism (10^5–10^6 CFU/mL). The inhibition zones were measured after 24 hours of incubation at 37°C and expressed in mm.

2.4. Phenotypic and Growth Characteristics of Potential Isolates. Morphological, biochemical, culture, and physiological characterization of potential isolates were determined. Formation of aerial, substrate mycelium and spore arrangements on mycelium were observed with a light microscope (Reichert-Jung series 150 model) and monitored under a phase contrast microscope (Nikon ECLIPSE E600, USA) at 100x magnification. Various colony characteristics such as mycelia color, size, shape, and diffusible pigment production were recorded. Biochemical characterization, namely, Gram reaction, oxidase, catalase, and H_2S, and indole production; urease, nitrate, and nitrite reduction; Red Methyl-Voges Prauskuer reactions; ONPG, citrate, and mannitol utilization were also performed as suggested by Holt et al. [27]. NaCl range tolerance and optimal requirement for growth were determined using Bennett medium agar supplemented with different concentrations of NaCl (0, 5, 10, 15, 20, 25, and 30%).

Temperature and pH range for growth were also determined using Bennett medium [28].

2.5. DNA Extraction of Potential Isolates and PCR Amplification of 16S rRNA. The six potential isolates were grown for 4 days at 37°C with agitation in 15 ml of Bennett medium. Biomass was harvested by centrifugation at 4,000 rpm for 15 min and washed twice with sterile saline water. The method of Rainey et al. [29] was used for the extraction and purification of genomic DNA. The 16S rDNA gene of the six isolates was amplified by polymerase chain reaction (PCR) using primers fD1 (5′AGAGTT TGATCCTG GCTCAG 3′) and Rs16 (5′AAG GAG GTG ATC CAA GCC 3′) [30]. The final volume of the reaction mixture of 50 μl contained tampon buffer (10x) with 50 mM $MgCl_2$, deoxynucleoside triphosphates (10 mM dNTP), 10 μM (each) S1 and S2 primers, 2 μL (80 ng) DNA, and 0.1 μL Taq DNA polymerase (5 U/μL). Amplification was made using a Basic PCR protocol which consisted of an initial denaturation at 95°C for 10 min, followed by 30 amplification cycles of 94°C for 45 s, 52°C for 30 s, and 72°C for 1 min and 30 s and a final extension step of 72°C for 10 min [31]. The amplification result was detected by agarose gel (1%) electrophoresis and visualized by ultraviolet fluorescence after ethidium bromide staining [32]. The purification of DNA 16S fragment from PCR on agarose gels was performed using the PureLink Quick Gel Extraction and PCR Purification Combo Kit. The same primers were then used separately in two sequencing reactions from the two ends of the amplified fragment (about 1.5 kbp). The two sequences obtained were compared for similarity with those contained in genomic database banks, using the NCBI BLAST [33].

2.6. Phylogenetic Analysis. Sequence data were established with the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and studied for sequence homology with the archived 16S rDNA sequences from GenBank at https://www.ncbi.nlm.nih.gov/nucleotide, using the BLAST search program [34]. Different sequences were aligned with CLUSTAL W [35, 36] and a phylogenetic tree was constructed using the neighbor-joining DNA distance algorithm within the MEGA6 (Molecular Evolutionary Genetics Analysis Version 6.0) (http://www.megasoftware.net/) software [37]. The 16S rRNA gene sequences of the six potential thermoactinomycete strains have been deposited in the GenBank database under the accession numbers of KP229518–KP229523.

2.7. Production, Extraction, and Liquid Chromatography-High Resolution Mass Spectrometry Analysis of Antimicrobial Products from Paludifilum halophilum. Paludifilum halophilum strain SMBg3 was cultured on Bennett medium supplemented with 10% NaCl, for 7 days at 37°C. Mycelium was scraped and inoculated into four Erlenmeyer flasks (1 L) containing 250 mL of the same medium. After seven days of incubation, the total broth was mixed, centrifuged at 4000g for 15 min, and then filtered through Whatman number 1 filter paper. The pellet was transferred aseptically into a conical flask and an equal volume of ethyl acetate was added to the filtrate and shaken vigorously for 2 hours for the complete extraction of the antibacterial compounds. The ethyl acetate phase containing the active principal was separated from the aqueous phase and was evaporated to a residue using Rota vapor (Heidolph: P/N Hei-VAP Value/G3: 560-01300-00). One mg of the residue was accurately weighed and dissolved in 300 μL of ethyl acetate and this solution was filtered through 0.2 μm PTFE filter into HPLC vial where it is submitted to LC/MS analysis. High resolution mass spectrometric data were obtained using a ThermoLTQOrbitrap coupled to an HPLC system (PDA detector, PDA autosampler, and pump). The following conditions were used: capillary voltage of 45 V, capillary temperature of 260°C, auxiliary gas flow rate of 10–20 arbitrary units, sheath gas flow rate of 40–50 arbitrary units, spray voltage of 4.5 kV, and mass range of 100–2000 amu (maximal resolution of 30000). For LC/MS, a Sunfire C18 analytical HPLC column (5 μm, 4.6 mm × 150 mm) was used with a mobile phase of 0 to 100% MeOH over 30 min at a flow rate of 1 mL/min.

3. Results and Discussion

3.1. Physicochemical and Microbiological Analysis of Samples. The physicochemical parameters of the water above the sediment surface and the microbiological parameters of the water and superficial sediment from which cores were collected during the three campaigns are summarized in Table 1. M1 pond was characterized by an intermediate salinity ranging from 19 to 21%, whereas the TS18 pond had a higher salinity that varied between 31 and 36%. Temperature was slightly higher in pond TS18 than in M1, while pH was slightly alkaline in M1 and close to neutrality in TS. Moreover, dissolved organic carbon was found to be significantly higher in TS18 than in M1. It was noticed that superficial sediment samples from M1 contained gypsum deposit, while those of TS were constituted with halite. The maximum values of total cell counts (5.9×10^9 cells/g) were detected in the sediment of the TS18 pond and were 2-3-fold higher than in M1 pond (Table 1). In addition, when comparing water and sediment samples, it was found that, for both prospected ponds, the maximum values of total cell counts were detected in sediments and were 3- to 10-fold higher than those obtained in waters (Table 1). This could be attributed to greater organic matter abundance [38] and lower predation rates [19, 39].

The cultivable actinomycete density during the 3 sampling campaigns showed different patterns in M1 and TS18 ponds, with counts between 2.5 and 7 times higher in TS18 than in M1 (Table 1). Intriguingly, for both M1 and TS18, no cultivable actinomycetes could be detected in waters, while their counts in superficial sediments were much lower than those reported in previous studies [40, 41].This could be explained, in part, by the high salinity of the prospected ponds and the severe selective pressure of our isolation procedure, which allows the growth of only halophilic strains able to support at least a salinity of 15%. In fact, the occurrence of actinomycetes in the hypersaline environment has been reported in several previous studies, stating the decrease in actinomycetes colonies forming units counts with

Table 1: Range of physicochemical characteristics of water above the two ponds' sediment surface and microbiological parameters during the three campaigns.

Parameters	Pond M1	Pond TS18
Physicochemical		
pH	8.3–8.5 ± 0.1	7.2–7.8 ± 0.2
Salinity (%)	19–21 ± 1	31–36 ± 1
Temperature (°C)	14–19 ± 1	16–22 ± 1
Dissolved organic carbon (mg l^{-1})	2.7–3.1 ± 0.5	5.8–7.4 ± 0.5
Microbiological		
Water total cell count (10^8 cells ml^{-1})	1.2–1.8 ± 0.01	3.2–19 ± 0.05
Sediment total cell count (10^8 cells g^{-1})	13–19 ± 0.02	36–59 ± 0.1
Water cultivable actinomycete count (UFC ml^{-1})	0	0-1
Sediment cultivable actinomycete count (UFC g^{-1})	3–30 ± 1	20–70 ± 3
Number of actinomycete isolates from water	0	0
Number of actinomycete isolates from sediment	3	20

Each data is mean of three independent analyses ± standard deviation; P value < 0.05.

the increase in salinity [41, 42]. In addition, the ability of actinomycete cells to enter a viable but nonculturable state in response to stressful conditions, in which bacteria lost their ability to form colonies in the surface of solid media, could not be discarded [43, 44].

3.2. Isolation of Strains and Screening of Antimicrobial Activities. Given that less than 1% of bacteria from saline environments can be cultured, the use of appropriate isolation media is critical for improving the recovery of Actinobacteria [45]. Six isolation media were chosen in this study to select for Actinobacteria. During the 3 sampling campaigns and based on colony morphology, growth characteristics, and macroscopic examination, a total of twenty-three actinomycete strains were collected on all isolation media with 3 being isolated from M1 and 20 from TS18 (Table 1). Bennett medium exhibited the highest recovery producing 9 isolates, followed by GTY medium with 7 strains while no strain was recovered on ISP2 medium. Most isolates showed, on Bennett medium at 10% NaCl, aerial mycelia with color varied from yellow and light yellow to beige white and fluorescent spores arranged in chains.

The 23 strains were tested for their ability to produce antimicrobial substances. The result of antagonistic interactions between the actinomycete strains, taken in pairs, allowed the detection of 18 active strains named STS3, STB1, STG6, STG1, STC3, STS2, STC4, STG4, STB7, SMC3, SMBg3, STG8, STC5, STG2, STB6, STB8, STB2, and STG5 producing antimicrobial compounds against one or more target-actinomycete strains (Table 2). The halo diameter was used to monitor each strain level of antimicrobial substance produced. Our results showed that the inhibition zones diameter versus the target strain ranged from 13 to 33 mm and their activity spectrum comprised between one and 8 target strains, which suggested that the produced substances could be of a different nature (Table 2). Most strains (thirteen of the eighteen) have a wide spectrum of inhibition, with at least two sensitive strains (Table 2).

The isolates antibacterial potential was also analyzed against seven pathogen strains and the antibacterial activity extent was varied among the actinobacterial isolates (Table 3). Nineteen out of the 23 strains of halophilic actinomycetes exhibited appreciable inhibitory activity against Gram-negative and/or Gram-positive bacteria. Among them, 5 strains, named STB2, STC3, STG2, SMB5, and SMC3, acted only against Gram-negative bacteria and 6 strains (STG3, STG4, STG6, STB1, STB3, and STB4) against only Gram-positive bacteria. The 8 remaining strains (STB6, STB8, STS2, STS3, STC5, STC4, STG1, and SMBg3) revealed excellent antibacterial activity against both Gram-positive and Gram-negative bacteria. Interestingly, when the antagonistic activity was lacking for one strain, either it was completely inactive on human pathogens or its activity was strongly reduced. In the literature, while the antagonistic properties of halotolerant and moderate halophilic actinomycetes have often been reported in the literature at low or medium salinity [41, 46, 47], those of halophilic isolates at salinity close to saturation have never been mentioned. This is the first report showing that the crystallizer and noncrystallizer ponds of Sfax marine saltern harbored potential halophilic actinomycetes producing antimicrobial compounds against Gram-positive and Gram-negative pathogen bacteria. Based on their broad (STB8, STS2, STS3, STC4, and SMBg3) or narrow (SMC3) activity spectrum, 6 strains among all the actinomycete isolated were, therefore, subjected to detailed taxonomic studies.

3.3. Characterization of Potential Isolates. In order to estimate the relatedness between the 6 potent isolates, the physiological, biochemical, and growth characteristics of each strain were compared. Results in Table 4 revealed that the colonies of the six isolates were circular and aerial mycelium was observed for only STC4, SMC3, STB8, and SMBg3 with fluorescent spores arranged in chains. However, substrate mycelium was between pale yellow for SMBg3 and SMC3, white for STS3 and STS2, colorless for STC4, and transparent

Table 2: Antagonistic interactions between the isolate strains.

Producer strain	Target strain																							
	STS2	STG1	STB6	STG5	STB8	STG4	STC5	STG8	STB7	STG6	STG3	STG2	STS3	STC4	STB2	STC3	STB1	STB3	STB4	SMC3	STB9	SMB5	SMBg3
STG3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
STS3	13	18	—	28	—	18	—	—	—	—	—	18	—	—	—	—	—	—	—	—	14	16	—
STB1	—	—	—	—	—	—	—	—	—	—	—	15	—	—	—	—	—	—	—	—	—	—	—
STB3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
STB4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
STG6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	23	25	—
STG1	—	—	12	—	—	17	—	—	—	—	—	22	15	—	15	—	—	—	—	14	—	—	—
STC3	—	29	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	16	22	—
STC4	14	34	14	—	23	—	—	—		—	—	—	—	—	—	—	—	—	—	15	16	18	—
STG5	—	—	15	—	—	17	—	—	12	—	—	19	17	—	—	—	—	—	—	13	16	18	—
STB6	—	—	—	17	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		—	—
STB8	16	—	13	—	—	14	19	—	—	18	—	—	—	—	—	—	—	—	—	—	22	17	16
STB2	13	—	—	—	14	12	—	—	—	—	—	—	—	—	—	—	—	—	—	—	17	12	—
STS2	—	22	—	—	—	14	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
STG4	—	—	—	—	—		—	—	—	—	—	—	—	—	—	—	—	—	—	—	24	27	—
STB7	—	—	18	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—	17	—	15	—
STC5	—	25	—	—	—	—	—	—	—	—	—	—	15	—	—	—	—	—	—	—	16	17	—
SMC3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		—	18	—
STG2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	18	—	—
STG8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	16	—	—
STB9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
SMBg3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	32	33	—
SMB5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Results shown in the above table are average of triplicate parallel experiments.

Table 3: Antimicrobial activities of halophilic actinomycetes isolated from solar salterns of Sfax.

Strains	*A. tumefaciens*	*S. aureus*	*S. enterica*	*M. luteus*	*E. coli*	*L. ivanovii*	*P. aeruginosa*
STB2	—	—	—	—	12	—	15
STB6	—	—	14	—	17	13	16
STB8	12	11	16	—	14	—	21
STG5	—	—	—	—	—	—	—
STS2	14	—	12	12	16	14	—
STS3	15	12	14	—	17	—	—
STC4	12	14	14	14	14	—	—
STC5	—	—	—	—	15	12	—
STC3	—	—	—	—	13	—	19
STG3	—	—	—	—	—	11	—
STG4	—	23	—	—	—	12	—
STG6	14	—	—	—	—	13	—
STB1	—	—	—	—	—	11	—
STB3	—	—	—	—	—	11	—
STB4	—	—	—	—	—	11	—
STG8	—	—	—	—	—	—	—
STG1	15	—	—	12	15	—	—
STB7	—	—	—	—	—	—	—
STG2	—	—	16	—	15	—	—
STB9	—	—	—	—	—	—	—
SMBg3	15	15	16	—	15	—	16
SMB5	—	—	—	—	20	—	—
SMC3	—	—	—	—	13	—	—

Results shown in the above table are average of triplicate parallel experiments.

for STB8. Gram and catalase reactions were positive for all strains. All the isolates could not metabolize mannitol and ONPG or produce H_2S. Only STC4 and STB8 strains were nitrate reductase +, while RM and VP reactions were negative for all isolates, except STB8 which was RM positive. Isolates were also screened for their growth at various NaCl, temperature, and pH levels. All isolates exhibited growth in the NaCl range of 5–20% with an optimum at 10% NaCl and at a temperature range of 30–55°C with an optimum of 45°C, while pH range for growth was between 5.0 and 11.0 with an optimum at 8.0–8.5 for all strains. These results showed that all isolates in this study were halophilic and thermotolerant. Carbon source utilization is also given in Table 4, showing that the six strains could metabolize glucose and starch, but not sucrose. However, saccharose, maltose, and xylose were metabolized weakly by STS2 strain and strongly by the others. Our results showed also that the actinomycete strains constitute potential producers of amylase (69% of the total isolates), followed by protease (52%), cellulase, DNase (39%), and lipase (4%). This is in agreement with our previous studies conducted on the same ponds which found that the most frequent hydrolytic activity among archaeal isolates was observed for amylase and protease [21]. Assuming that these frequencies are related to the nature of the organic matter in the pond, these results may suggest that carbohydrates and proteins in the sediment are the major carbon sources for the halophilic prokaryotes inhabiting the two ponds [19].

To ascertain the phylogenetic relationships of potential strains, their almost-complete 16S rRNA gene sequences (1352–1483 bp) were determined. A comparative sequence analysis using the BLAST program and a phylogenetic analysis using neighbor-joining revealed that the six strains were very close and formed a distinct sublime within the Thermoactinomycetaceae family (Figure 2). The six strains shared the highest 16S rRNA sequence similarity with respect to the strain types of *Salinithrix halophila* CECT 8506^T (94%), *Desmospora activa* DSM 45169^T (94%), *Kroppenstedtia guangzhouensis* KCTC 29149^T (95%), *Kroppenstedtia eburnea* DSM 45196^T (95%), and *Melghirimyces algeriensis* (95%). These similarity percentages lower than 97% suggested that the isolated actinomycete strains could represent new members of the Thermoactinomycetaceae family and their sequences were published in the GenBank database under the accession numbers of KP229518–KP229523. To go further in the taxonomic position of these strains in the phylum Actinobacteria, we recently performed a polyphasic taxonomic study on strain SMBg3 [16]. Our results revealed that this strain occupied an independent phylogenetic lineage distinct from all other reference genera within the family Thermoactinomycetaceae. On the basis of these data and

Table 4: Phenotypic characteristics of potential actinomycete isolates.

Characteristics	STS2	STS3	STC4	SMBg3	SMC3	STB8
Pond/growth medium	TS/SCA	TS/SCA	TS/SCA	M1/Bg	M1/CA	TS/Bt
Morphological characteristics						
Colony aspect	Circular	Circular	Circular	Circular	Circular	Circular
Colony size (mm)	4–8	4–8	2–4	4–8	4–8	1–5
Aerial mycelium	Absent	Absent	Yellow	Pale yellow	Yellow	Yellow
Substrate mycelium	White	White	Colorless	Yellow pale	Yellow pale	Transparent
Spore chains/fluorescence	Short chains/fluorescent	Long chains/very fluorescent	Short chains/fluorescent	Long chains/fluorescent	Short chains/fluorescent	Long chains/fluorescent
Biochemical characteristics						
Gram staining	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Oxidase						
Mannitol	−	−	−	−	−	−
Citrate	−	−	−	−	+	−
H_2S production	−	−	−	−	−	−
Nitrate reductase	−	−	+	−	−	+
Nitrite reductase	−	−	−	−	−	−
ONPG	−	−	−	−	−	−
Urea	−	−	−	−	+	+
Indole production	−	−	−	−	+	−
RM	−	−	−	−	−	+
VP	−	−	−	−	−	−
Growth characteristics						
% NaCl range (optimum)	5–15 (10)	5–20 (10)	5–20 (10)	5–20 (10)	5–20 (10)	5–15 (10)
Temperature range (optimum)	30–55 (45)	30–55 (45)	30–55 (45)	30–55 (45)	30–55 (45)	30–55 (45)
pH range (optimum)	5–11 (8)	5–11 (8)	5–11 (8)	5–11 (8)	5–11 (8)	5–11 (8)
Carbon source utilization (1%)						
Glucose	+	+	+	+	+	+
Sucrose	−	−	−	−	−	−
D(+) Saccharose	+/−	+	+	+	+	+
D Maltose	+/−	+	+	+	+	+
D-Xylose	+/−	+	+	+	+	+
Mannitol	−	+	+	+/−	+	+
Starch	+	+	+	+	+	+

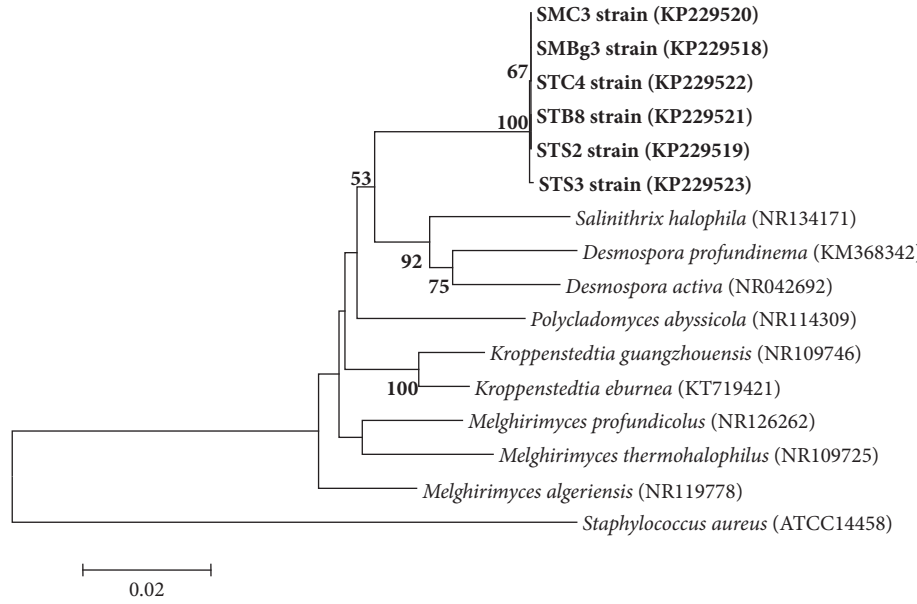


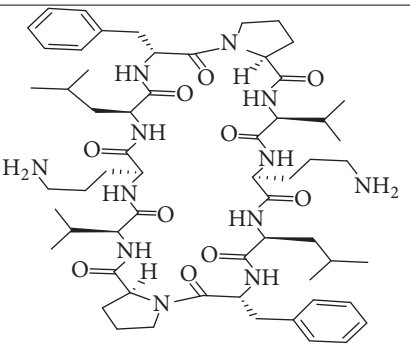
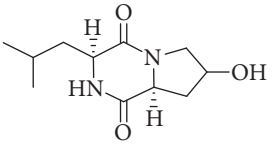
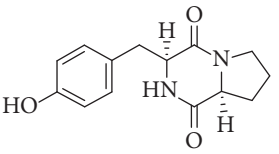
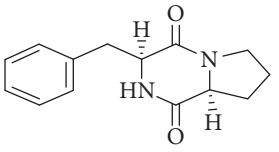
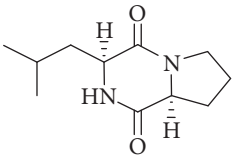
Figure 2: Neighbor-joining tree based on 16S rDNA sequences showing the relations between the halophilic actinomycete strains (STS2, STC4, STB8, SMBg3, STC3, and STS3) and type species of the family Thermoactinomycetaceae. The accession numbers of strain sequences are given in parentheses. The numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 1000 resampled datasets; only values over 50% are given. Bar: 0.02 nucleotide substitutions per nucleotide position. *Staphylococcus aureus* (ATCC14458) is given as outgroup.

other phenotypic and chemotaxonomic characteristics, strain SMBg3 was assigned to a new genus in the family Thermoactinomycetaceae for which the name *Paludifilum* was proposed and the type strain of the type species SMBg3^T was named *Paludifilum halophilum* and deposited in the DSMZ (=DSM102817^T) and CCUG (=CCUG 68698^T) public collections [16]. In fact, the occurrence of new genera of halophilic thermoactinomycetes has been reported in the hypersaline environments of distant geographical sites such as the Algerian salt lake [17] and the soil of hypersaline wetland in the north of Iran [18]. The saltern of Sfax can thus be considered as an additional geographical site harboring new members of halophilic thermoactinomycetes. Moreover, this is the first report revealing that these microbes could have an antimicrobial potential.

3.4. Production, Purification, and LC/MS Characterization of Antimicrobial Compounds Produced by Paludifilum halophilum. In order to unveil the preliminary characteristics of antimicrobial compounds produced by *Paludifilum halophilum* strain SMBg3, the strain was cultivated in large scale and active substances were extracted by ethyl acetate as mentioned above and tested against three Gram-positive and four Gram-negative bacteria at a 2.5 mg/disc concentration. Our results showed good activity against *S. aureus* ATCC6538 (14 mm), *E. coli* W 25113 (15 mm), *A. tumefaciens* (14 mm), *S. enterica* ATCC43972 (16 mm), and *P. aeruginosa* ATCC49189 (16 mm). No inhibition was observed for *M. luteus* and *L. ivanovii*. In a previous study by Vijayakumar et al. [48], the ethyl acetate extract of *Streptomyces* sp. was highly active against *Vibrio cholerae* (26 mm), *Salmonella typhi* (24 mm), *Proteus vulgaris* (23 mm), *Candida albicans* (17 mm), *Klebsiella pneumoniae* (16 mm), *Proteus mirabilis* (15 mm), *Staphylococcus aureus* (15 mm), and *Escherichia coli* (14 mm). In Tunisia, little attention has been paid to the antimicrobial activity of actinomycetes. To date, only one study has been conducted by Trabelsi et al. [49] on different rhizospheric soils and showed that, among fifty-four isolates of actinomycetes collected, 42 strains were classified as *Streptomyces*, 4 strains were classified as *Micromonospora*, 1 was classified strain as *Pseudonocardia*, 1 strain was classified as *Actinomadura*, 1 was classified strain as *Nocardia*, and 5 strains were classified as non-*Streptomyces*. In addition, more than the half of the ethyl acetate extract of these isolates was shown to inhibit at least one tested pathogenic microorganisms in liquid culture.

The LC-MS profiles of the crude extract of *Paludifilum halophilum* strain SMBg3 are shown in Figure 3. The chromatograph showed five major constituents whose structures are provided in Table 5. Molecular formula and chemical structures of these compounds were deduced from the quasimolecular ion peak $[M + H]^+$ on the basis of their HRESI-MS spectrum. These five major constituents are the cyclic lipopeptide Gramicidin S (Rt: 9.08 min) and four cyclic dipeptides (CDPs) identified as cyclo(L-4-OH-Pro-L-Leu) (Rt: 4.08 min), cyclo(L-Tyr-L-Pro) (Rt: 4.83 min), cyclo(L-Phe-L-Pro) (Rt: 5.18 min), and cyclo(L-Leu-L-Pro) (Rt: 5.87 min). In fact, CDPs [also known as 2,5-dioxopiperazines; 2,5-diketopiperazines; cyclo(dipeptides); or dipeptide anhydrides] are well known for their economically beneficial

Table 5: LC-HR-ESI-MS analysis of the most interesting compounds extracted from liquid cultures of *Paludifilum halophilum* strain SMBg3.

Rt (min)	Accurate mass	Molecular formula*	Suggested compound	Notes	Structure
9.08	1141.71463	$C_{60}H_{92}O_{10}N_{12}$	Gramicidin S-1141	Lipopeptide	
4.08	227.13956	$C_{11}H_{18}N_2O_3$	Cyclo(L-4-OH-Pro-L-Leu)	Dipeptide	
4.83	261.12405	$C_{14}H_{16}N_2O_3$	Cyclo(L-Tyr-L-Pro)	Dipeptide	
5.18	245.12918	$C_{14}H_{16}N_2O_2$	Cyclo(L-Phe-L-Pro)	Dipeptide	
5.87	211.14438	$C_{11}H_{18}N_2O_2$	Cyclo(L-Leu-L-Pro)	Dipeptide	

*Formula deduced from the quasimolecular ion peak $[M + H]^+$.

biological activities and therefore are among the most common peptide derivatives found in nature [50]. They have been isolated from microorganisms, sponges, and from a variety of tissues and body fluids [51–53]. The cyclic form of the dipeptide is often more stable in vivo than its linear counterpart, making them far more promising in terms of drug candidacy [54]. Both natural and synthetic diketopiperazines have a wide variety of biological activities, including antitumor [55] (Nicholson et al. 2006), antiviral [56], antifungal [57], and antibacterial [58] activities. For instance, the cyclo(Pro-Tyr) was first isolated from *Alternaria alternate* [53] and then from *Streptomyces* sp. TN25644. Cain et al. [59] reported that cyclo(Pro-Tyr) exhibits no activity against strains of *Micrococcus luteus, Mycobacterium smegmatis, Sacharomyces cerevisiae, Candida neoformans, Candida albicans*, and *Aspergillus niger*. In contrast, Smaoui et al. [60] reported a strong antimicrobial activity of cyclo(Pro-Tyr) against *Micrococcus luteus* LB 14110, *Salmonella enterica* ATCC43972, and *Fusarium* sp.

4. Conclusions

This study deals with the isolation, characterization, and antimicrobial potentiality of a collection of halophilic actinomycete strains isolated from the solar saltern of Sfax. Our results revealed, for the first time, that the superficial sediment of this ecosystem is a source of novel halophilic actinomycetes belonging to the Thermoactinomycetaceae family with unexplored potential for antimicrobial discovery. For the potent strain SMBg3, assigned to a new genus in the family and named *Paludifilum halophilum*, we have shown that the observed antimicrobial activity is most likely explained by the production of Gramicidin S and four cyclic dipeptides identified as cyclo(L-4-OH-Pro-L-Leu), cyclo(L-Tyr-L-Pro), cyclo(L-Phe-L-Pro), and cyclo(L-Leu-L-Pro). The potent inhibitory effect of these compounds covered the growth of the plant pathogen *A. tumefaciens* and the human pathogens *S. aureus, S. enterica, E. coli, and P. aeruginosa*. To the best of our knowledge, this is the first time that the bioactivity of cyclic antimicrobial peptides

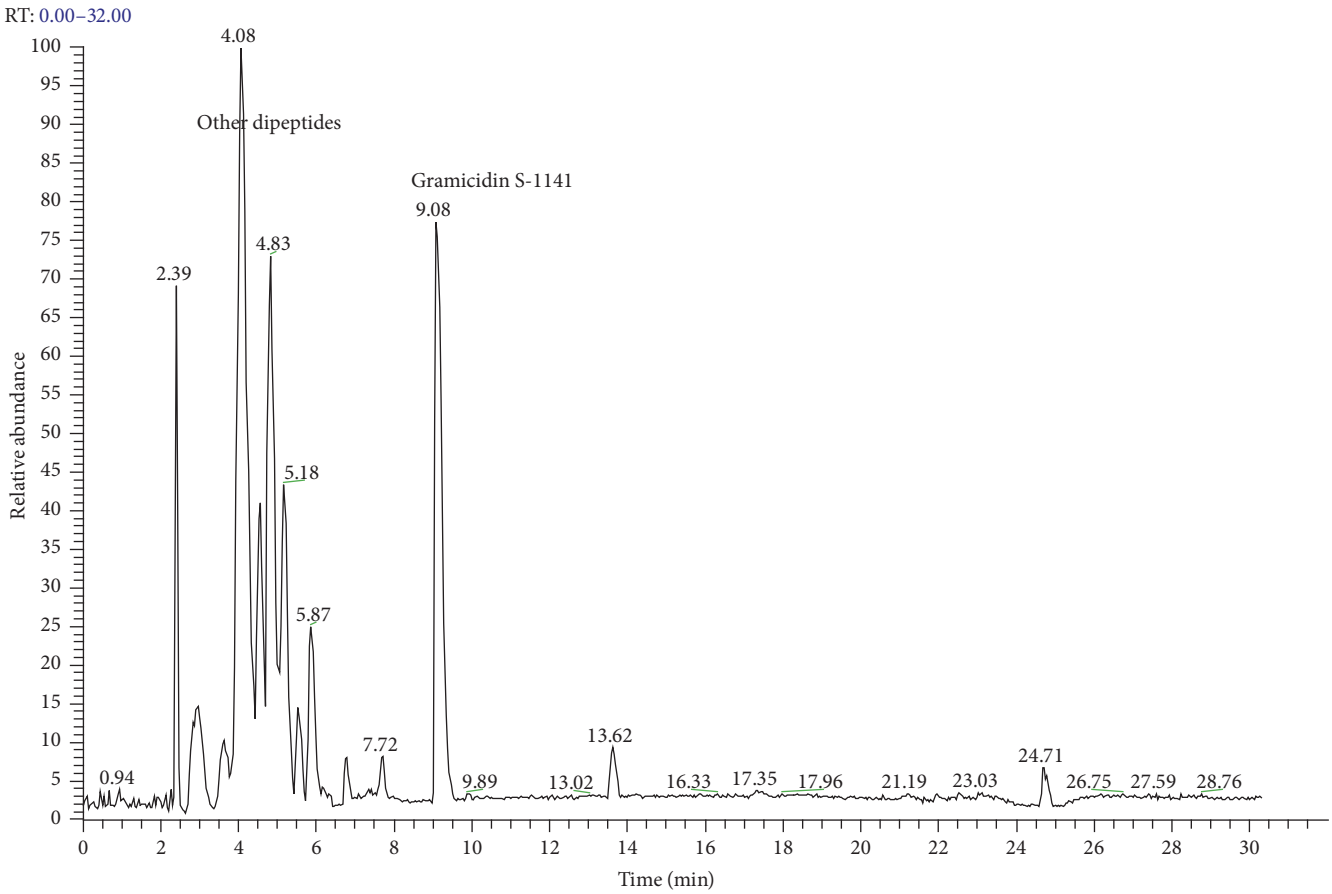


Figure 3: LC/MS chromatogram of the crude extract from *Paludifilum halophilum* strain SMBg3.

from halophilic thermoactinomycete against agriculturally and medically important bacteria is reported. Our work is now in progress to purify these cyclic antimicrobial peptides for further characterization.

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

Kinetics of Tyrosinase Inhibitory Activity Using *Vitis vinifera* Leaf Extracts

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Natural medical plant is considered as a good source of tyrosinase inhibitors. Red vine leaf extract (RVLE) can be applied to a wide variety of medical disciplines, such as treatments for chronic venous insufficiency over many decades. This study investigated the tyrosinase inhibitory activity of RVLE containing gallic acid, chlorogenic acid, epicatechin, rutin, and resveratrol which are effective for skin hyperpigmentation. The five components contents are 1.03, 0.2, 18.55, 6.45, and 0.48 mg/g for gallic acid, chlorogenic acid, epicatechin, rutin, and resveratrol. The kinetic study showed the tyrosinase inhibitory of RVLE via a competitive reaction mechanism. RVLE solution has an IC_{50} (the half inhibitory concentration) value of 3.84 mg/mL for tyrosinase inhibition, that is, an effective tyrosinase inhibitory activity, and can be used as a whitening agent for cosmetic formulations in the future.

1. Introduction

A wide variety of skin care products, tagged with effective ingredients for skin hyperpigmentation, become the best-selling products in the cosmetics market across Asia [1]. These products containing extracts of plants, like *Glycyrrhiza uralensis* or *Morus alba*, usually become popular cosmetics. Numerous studies aimed to search for effective materials for skin hyperpigmentation in an effort to reduce dark spots and hyperpigmentation [2–4]. This inhibitory activity of tyrosinase (EC 1.14.18.1) has been extensively studied over the past years [5–7]. Tyrosinase is the rate limiting enzyme of melanogenesis and the main target of antimelanogenesis [8] and catalyzes the oxidation of L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA), which forms dopachrome. These catalyzed reactions result in the formation of melanin, which is responsible for skin pigmentation [9].

Tyrosinase inhibitors can be classified into four types, namely, competitive, uncompetitive, mixed type (competitive/uncompetitive), and noncompetitive inhibitors. Kojic acid shows a typical competitive inhibitory effect of tyrosinase [10].

Studies on tyrosinase inhibitors, such as hydroquinone, azelaic acid, kojic acid, and arbutin, can effectively expand the scope of research on hypermelanosis [11]. However, there exists certain degree of risk when using a great number of well-known tyrosinase inhibitors. For example, dihydroxybenzene may be irritating, mutagenic, and cytotoxic to sensitive skin, while arbutin and kojic acid may result in contact dermatitis and erythema [12]. Therefore, natural medical plants are considered as a good, and alternative, source of tyrosinase inhibitors. Numerous natural tyrosinase inhibitors are identified and reviewed [13, 14].

A red vine leaf is composed of the dried leaves from cultivars of the plant *Vitis vinifera* L. [15]. Red vine leaf extract (RVLE) is herbal medicine involving numerous flavonoids as the major active ingredients thereof [16]. It contains not less than 4% of total polyphenols and 0.2% of anthocyanins [15]. Numerous investigations reported medical applications of RVLE [17], for example, treatments for chronic venous insufficiency over many decades [16, 18]. Besides, it could also improve endurance capacity by facilitating fatty acid utilization in skeletal muscle in mice [19].

Although RVLE has many pharmacological effects, there is no report on the use of RVLE as a tyrosinase inhibitor. Accordingly, this study aims to investigate the kinetics of tyrosinase inhibitory activity using red vine leaf extracts and to develop an alternative natural cosmetic material.

2. Materials and Methods

2.1. Material. Mushroom tyrosinase, chlorogenic acid, epicatechin, schisandrin, and sodium phosphate monobasic were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-3,4-Dihydroxyphenylalanine (L-dopa) and kojic acid were purchased from Acros (New Jersey, USA). Acetonitrile was purchased from Aencore (Surrey Hills, Australia). Sodium phosphate dibasic anhydrous was purchased from J. T. Baker (Petaling Jaya, Selangor, Malaysia). Methanol was purchased from Merck (Darmstadt, Germany). RVLE (Elastvein®) was purchased from Healthmate (Changhua, Taiwan). Acetic acid was purchased from Panreac (Barcelona, Spain), and gallic acid was purchased from Alfa Aesar (Ward Hill, MA, USA). Rutin was purchased from Extrasynthese (Genay, France). Resveratrol was purchased from TCI (Tokyo, Japan).

2.2. Preparation of RVLE Samples. The solvent used for RVLE preparation was deionized water. Two grams of RVLE was sonicated in an ultrasonic bath (Chrom Tech) for 40 min with 98 mL of deionized water. The suspension was centrifuged at 6000 rpm (HERMLE 2206A) for 15 min. The supernatant was collected and run through a 0.45 μm filter. One milligram of schisandrin was dissolved in 10 mL of 70% methanol and then filtered through a 0.45 μm filter. The filtrate was collected as an internal standard solution. Before running the HPLC, 180 μL of the RVLE solution was mixed with 10 μL of the internal standard solution as a sample solution for analysis [20].

2.3. Calibration and Validation. Five purchased marker standard solutions, 20 mg of gallic acid, 100 mg of chlorogenic acid, 100 mg of epicatechin, 10 mg of rutin, and 10 mg of resveratrol, were individually dissolved with 10 mL of methanol and stored in a refrigerator for further use. Before adding the internal standard solution, a stock solution was diluted with methanol into a series of standard solutions (gallic acid standard: 60, 30, 15, 7.5, and 5 μg/mL; chlorogenic acid standard: 8.57, 6, 4.29, 3.53, and 3 μg/mL; epicatechin standard: 750, 375, 187.5, 93.75, and 62.5 μg/mL; rutin standard: 100, 50, 33.33, 25, and 20 μg/mL; resveratrol standard: 15, 7.5, 3.75, 1.875, and 1.25 μg/mL). Each solution was analysed twice by HPLC. Peak areas were plotted versus concentrations to establish a calibration curve of each standard. The recovery was determined by comparing the amount of the marker standard added with that of the marker standard found. The detection limit was determined by a signal to noise (S/N) ratio of at least 3 : 1. Relative standard derivation (RSD) for reproducibility derived from the variation of the peak-area ratio or retention time in six replicate samples [21].

2.4. HPLC Analysis. An HPLC system (Agilent 1200 Infinity Series, Agilent, USA) is equipped with a quaternary pump, an autosampler, a vacuum degasser, and a diode array detector. A reverse phase column (Cosmosil 5C18-AR II, 5 μm, 25 cm × 4.6 mm ID, Nacalai Tesque, Kyoto, Japan) was used. The mobile phase was a mixture of (A) 0.5% acetic acid and (B) methanol/acetonitrile (2/1, v/v). The mobile phase composition was as follows: 0 min, 90% of (A); 0–10 min, 80% of (A); 10–20 min, 60% of (A); 20–30 min, 40% of (A); and 30–40 min, 0% of (A). The flow rate was 0.8 mL/min, and the wavelength of the detector was set at 280 nm [21].

2.5. Analysis of Tyrosinase Inhibitory Activity. Put 40 μL of RVLE solution (3 mg/mL) in a 96-well plate. Then add 40 μL of tyrosinase solutions (0.693 (2.5 U/mL), 1.386 (5 U/mL), 2.772 (10 U/mL), 5.544 (20 U/mL), 6.93 (25 U/mL), and 11.088 (40 U/mL) μg/mL, resp.) in a sodium phosphate buffer at pH 6.8 (PBS) and 120 μL of the 0.625 mM of L-dopa solution (dissolved in PBS). Put another 40 μL of RVLE solution (2, 3, 4, 5, and 6 mg/mL) in a 96-well plate. Then add 40 μL of tyrosinase solution (6.93 μg/mL) and 120 μL of 0.625 mM of L-dopa solution. These mixed solutions were kept at 37°C for 30 min to find the suitable concentrations of tyrosinase used for the tyrosinase inhibitory activity of RVLE. The absorbance was measured at 475 nm [22] using a Microplate-Reader (Sunrise Basic, Grödig, Austria). Kojic acid (0.01, 0.02, 0.04, 0.06, and 0.08 mg/mL) was used as a positive control, and the solvent control used as a blank was deionized water. The tyrosine inhibition (%) was calculated by

$$\text{The inhibition rate}\,(\%) = \left(1 - \frac{\Delta \text{OD}_{\text{sample}}}{\Delta \text{OD}_{\text{control}}}\right) \times 100\%, \quad (1)$$

where $\Delta \text{OD}_{\text{sample}}$ and $\Delta \text{OD}_{\text{control}}$ represent the absorbance of the sample and the control measured at 475 nm, respectively. An IC_{50} (the half inhibitory concentration) value was determined by regression of a dose-response curve at which 50% target activity was lost.

2.6. Kinetic Properties of RVLE. A Lineweaver-Burk plot was made by plotting the inverse numbers of the reaction rate V and the concentration of substrate [S]:

$$\frac{1}{V} = \frac{K_m}{V_{\max}} \times \frac{1}{[S]} + \frac{1}{V_{\max}}. \quad (2)$$

A linear regression model created in a double reciprocal plot can be used to determine the Michaelis constant K_m and the maximum velocity $V_{\max}$, for the reason that the curves therein have the x-intercept $1/K_m$, the y-intercept $1/V_{\max}$, and the slope of $K_m/V_{\max}$. In addition, a Lineweaver-Burk plot can be used to rate the performance of an inhibitor as competitive, noncompetitive, or even uncompetitive [23].

Before the kinetic studies, the dose-dependent relationship between RVLE and tyrosinase was checked first. Put 40 μL of RVLE solution (0.1875, 0.375, 0.75, 1.5, and 3 mg/mL) in a 96-well plate. Then add 40 μL of tyrosinase solutions (0.693, 1.386, 2.772, 5.544, and 11.088 μg/mL). The substrate was L-dopa by 120 μL 0.1 mM in a sodium phosphate buffer at pH 6.8.

For kinetic studies, another 40 μL of RVLE solution (0, 0.375, 0.75, 1.5, and 3 mg/mL) was put in a 96-well plate, and

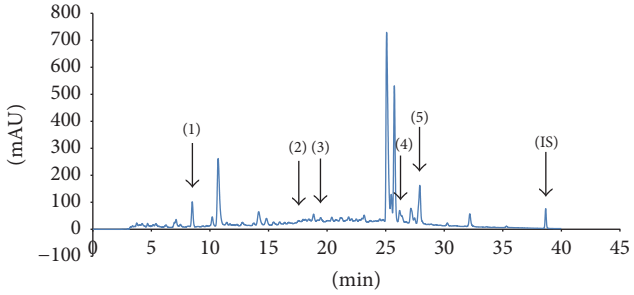

Figure 1: HPLC chromatograms of RVLE solution: (1) gallic acid, (2) chlorogenic acid, (3) epicatechin, (4) rutin, and (5) resveratrol and (IS) schisandrin.

then 40 μL of tyrosinase solutions (2.772 μg/mL) was added. The substrate was L-dopa solution which was made by 120 μL of dissolving L-dopa (0.078, 0.1, 0.156, 0.3125, and 0.625 mM, resp.) in a sodium phosphate buffer at pH 6.8. A Lineweaver-Burk plot was made by plotting the inverse numbers of the reaction rate (V) and the concentration of L-dopa (2) [23].

2.7. Statistical Analysis. Statistical evaluation was performed by running one-way analysis of variance (ANOVA) with SASR software (version 6.08, SAS Institute Inc., Cary, NC, USA). All data were presented as means and standard deviations (mean (SD)). Differences were considered statistically significant in case of a p value less than 0.05.

3. Results and Discussion

3.1. HPLC Analysis. Figure 1 shows the representative HPLC chromatograms of RVLE solution. The bioactive components of RVLE solution are gallic acid, chlorogenic acid, epicatechin, rutin, and resveratrol, which were effective compounds for skin hyperpigmentation confirmed by previous studies [24–27]. These components were identified by a comparison of HPLC chromatograms with standards. Based on the chromatographic analysis results, the five components contents are 1.03, 0.2, 18.55, 6.45, and 0.48 mg/g for gallic acid, chlorogenic acid, epicatechin, rutin, and resveratrol, respectively.

3.2. Inhibitory Ability of RVLE Solution. Figure 2 shows the inhibition of tyrosinase activity using RVLE solution as an inhibitor. RVLE solution reduced the tyrosinase activity in a dose-dependent manner. The linear regression line has a slope of 12.216 and y-intercept of 3.0097. The IC_{50} value of RVLE solution was evaluated as 3.84 mg/mL. For comparison, kojic acid was used as a positive control in this study. As in Figure 3, the inhibition of tyrosinase activity increased with the added amount of kojic acid, and its IC_{50} was 0.014 mg/mL. The tyrosinase inhibitory activity of *Vitis vinifera* L. was reported in the previous study using the extract of dried stems of the grape tree [28]. Although RVLE solution does not outperform kojic acid as an inhibitor, it was still generally recognized as a safe natural ingredient and could be safely used in cosmetic products [29].

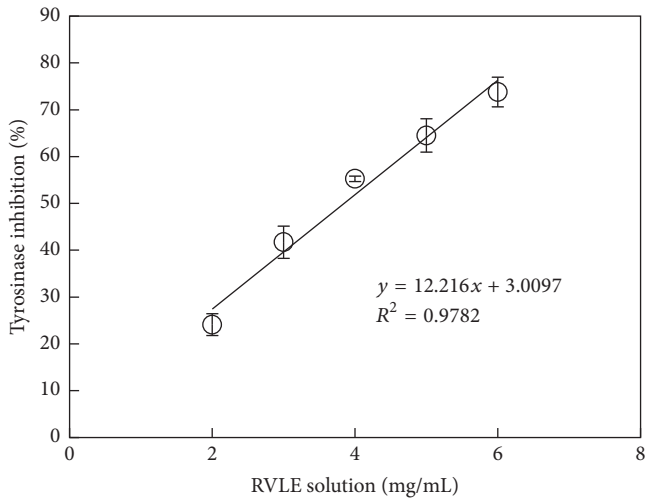

Figure 2: Inhibition of the tyrosinase activity using RVLE solution as an inhibitor (40 μL of tyrosinase solution (6.93 μg/mL) and 120 μL of 0.625 mM of L-dopa solution were added for each measurement).

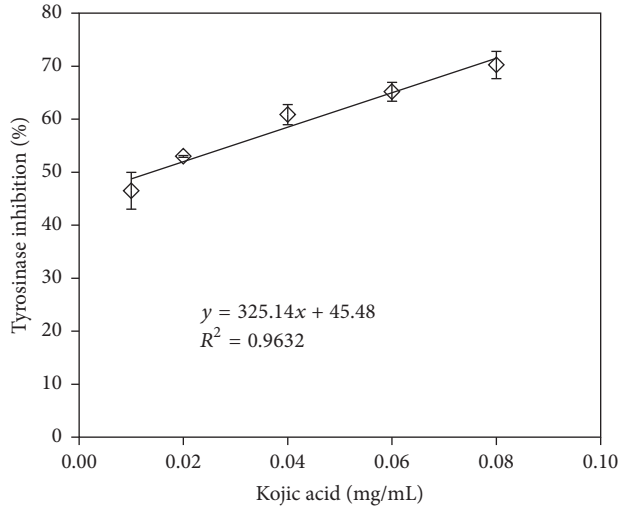

Figure 3: Inhibition of the tyrosinase activity using kojic acid as an inhibitor.

3.3. Kinetic Study of Tyrosinase Inhibitory Activity. RVLE solution showed the ability to inhibit the formation of dopachrome, which can be detected using a spectrophotometer at a wavelength of 475 nm. With 0.1 mM of L-dopa as a substrate, tyrosinase activity increased with the added amount of tyrosinase. As illustrated in Figure 4, there exists a linear relationship between the tyrosinase activity and the tyrosinase concentration, while the tyrosinase activity decreases with the added amount of RVLE solution. The RVLE solution is hence validated to inhibit tyrosinase activity successfully. In addition, low slope curves were seen as the amount of RVLE solution increased.

The inhibitory mechanism of RVLE could be further investigated by means of this kinetic study. Figure 5 shows a Lineweaver-Burk double reciprocal plot with the concentration of RVLE solution as a parameter. With L-dopa as

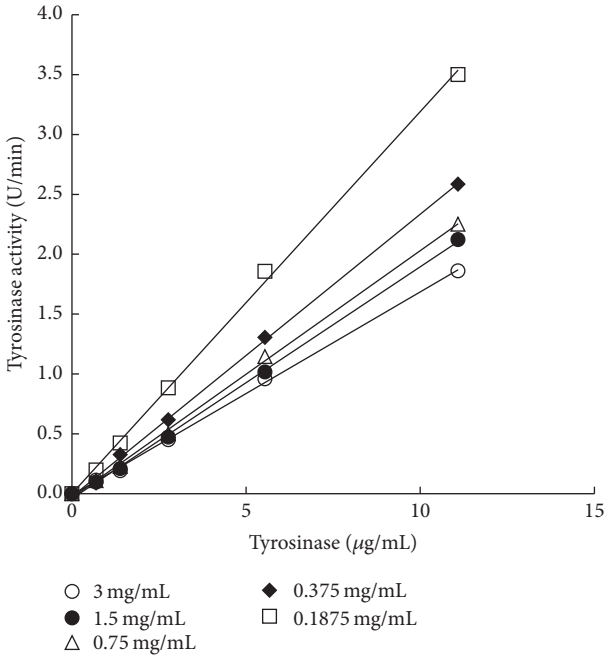

Figure 4: Influence of the RVLE concentration on the tyrosinase activity with 0.1 mM of L-dopa as a substrate.

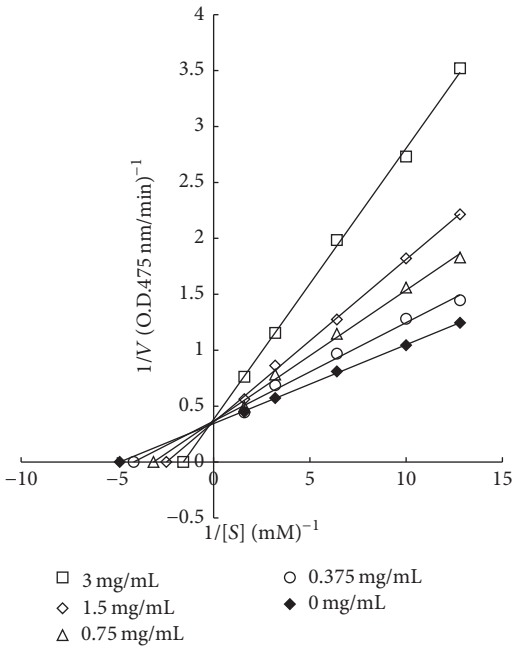

Figure 5: Lineweaver-Burk double reciprocal plot of RVLE solution with the concentration as a parameter (V: absorbance change rate, $\Delta OD_{475\,nm}$/min; [S]: concentration of L-dopa).

a substrate, the curves share the same y-intercept $1/V_{max}$, while the x-intercept $-1/K_m$ increased with the concentration of RVLE solution, as illustrated in Figure 5. In other words, V_{max} = 0.36 mM for all cases, but K_m increased due to the introduction of the inhibitor. In this context, the RVLE binding by tyrosinase had an effect on the L-dopa binding. Therefore, L-dopa and RVLE bound at the same sites on the tyrosinase. According to Figure 5, the inhibitory activity was rated as competitive.

4. Conclusions

In this study, it was concluded that a red vine leaf extract (RVLE) solution successfully reduced the tyrosinase activity. It provided an IC_{50} value of 3.84 mg/mL for tyrosinase inhibition, and the tyrosinase inhibitory activity was rated as competitive. The bioactive components of RVLE solution contained gallic acid, chlorogenic acid, epicatechin, rutin, and resveratrol. Therefore, RVLE solution could be used in cosmetic formulations as a natural whitening agent.

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

The Potential of a Brown Microalga Cultivated in High Salt Medium for the Production of High-Value Compounds

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Amphora sp. was isolated from the Sfax Solar Saltern and cultivated under hypersaline conditions. It contains moderate rates of proteins, lipids, sugars, and minerals and a prominent content of bioactive compounds: polyphenols, chlorophyll a, carotenoids, and fatty acids. The analysis of fatty acids with GC/MS showed that the C16 series accounted for about 75% of *Amphora* sp. lipids. Saturated fatty acids whose palmitic acid was the most important (27.41%) represented 41.31%. *Amphora* sp. was found to be rich in monounsaturated fatty acids with dominance of palmitoleic acid. It also contains a significant percentage of polyunsaturated fatty acids with a high amount of eicosapentaenoic acid (2.36%). Among the various solvents used, ethanol at 80% extracted the highest amounts of phenols and flavonoids that were 38.27 mg gallic acid equivalent and 17.69 mg catechin equivalent g^{-1} of dried extract, respectively. Using various *in vitro* assays including DPPH and ABTS radicals methods, reducing power assay, and β-carotene bleaching assay, the 80% ethanolic extract showed high antioxidant activity. A strong antibacterial activity was checked against Gram-positive bacteria (*Staphylococcus aureus* and *Micrococcus luteus*) and Gram-negative bacteria (*Klebsiella pneumoniae* and *Salmonella enterica*). These results are in favor of *Amphora* sp. valorization in aquaculture and food and pharmaceutical industries.

1. Introduction

Microalgae are considered as an important source of bioactive compounds with a wide range of applications and commercial use. They produce proteins, lipids, vitamins, pigments, and other molecules exploited for health, for food and feed additives, and for energy production. Due to their wide diversity and high storage capacity for lipids, particularly eicosapentaenoic acid (EPA) and amino acids, microalgae have pharmaceutical and cosmetic applications [1, 2]. Furthermore, the protective effects of natural antioxidants, against chemically induced oxidative damage, have been paid attention nowadays, especially when the generation of free radicals is involved. Benthic diatoms have become good sources of natural antioxidants, as revealed by a number of studies [3–6]. Moreover, microalgae represent a large and underexplored resource of antimicrobial compounds [7, 8]. Potentially, microalgal extracts are already being tested as additives for food and feed formulations, in an attempt to replace the currently used synthetic antimicrobial compounds, including subtherapeutical doses of antibiotics employed as a prophylactic measure in animal breeding [9]. Microalgae are ubiquitously distributed throughout the biosphere, where they have been adapted to survival under a large spectrum of environmental stresses, such as heat, cold, anaerobiosis, salinity, photooxidation, osmotic pressure, and exposure to ultraviolet radiation [10]. Hence, they may grow essentially under most of the environmental conditions available, ranging from freshwater to extreme salinity. They possess the extra advantage of substantial metabolic plasticity,

dependent on their physiological state. Likewise, their secondary metabolism can easily be triggered by most forms of externally applied stress [7].

Among the large variety of microalgae, Cyanophyceae, Chlorophyceae, Bacillariophyceae (diatoms), and Chrysophyceae are the most studied for biodiesel production [11]. It is estimated that diatoms produce about 25% of the global primary biomass [12]. In the Sfax Solar Saltern, Bacillariophyceae dominated with Dinophyceae in the least salty ponds but they are rarely abundant in hypersaline environments [13].

Unlike the universal macromolecules of primary metabolism (e.g., monosaccharides, polysaccharides, amino acids, proteins, and lipids), which are commonly present in all organisms, secondary metabolites have a far more limited distribution in nature. They are not necessarily produced under all conditions and can only be found in specific organisms or a group of organisms. The organism can produce these compounds either to protect itself within its own living ecosystem or to play a basic role in its everyday existence. This reveals bioactive molecules in unrelated biological systems [14]. The medicinal and pharmacological actions are often dependent on the presence of bioactive compounds called secondary metabolites [15]. The growth of algae is relatively rapid, and it is possible to control the production of their bioactive compounds such as polyphenols and pigments by modifying culture conditions [10].

In the present work, *Amphora* sp. (Bacillariophyceae) was cultivated in a hypersaline medium and the physicochemical properties, fatty acids profile, bioactive compounds, and some biological activities were determined.

2. Materials and Methods

2.1. Microalga. *Amphora* sp. is a Bacillariophyceae that was isolated via micromanipulation and serial dilution from a water sample collected in the pond C4-1 of the Sfax Solar Saltern with average salinity of 107 p.s.u. (practical salinity unit). The Sfax Solar Saltern is located in the central eastern cost of Tunisia, at about 34°39′N and 10°42′E. The ponds are shallow (20–70 cm depth) and have various salinities ranging from 40 to 400 p.s.u. [16]. They are connected by pipes and channels along a 12 km section of sea cost.

2.2. Culture Conditions. The microalga was cultivated in batch in autoclaved artificial seawater, which was enriched with F/2 nutrient medium, sodium silicate (Na_2SiO_3), and trace metals solution [17, 18]. Cultures were conducted for 15 days at the salinity of 100 p.s.u., 25°C, light : dark (L : D) cycle of (16 h : 8 h), and cool white fluorescent light intensity of 60 μmoles photons m^{-2} s^{-1}. The biomass was separated from the culture media by centrifugation (4500 ×g, 10 min), and the pellet was washed with distilled water and centrifuged again at 4500 ×g for 10 min (the washing was repeated two times). The pellet was freeze-dried and stored at −70°C.

2.3. Determination of Dry Matter, Ash, Proteins, Lipids, and Total Sugars. The dry matter and ash content were determined according to the AOAC standard methods [19]. Total nitrogen contents of *Amphora* sp. crude and undigested protein substrates were determined using the Kjeldahl method according to the AOAC method number 984.13 [19] with equipment of BÛCHI Digestion Unit K-424 (Switzerland). Crude proteins were estimated by multiplying the total nitrogen content by the factor of 6.25.

Lipids content was determined gravimetrically after the Soxhlet extraction of dried samples with hexane for 2 hours using Nahita Model 655 (Navarra, Spain).

As regards sugars, they were estimated by phenol-sulfuric acid method [20] using glucose as a standard.

2.4. Determination of Pigments Content: Chlorophylls and Carotenoids. Pigments were determined according to the method described by Lichtenthaler [21]. Two milliliters of culture was centrifuged at 5500 ×g for 5 min, and the supernatant was discarded and the pellet was mixed with 99.9% methanol and incubated in the dark for 24 hours at 45°C. After incubation, pigments content was determined measuring absorbances at 470, 652.4, and 665.2 nm, which were corrected for turbidity by subtracting absorbance at 750 nm.

2.5. Determination of Mineral Content. The analysis of sodium, potassium, calcium, magnesium, iron, copper, and zinc contents in *Amphora* sp. was carried out using the inductively coupled plasma optical emission spectrophotometer (ICP-OES) Model 4300 DV, PerkinElmer (Shelton, CT, USA), according to the method of AOAC 1999 [22]. Measurements were performed in triplicate and the results were the average of three values.

2.6. Determination of Fatty Acids Profile by GC-MS. Fatty acids methyl esters (FAME) were prepared by basic transesterification protocol. The extracted lipids were collected in 10 mL heptane and introduced into a 50 mL flask. A volume of 10 mL of KOH solution (11 g L^{-1}) in methanol was added with a few antibumping beads. The mixture was boiled under reflux condenser for 10 min. Then, 5 mL of boron trifluoride (BF_3), methanol complex (150 g L^{-1}) was introduced through the condenser by a graduated syringe, and the mixture was boiled for 2 min. The mixture was cooled under room temperature and 15 to 20 mL of saturated sodium sulfate solution was added and shaken well. Furthermore, this solution was added until the liquid level reached the neck of the flask. After the phase separation, the upper layer (n-heptane) was collected by a Pasteur pipette and evaporated under nitrogen flow. The FAME were redissolved in 500 μL hexane for GC-MS analysis.

The analysis of FAME was performed with a 6890 GC/5973 MSD GC/MS system from Agilent Technologies, equipped with an HP-INNO Wax Polyethylene Glycol capillary column (30 m length; 250 μm diameter; 0.25 μm film thickness). Helium was used as a gas carrier with a flow rate through the column of 1 mL/min. Column temperature was held initially for 1 min at 200°C and increased to 250°C at 10°C min^{-1} and was then held at 250°C for 15 min. The injector was kept at 260°C using a splitless mode, and then a sample of 1 μL was injected. The ion source temperature

was set at 230°C. The mass spectra were obtained in full scan mode MS, and scan range was (m/z): 50–600 atomic mass units (AMU) under electron impact (EI) ionization (70 eV). Data were exploited using the National Institute of Standards and Technology (NIST) Mass Spectral Search Program (version 2.0g).

2.7. Determination of the Total Phenolic Content. The total phenols content in microalga was determined by the Folin–Ciocalteu method [23]. Briefly, 0.2 mL of extract was mixed with 1 mL of Folin–Ciocalteu reagent (diluted 1 : 10, v/v) followed by the addition of 0.8 mL of sodium carbonate (7.5%, w/v). After incubation in the dark, the absorbance was measured at 760 nm. The total phenolic content of algal extract was expressed as mg of gallic acid equivalent per g of dry extract (mg GAE g^{-1} extract) using a calibration curve with gallic acid. All samples were analyzed in triplicate.

2.8. Determination of the Total Flavonoid Content. The total flavonoid content was determined according to the modified method of Zhishen et al. [24]. Briefly, 0.4 mL of the extract was mixed with 120 μL of 5% sodium nitrite and 120 μL of 10% aluminum chloride followed by the addition of 0.8 mL of 1 M sodium hydroxide. After the incubation of reaction mixture at room temperature for 6 min, absorbance was measured at 510 nm. The total flavonoids content in the extract was expressed in terms of catechin equivalent (mg g^{-1} of dry extract). All samples were analyzed in triplicate.

2.9. Extraction of Antioxidants. The extracts from the biomass samples were obtained by two solid-liquid extraction procedures inspired from the method of Goiris et al. [25]. In a first step, both apolar and polar compounds were extracted by 80% ethanol. For this, 2 g of freeze-dried biomass was ground using a pestle and mortar and extracted under agitation in the dark with 20 mL ethanol/water (4/1 v/v) mixture at 50°C for 1 hour. After centrifugation (4500 ×g, 10 min), the pellet was resuspended in 2 mL of the ethanol/water mixture and extracted for a second time with maceration. The two extracts were pooled and stored at −20°C prior to analysis. The second procedure aimed to separate polar from apolar compounds after sequential extraction in solvents with increasing polarity: hexane, ethyl acetate, and water. Freeze-dried biomass (2 g) was ground in a mortar and extracted with 20 mL of hexane for 1 hour at 50°C. After centrifugation, the pellet was resuspended in hexane and extracted for a second time, and both extracts were combined. The biomass pellet was subsequently extracted twice with 2 × 20 mL ethyl acetate using the same procedure and finally with 2 × 20 mL of water at 50°C for 1 hour. All extracts were concentrated under reduced pressure by a rotary evaporator to a dry condensed residue. The dried samples were weighed and then stored at −20°C prior to analysis.

2.10. Antioxidant Property. Four *in vitro* assays were used to evaluate the antioxidant property of the 80% ethanolic extract of *Amphora* sp.

2.10.1. 2,2-Diphenylpicrylhydrazyl (DPPH) Free Radical Scavenging Assay. Free radicals scavenging activity was assessed according to Blois [26] with some modifications: 1 mL of the extract at different concentrations (0.06–1 mg mL^{-1}) was mixed with 1 mL of 0.1 mM DPPH in ethanol and 450 μL of 50 mM Tris-HCl buffer (pH 7.4). The solution was incubated at 37°C for 30 min, and the reduction of DPPH free radicals was evaluated by reading the absorbance at 517 nm. The DPPH scavenging activity is given as percentage and calculated according to the following equation:

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

where A_{control} is the absorbance of the reaction medium and A_{test} is the absorbance of the reaction test containing the extract. The antioxidant activity of each extract was expressed as IC_{50}, defined as the concentration of extract required to cause a 50% decrease in initial DPPH concentration. Butylated hydroxytoluene (BHT) was used as positive control, and each sample was analyzed three times.

2.10.2. ABTS Radical Scavenging Activity. The Trolox equivalent antioxidant capacity (TEAC) assay, measuring the reduction of the 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ABTS radical cation by antioxidants, was derived from the method of Katalinic et al. [27] with minor modifications. Briefly, ABTS radical cation (ABTS·+) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate, allowing the mixture to stand in the dark at room temperature for 12–16 hours before use. ABTS·+ solution was diluted with saline phosphate buffer (PBS, pH 7.4) to absorbance of 0.70 (±0.02) at 734 nm. After the addition of 2 mL of diluted ABTS·+ solution to 100 μL of each extract, or Trolox standard, the reaction mixture was incubated for 30 min in a glass spectrophotometer cell at 30°C. The decrease in absorbance was recorded at 734 nm. Measurements were performed in triplicate. Radical scavenging activity was calculated using the formula below:

$$\%\ \text{Inhibition} = \left[\frac{\left(A_{\text{control}} - A_{\text{test}}\right)}{A_{\text{control}}}\right] \times 100, \quad (2)$$

where A_{control} is the absorbance of the reaction medium and A_{test} is the absorbance of the reaction medium with the extract. Each sample was analyzed three times.

2.10.3. Ferric Reducing Antioxidant Power (FRAP). The reducing power of the extract was determined according to the method described by Yildirim et al. [28]. Briefly, 0.5 mL of 80% ethanolic extract solution at various concentrations (0.1–0.5 mg mL^{-1}) was mixed with 1.25 mL of 0.2 M phosphate buffer (pH 6.6) and 1.25 mL of 1% (w/v) potassium ferricyanide $K_3[Fe(CN)_6]$. The mixture was incubated at 50°C for 20 min, and then 1.25 mL of 10% (w/v) trichloroacetic acid was added to the mixture which was then centrifuged at 3500 ×g for 10 min. The supernatant (1.25 mL) was mixed with 1.25 mL distilled water and 0.25 mL of 0.1% $FeCl_3$ (w/v). The absorbance was measured at 700 nm. Ascorbic acid was used as a reference. The increase of the absorbance in the

reaction indicates the increase of the iron reduction. The values presented are the mean of triplicate analysis.

2.10.4. β-Carotene-Linoleic Acid Assay. The ability of the extract to prevent the bleaching of β-carotene was assessed as described by Koleva et al. [29]. A stock solution of β-carotene/linoleic acid mixture was prepared by dissolving 0.5 mg of β-carotene in 1 mL of chloroform, 25 μL of linoleic acid, and 200 μL of Tween 80. The chloroform was completely evaporated under vacuum in a rotary evaporator at 40°C, and then 100 mL of distilled water was added and the resulting mixture was vigorously stirred. The emulsion obtained was freshly prepared before each experiment. Aliquots (2.5 mL) of the β-carotene/linoleic acid emulsion were transferred to test tubes containing 0.1 mL of extract at different concentrations (0.1–0.5 mg mL^{-1}). Following incubation for 2 h at 50°C, the absorbance of each test was measured at 470 nm. Vitamin C was used as a positive standard. The control tube contained no sample. Antioxidant activity in β-carotene bleaching model expressed as percentage was calculated with the following equation:

$$\beta\text{-carotene bleaching inhibition (\%)} = \left[1 - \frac{A_0 - A_t}{A'_0 - A'_t}\right] \times 100, \quad (3)$$

where A_0 and A'_0 are the absorbance of the sample and the control, respectively, measured at time zero and A_t and A'_t are the absorbance of the sample and the control, respectively, measured after 2 hours. Tests were carried out in triplicate.

2.11. Antibacterial Assays. The operative pathogens were Gram-positive (*Bacillus cereus* (ATCC11778), *Bacillus subtilis* (JN934392), *Micrococcus luteus* (ATCC4698), and *Staphylococcus aureus* (ATCC6538)) and Gram-negative (*Klebsiella pneumoniae* (ATCC13883), *Salmonella enterica* (ATCC43972)) bacteria. The antimicrobial activity of 80% ethanolic extract was evaluated using agar well diffusion method. The wells were then filled with 60 μL of the extract at 20 mg/mL in 5% DMSO and 5% DMSO was used as negative control. Besides, plates were incubated for 24 hours at 37 ± 1°C for bacterial strains. Then, the diameters of inhibition zones (IZD) were measured.

2.12. Statistical Analysis. The data for biological and biochemical parameters are expressed as mean ± SD. Tests were carried out in triplicate. The IC_{50} values were calculated by Probit Analysis with a reliability interval of 95%.

3. Results and Discussion

3.1. Physicochemical Characterization of Amphora sp. Some physicochemical characteristics of *Amphora* sp. are presented in Table 1. The obtained results have shown that the biomass of *Amphora* sp. contains moderate amounts of lipids, proteins, carbohydrates, ashes, and minerals and an important percentage of chlorophyll a and carotenoids. The dry matter content of 7% is close to that found for other strains: 8% for *Amphora* sp. CTM 20023 [30] and 5.9% for *Amphora coffeaformis* [31]. However, the lipids content of *Amphora* sp. of 11.14% DW was relatively lower than the values published for other strains of *Amphora* [32], and it was higher than that of *Amphora coffeaformis* (6.9% DW) [31]. A study on eight marine species of diatoms revealed various lipid contents ranging from 2.4 to 21.3% [33].

Table 1: Physicochemical characteristics of *Amphora* sp.

Component	*Amphora* sp.
Dry matter (% FW)	7 ± 0.45
Proteins (% DW)	27.62 ± 0.3
Lipids (% DW)	11.14 ± 0.19
Total sugars (% DW)	12.60 ± 0.76
Ashes (% DW)	37.78 ± 0.43
Chlorophyll a (% DW)	4.945 ± 0.2
Chlorophyll b (% DW)	0.666 ± 0.05
Carotenoids (% DW)	1.083 ± 0.05
Sodium (g Kg^{-1} DW)	1.125 ± 0.2
Potassium (g Kg^{-1} DW)	0.485 ± 0.05
Calcium (g Kg^{-1} DW)	0.584 ± 0.05
Magnesium (g Kg^{-1} DW)	0.747 ± 0.1
Iron (g Kg^{-1} DW)	0.016 ± 0.002
Copper (g Kg^{-1} DW)	0.008 ± 0.001
Zinc (g Kg^{-1} DW)	0.008 ± 0.001

Data are expressed as mean ± standard deviation of triplicates. FW: fresh weight; DW: dry weight.

The proteins level for *Amphora* sp. (27.62%) was lower than that found for other strains of *Amphora*; 54% for CTM 20023 [30] and 30–40% for *Amphora* sp. [32]. Indeed, the proteins level was in agreement with the published values range of 12–42% for some microalgae [34] and higher than the contents found for *Amphora coffeaformis* [31].

The total sugars content of *Amphora* sp. was 12.60% DW, which is consistent with that of some microalgae (5–23% DW) [34]. Nevertheless, *Amphora coffeaformis* and some diatoms contain a high amount of sugars in the range of 13.5–16.4% [31].

With respect to the ash content of *Amphora* sp. (37.78% DW), it is in line with that found for another *Amphora* strain (30% DW) cultivated at salinity ranging between 15 and 35 p.s.u. [32]. Ash content exceeds 50% (55.8 to 67.9%) of the dry weight for some diatoms [31]. *Amphora* sp. has moderate amounts of sodium, potassium, calcium, and magnesium.

Moreover, *Amphora* sp. was found to be rich in chlorophyll, mainly chlorophyll a, and carotenoids. In fact, its chlorophyll content reached almost 5% of the dry matter, which is in agreement with other research works [30]. Furthermore, the carotenoids content was prominent (1.083% DW). Bacillariophyceae exhibited high levels of carotenoids, namely, β-carotene and xanthophylls, thus indicating a powerful antioxidant activity [35].

3.2. Fatty Acids Composition of Amphora sp. In salinity of 100 p.s.u., on F/2 medium, the fatty acids profile of *Amphora* sp. was composed of saturated, monounsaturated,

Table 2: Fatty acids composition of *Amphora* sp. (% of total fatty acids).

Fatty acids	*Amphora* sp.
C14:0	3.623 ± 0.3
C15:0	3.418 ± 0.3
C16:0	27.427 ± 0.5
C17:0	1.664 ± 0.4
C18:0	1.974 ± 0.3
C20:0	0.734 ± 0.2
C24:0	2.468 ± 0.2
∑ SFA	**41.308 ± 0.8**
C14:1	3.386 ± 0.3
C16:1	45.089 ± 0.8
C17:1	0.521 ± 0.1
C18:1	3.658 ± 0.3
∑ MUFA	**52.654 ± 1.2**
C16:2	1.603 ± 0.2
C16:3	0.924 ± 0.3
C18:2	0.432 ± 0.1
C20:4	0.712 ± 0.2
C20:5	2.367 ± 0.3
∑ PUFA	**6.038 ± 0.5**

∑ SFA: total saturated fatty acids; ∑ MUFA: total monounsaturated fatty acids; ∑ PUFA: total polyunsaturated fatty acids.

and polyunsaturated fatty acids (SFA, MUFA, and PUFA), respectively (Table 2). The C16 fatty acids series (C16:0, C16:1, C16:2, and C16:3) represented more than 75%. Moreover, palmitic acid (C16:0) and palmitoleic acid (C16:1) accounted together for more than 72.51% of fatty acids. SFA were present at a high level, 41.31%, with 27.41% of palmitic acid. Hence, *Amphora* sp. could be a suitable producer of SFA, which are easily convertible to biodiesel [36]. *Amphora* sp. is rich in MUFA with the dominance of palmitoleic acid (45.09%) and contains an important percentage of PUFA (6.03%). High levels of palmitoleic acid and other bioactive fatty acids were also detected in the fusiform morphotype of the Bacillariophyceae *Phaeodactylum tricornutum* [37]. Eicosapentaenoic acid C20:5 (EPA) was produced in a noticeable level (2.367%). Indeed, it is known that EPA is an important PUFA for health protection from many pathologies, including cardiovascular diseases [38] and cancer [39].

3.3. Phytochemical Composition. The total phenols and flavonoids contents of *Amphora* sp. extracts are summarized in Table 3. The 80% ethanolic extract of this Bacillariophyceae showed the highest phenols and flavonoids contents, 38.27 ± 2.21 mg GAE g^{-1} extract and 17.69 ± 0.70 mg CE g^{-1} extract, respectively, compared to the extracts obtained by ethyl acetate, hexane, and water. The phenolic content of ethanol extracts of some Moroccan marine microalgae was found to range from 8.2 to 32 mg GAE g^{-1} extract [40]. The authors noted that the highest phenolic content was found in the extract of *Nannochloropsis gaditana* with 32 mg GAE g^{-1} extract. The extracts of *Dunaliella* sp., *Phaeodactylum tricornutum*, and *Navicula* sp. also contained high phenolic content of more than 15 mg GAE g^{-1} extract [40].

The flavonoid content in the 80% ethanolic extract of *Amphora* sp. was 3.22 mg CE g^{-1} DW, which is sixfold higher than that found for *Amphora* CTM 20023 [30].

The high levels of polyphenols and flavonoids in the *Amphora* sp. 80% ethanolic extract may be due to the culture conditions under the high salinity of 100 p.s.u. and the extraction conditions.

3.4. Antioxidant Property of Amphora sp. Due to its highest content of phenols and flavonoids, the 80% ethanolic extract was used to check antioxidant activity through four *in vitro* assays: the DPPH and ABTS radical scavenging capacities, the ferric reducing antioxidant power, and the β-carotene bleaching inhibition tests, using different concentrations of the extract. The inhibition percentages of the radicals DPPH and ABTS by the extract and standards (Trolox and BHT) were found to be concentration-dependent (0.065 to 1 mg mL^{-1} for DPPH test and 0.5 to 4 mg mL^{-1} for ABTS test) as shown in Figure 1. The deduced values of IC_{50} are presented in Table 4. A lower value of IC_{50} indicates a high antioxidant activity. *Amphora* sp. 80% ethanolic extract exhibited high antiradical power with an IC_{50} value of 0.23 ± 0.07 mg mL^{-1} for DPPH scavenging activity and an IC_{50} value of 2.61 ± 0.64 mg mL^{-1} for ABTS radical scavenging capacity. Some authors have found a strong correlation between the phenolic contents and the antioxidant activity [41].

The FRAP assay is based on the ability of phenols to reduce yellow ferric tripyridyl triazine complex (Fe(III)-TPTZ) to blue ferrous complex (Fe(II)-TPTZ) by the action of electron-donating antioxidants [42]. Figure 2(a) shows a significant reducing power compared to the vitamin C used as a reference. The reducing power was also dependent on the extract concentration.

The effect of antioxidants on DPPH free radical scavenging was considered to be emanating from their hydrogen-donating ability. In this investigation, the 80% ethanol extract was proven to exhibit a strong effect of DPPH free radical scavenging. In fact, it reached 74.34% of inhibition at 1 mg of extract, which is considered higher than the methanol extract of some Bacillariophyceae tested at 2 mg of the extract (22.7%, 31.6%, and 76.6% for *Amphora coffeaeformis*, *Navicula* sp., and *Achnanthes longipes*, resp.) [31]. The IC_{50} value for ABTS scavenging capacity of *Amphora* sp. extract was 2.6 mg mL^{-1}. Many research studies [43] have mentioned IC_{50} ABTS scavenging capacities for two brown algae of 5.29 mg mL^{-1} for *Sargassum binderi* and 15.2 mg mL^{-1} for *Turbinaria conoides*.

The β-carotene bleaching inhibition and the reducing power tests (absorbance at 700 nm) were measured at concentrations of extract between 0.1 and 0.5 mg mL^{-1}, showing its dependency on concentration (Figure 2(b)). The IC_{50} value of β-carotene bleaching test (Table 4) was strong (0.21 ± 0.05 mg mL^{-1}), with about twice the IC_{50} value of vitamin C used as the standard. The absorbance at 700 nm reached a value of 0.78 at a concentration of 0.5 mg mL^{-1} of the extract, which is considered high, indicating a good antioxidant activity. Therefore, *Amphora* sp. extract was confirmed to have a strong antioxidant capacity. The multiple regression analysis has proven that both carotenoid and phenolic

Table 3: Extraction yields and polyphenols and flavonoids contents in extracts from *Amphora* sp. with different solvents.

Solvent	Yield (% DW)	Polyphenols (mg GAE g^{-1} extract)	Flavonoids (mg CE g^{-1} extract)
80% ethanol	18.20 ± 0.21	38.27 ± 2.21	17.69 ± 0.70
Hexane	14.95 ± 0.25	11.47 ± 0.60	4.76 ± 0.15
Ethyl acetate	2.78 ± 0.12	14.04 ± 0.31	5.25 ± 0.30
Water	23.61 ± 0.30	13.34 ± 0.24	4.27 ± 0.20

Data are expressed as mean ± standard deviation of triplicates. GAE: gallic acid equivalent; CE: catechin equivalent.

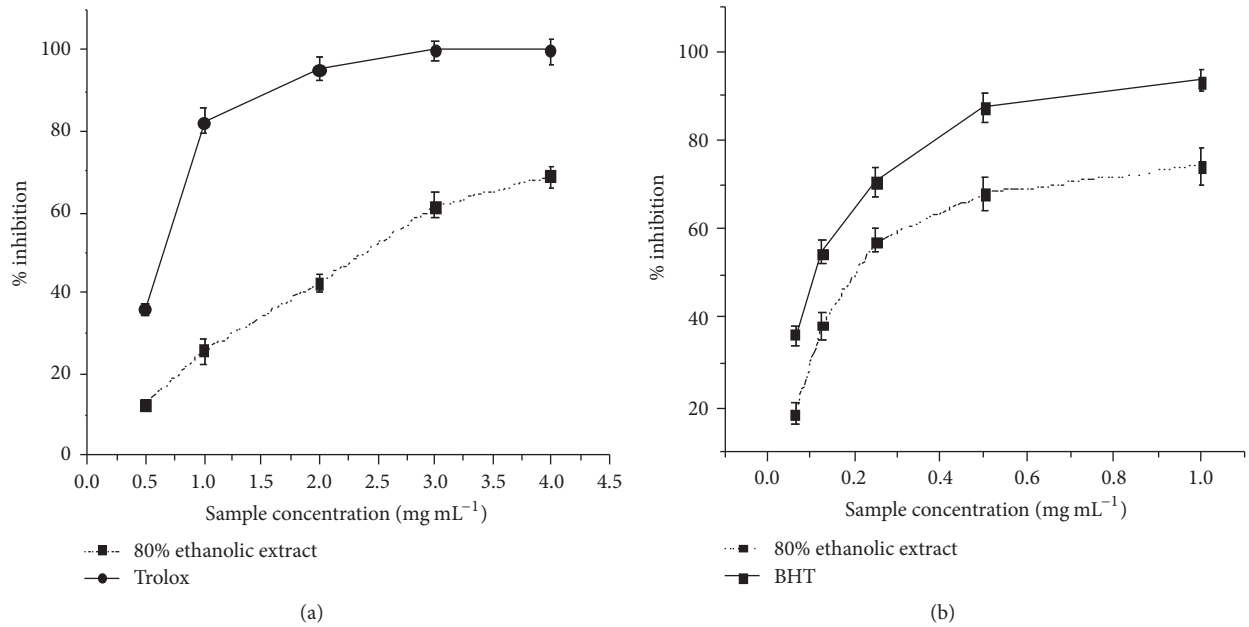

Figure 1: Anti-free radical effects of *Amphora* sp. 80% ethanolic extract for ABTS (a) and DPPH (b) compared to respective standards (Trolox and BHT).

Table 4: IC_{50} values for antioxidant capacities of *Amphora* sp. extract compared to standards.

Activity	Extract (mg mL^{-1})	Standard
DPPH radical scavenging activity	0.23 ± 0.07	0.11 ± 0.03
ABTS radical scavenging activity	2.61 ± 0.64	0.54 ± 0.08
β-Carotene bleaching assay	0.21 ± 0.05	0.09 ± 0.01

Data are expressed as mean ± standard deviation of triplicates.

contents significantly contribute to the explanation of the variation in FRAP and TEAC activity of the extracts. The regression coefficients indicate that phenols and carotenoids are of similar importance in explaining variation in the antioxidant activity [25].

3.5. Antibacterial Assay. In the present study, the antibacterial activity of *Amphora* sp. 80% ethanolic extract was screened against six bacteria and their potency was qualitatively and quantitatively analyzed by the diameters of the inhibition zones (IZD) as summarized in Table 5. The 80% ethanolic extract of *Amphora* sp., at a concentration of 20 mg mL^{-1}, was active against Gram-positive bacteria, *Micrococcus luteus* and *Staphylococcus aureus*, and inactive against both strains of *Bacillus* (*cereus* and *subtilis*). This extract was active against Gram-negative bacteria tested: *Klebsiella pneumonia* and *Salmonella enterica*. The extract exhibited strong activity against *Micrococcus luteus*, *Staphylococcus aureus*, and *Klebsiella pneumoniae*. The IZD for *Klebsiella pneumoniae* and *Staphylococcus aureus* of 16 mm and 20 mm, respectively, were stronger than those found for the ethanolic extract of a strain of *Amphora* sp. cultured in standard Walne's medium (10 mm and 10.5 mm), respectively [44]. This difference may be due to the culture conditions. According to some authors, the antimicrobial activity of algae depends on the species and the extraction efficiency of the active compounds as well as survival conditions [45].

The extract used in this study was found to be rich in a variety of compounds: lipids and fatty acids, carbohydrates, polysaccharides, polyphenols, flavonoids, pigments, and carotenoids, which can be indicative for having antibacterial activity as mentioned for several microalgae [46]. The antimicrobial activity of microalgae has been attributed to compounds belonging to several chemical classes including indoles, terpenes, acetogenins, phenols, fatty acids, and volatile halogenated hydrocarbons [47, 48]. The mechanisms of action of some antimicrobial agents were described; carotenoids digest the cell wall [49]; flavonoids

Table 5: Antibacterial activity of *Amphora* sp. 80% ethanolic extract.

Strains	Gram +/−	IZD (mm) 80% ethanol extract of *Amphora* sp.
Bacillus cereus (ATCC11778)	+	0 ± 0
Bacillus subtilis (JN934392)	+	0 ± 0
Micrococcus luteus (ATCC4698)	+	16 ± 0.5
Staphylococcus aureus (ATCC6538)	+	20 ± 2
Klebsiella pneumoniae (ATCC13883)	−	16 ± 0.5
Salmonella enterica (ATCC43972)	−	12 ± 1

The data are expressed as mean ± SD ($n = 3$). IZD: inhibition zone diameter.

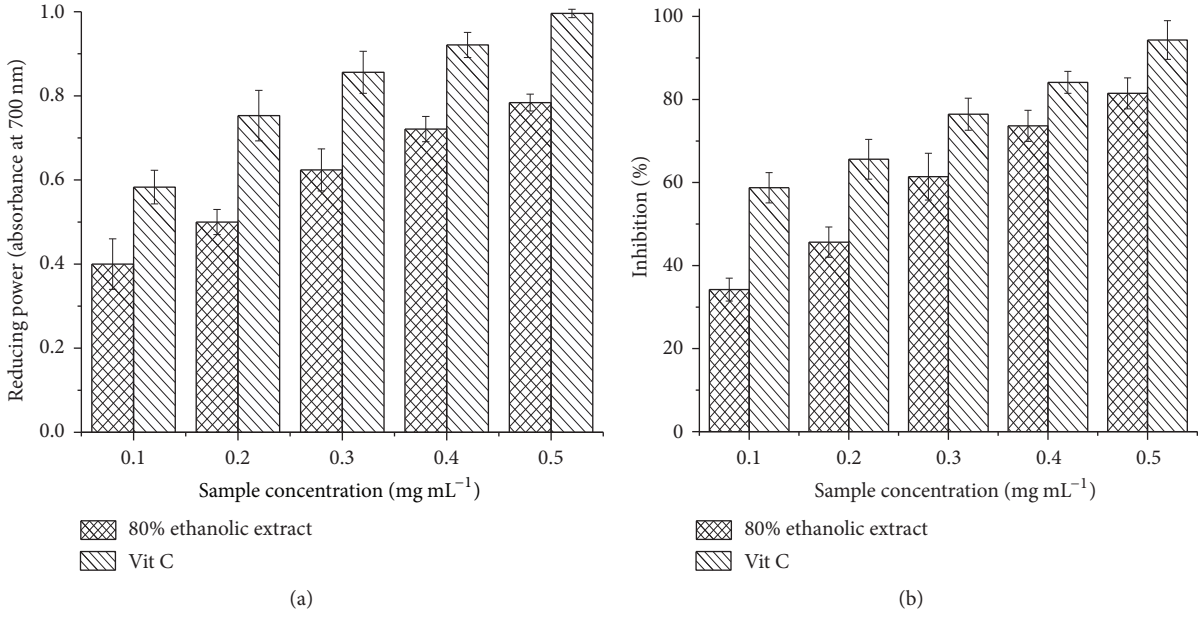

Figure 2: Ferric reducing antioxidant power (FRAP) (a) and beta-carotene bleaching test (b) for 80% ethanolic extract of *Amphora* sp. compared to vitamin C (Vit C) as a standard.

increase permeability of the inner bacteria [50]; polyphenols participate in enzyme inhibition, substrate deprivation, complexing with cell wall, and membrane disruption [8]; polysaccharides inhibit hyaluronidase [8], while fatty acids and lipids cause the disruption of the cellular membrane [51]. The antimicrobial activity of supercritical extracts obtained from the microalga *Chaetoceros muelleri* was related to its lipids composition [52]. Fatty acids were reported to be bioactive compounds having antibacterial activity in some diatoms *Phaeodactylum tricornutum* [51, 53] and *Skeletonema costatum* [54] and other microalgae *Haematococcus pluvialis* [55] and *Dunaliella salina* [56].

Microalgae extracts should be tested as additives for food and feed formulations, in an attempt to replace the currently used antimicrobial compounds of synthetic origins, including subtherapeutical doses of antibiotics employed as a prophylactic measure in animal breeding [9].

4. Conclusion

Amphora sp., a brown microalga cultivated in high salinity medium of 100 p.s.u., was confirmed to contain a moderate percentage of proteins, lipids, sugars, and minerals and important levels of polyphenols, flavonoids, chlorophyll, carotenoids, and bioactive fatty acids as EPA. Besides, this microalga has important antioxidant and antibacterial activities. Prominent contents of bioactive compounds in this microalga are in favor of its possible valorization in food additives and pharmaceutical or cosmetic products. Moreover, its richness in saturated fatty acids allows its eventual use for biodiesel production.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Saoussan Boukhris and Khaled Athmouni contributed equally to this work.

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Research Article

Nutritional Composition and Phytochemical, Antioxidative, and Antifungal Activities of *Pergularia tomentosa* L.

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Crude extracts from a medicinal Tunisian plant, *Pergularia tomentosa* L., were the investigated natural material. Butanolic extract of roots analyzed with IR spectra revealed the presence of hydroxyl, alcoholic, and carboxylic groups and sugars units. Analysis of some secondary metabolites, total phenolic, flavonoids, flavonols, and procyanidins, was performed using different solvents following the increased gradient of polarity. Fruits and leaves contained the highest amounts of all these compounds. Antioxidant properties were evaluated by the determination of free radical scavenging activity and the reducing power of methanolic extracts. Fruits and leaf extracts were the most powerful antioxidants for the two-assay in vitro system. Stems and fruits extracts exhibit an antifungal activity against *Fusarium oxysporum f. sp. lycopersici* which could become an alternative to synthetic fungicide to control *Solanum* species fungal diseases.

1. Introduction

Bioactive molecules obtained from medicinal wild plants and their curative potentials are well documented [1]. Recent studies showed that a large number of herbs products including polyphenolic substances can be considered as the most abundant plant secondary metabolites with highly diversified structures. This source of phytochemicals was related to a variety of biological activities including antioxidant potential [2]. Oxidative stress and cell damage occur in the human body when the Reactive Oxygen Species were in imbalance with the antioxidants. Those compounds were associated with cardiovascular disease, neurological disorders cancer, diabetes, and other diseases [3]. The use of synthetic antioxidants presents undesirable effects on health on long-term life [4]. Due to the fact that they have been considered as a major group of chemicals contributing to the antioxidant potential of plant extracts, phenolic compounds present in medicinal plants could be consumed in the diet and they have limited or no side effects [5, 6]. Their ingestion reduces the risk of certain cancers and reverses the human oxidative damage by acting as exogenous antioxidant system [7, 8].

Plants rich in phenols, flavonoids, tannins, vitamins, and many more phytochemicals were searched for the development of ethnomedicines and were responsible for several pharmacological activities [9]. Phytochemicals extracted from plants have been recognized as some of the most promising compounds for the development of novel ecofriendly compounds due to their high degradability and low incidences of negative impacts in comparison with synthetic fungicides chemicals giving rise to residual toxicity, hormonal imbalance, and carcinogenicity [4, 10]. As phytopathogenic fungi, *Fusarium* spp. is the largest expansion in "Tubercullariaceae" family. It causes wilts and cankers, stalk rots, leaf wilting, maize horticultural, field, and ornamental plants and affects tomato and many cucurbits [11, 12].

Pergularia tomentosa L., commonly known as Bou Hliba in Tunisia, is a fetid smelling lactiferous twinner [13]. At the slightest touch, leaves and fruits secret a sticky white fluid [14]. The plant was widely distributed across the Horn of Africa [15] to Sinai, Jordan, and Saudi Arabica [16]. Crushed *P. tomentosa* was administered in the case of diarrhea and the sap of leaves was used as ocular instillation and regarded as a sovereign remedy for the ills of the head

[17]. The roots were used for the treatment of bronchitis, constipation, and skin diseases and leaves for bronchitis and tuberculosis [18]. In Egypt, the plant was used as a poultice, depilatory, laxative, antihelmintic, and abortifacient [19]. The latex of stems and leaves irritates the skin and eyes and can cause inflammation [20]. The plant was reported to contain cardiotonic glycosides such as desglucouzarin, coroglaucigenin, and uzarigenin in the leaves [21]. Roots contains uzarigenin, pergularoside, ghalakinoside, and calcatin and their derivates, 6′-hydroxycalactin, 6′-hydroxy-16α-acetoxycalactin, 16α-hydroxycalactin [22], 3′-O-β-D-glucopyranosylcalactin, 12-dehydroxyghalakinoside, and 6′-dehydroghalakinoside [23]. The plant was reported to have molluscicidal activity [24] and its cardenolides induce cell death by apoptosis of Kaposi in the case of cancer [23].

The screening of the nutritional composition as the relevance of the presence of phytochemical and antioxidative potentials in a wild plant can lead to valorizing its implication in human diet, animal fodder, and sol fertilization. The fact that it can have antifungal activity affects beneficially the health of the product consumer and the economic capacity of the farmer. In this context, the present studies aimed to provide the nutritional composition and phytochemical analysis of principal polyphenols extracted from *Pergularia tomentosa* L., a therapeutically important medicinal plant. The antioxidant properties of four different organs (roots, stems, leaves, and fruits) were explored using a set of in vitro antioxidant assays including scavenging of DPPH as well as reducing power assay. Antifungal activity against *Fusarium oxysporum f. sp. lycopersici* was tested for the extracts obtained by the fractional method following an increased gradient of polarity.

2. Materials and Methods

2.1. Plant Materials. *Pergularia tomentosa* was collected from the surroundings of the region of Bïr Ben Ayed (south Sfax, Tunisia, arid climate). It was growing in the borders of olives farms. The collection was in early morning in the beginning of March. Plants were in flowering stadium and have simultaneously green and mature fruits. Handling of the sample amount from the farm was manually and was done by wearing plastic gloves to protect the skin from the latex secreted by fruits, stems, and leaves at the slight touch. The specimen was identified at the Herbarium of the Sfax Faculty of Sciences by Professor Ben Abdallah Ferjani. Roots, stems, leaves, and fruits were finely ground using an electric blender and stored in plastic containers at room temperature and in darkness until required for use.

2.2. Reagents. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), α-tocopherol, Folin-Ciocalteu reagent, potassium ferricyanide, and sodium carbonate were purchased from Sigma-Aldrich, USA. All solutions were freshly prepared in distilled water. The solvents used for extraction and partition were from Sigma-Aldrich.

2.3. Morphological and Phenological Characterizations. Morphological characterization was determined by simple observation. Physical characteristics of the whole plant, even for each organ, were determined on 10 samples taken at random. The dimensions of the different organs were determined using a caliper. Weights of fruits and seeds were determined using an analytical balance of an accuracy of ±0.001.

2.4. Preparation of Different Extracts

2.4.1. Infusion. To estimate protein content, pH, titratable acidity, total sugars, reducing sugars, phenolic compounds, and antifungal activity, aqueous extracts were prepared. 5 g of the dry matter was incubated in bowling water for 15 to 20 min. The filtrate was retained and the infusion was repeated three times. The totality of filtrates was lyophilized.

2.4.2. Extraction following Increased Polarity. To extract phenolic compounds, each organ was macerated with ethanol. 50 g of was macerated with 200 mL of 80% ethanol during 48 hours at 40°C and with continuous agitation. The residue obtained after the filtration of the mentioned mixture was extracted twice with the same solvent and at the same conditions. This mixture was filtrated and the obtained filtrate was evaporated using a rotary evaporator at 40°C until discarding the totality of ethanol. The obtained residue after evaporation under reduced pressure was partitioned following an increased gradient of polarity, with successively hexane, chloroform, ethyl acetate, and n-butanol (Figure 1). Only the n-butanol extract from stems showed two phases: organic and aqueous.

2.4.3. Extraction with Methanol. To determine the antioxidant activity, the extraction was performed with methanol. 50 g from each ground plant organ was soaked in pure methanol. Extraction was repeated three times, considering every time that the plant material should be submerged wholly by the sufficient quantity of fresh methanol. Each extraction lasts 3 days with continuous agitation in shaker at 37°C. All the combined extracts were concentrated on a rotary evaporator under vacuum at 40°C. To evaluate the antifungal activity of *P. tomentosa,* extracts I, II, III, and IV of stems, leaves, roots, and fruits (Figure 1) were used in addition to the aqueous extract of each organ.

To compare the proportions sample/extract, the extraction yield was calculated according to the following formula:

$$\text{Yield (\%)} = \frac{\text{residu weight obtained after solvent evaporation}}{\text{initial sample weight}} \times 100. \quad (1)$$

2.5. Screening of Nutritional Composition. The moisture content was determined by drying in an oven at 103 ± 2°C (NF

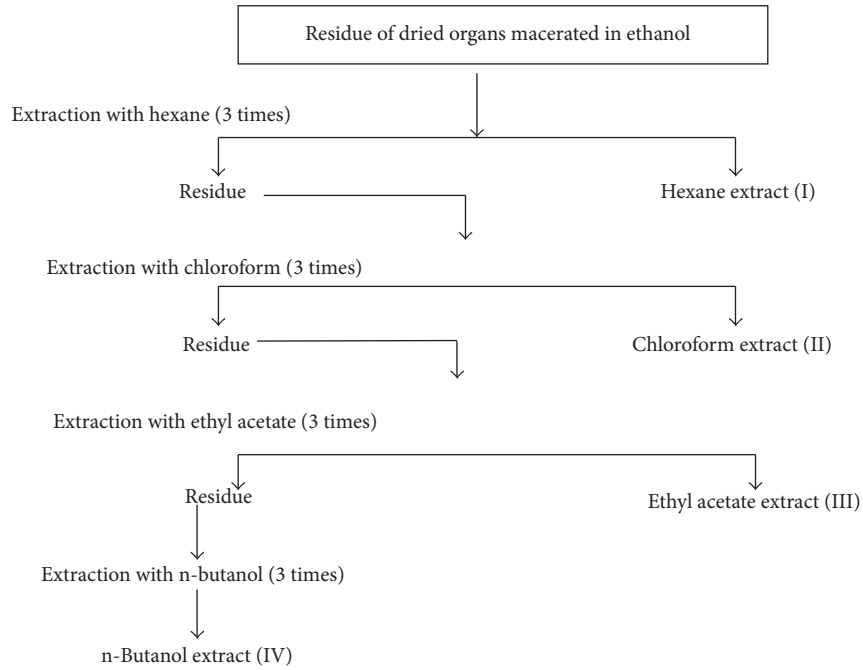

Figure 1: Diagram of fractional extraction of phenolic compounds from roots, stems, leaves, and fruits of *Pergularia tomentosa* L.

V05-108, 1970). The protein content was estimated by the Kjeldhal method [25] based on the following equation:

$$N\,(\%) = \frac{\left(N - N'\right) \times 0.05 \times 1.4 \times \left(V/V'\right)}{P}, \quad (2)$$

where V is volume of the mineralized solution (mL), V' is added volume of sodium solution (mL), N is read amount of sulfuric acid after titration (mL) with the sulfuric acid (0.05 N), N' is volume of sulfuric acid added after control titration (mL), and P is sample weight (g).

The amount of proteins was calculated by the multiplication of the rate of the total azote N (%) by the coefficient 6.25.

Total fat was evaluated by the Soxhlet method (NF EN ISO 734-1, 2000) using hexane as a solvent for the extraction. The ash content was determined by the incineration of samples in a muffle furnace at 550 ± 5°C until a whitish color (NF V 05-113, 1972). Total carbohydrates were calculated as the residual difference after subtracting proteins, ash, moisture, and lipid content [26]. Total energy (nutritive value) of each sample was estimated in kcal by multiplying the values obtained for protein, fat, and available carbohydrate by 4.00, 9.00, and 4.00, respectively, and by adding up the values [27]. The pH was measured according to the potentiometric method (NT 52.21, 1982). The titratable acidity was determined in the presence of sodium hydroxide (0.1 N) for the titration and phenolphthalein as a color indicator (NF V 05-101, 1974). The titratable acidity was expressed in acetic citric g/100 g sample and based on the following equation:

$$A\,(\%) = \frac{250 \times V_1 \times 0.07}{V_0 \times W \times 10} \times 100, \quad (3)$$

where W is sample weight (g), V_0 is volume of the test sample (mL), V_1 is volume of the hydroxide sodium solution at 0.1 N (mL), and *0.07* is conversion coefficient of the titratable acidity as equivalents of citric acid.

Total sugars were assayed by the phenol-sulfuric method based on the absorbance at 490 nm of phenol-carbohydrate complex [28]. The calculation of their amount was based on the equation of the calibration curve: $y = 0.434x$ (determination coefficient $R^2 = 0.972$). Reducing sugars were estimated with 3,5-dinitrosalicylic acid (DNS) [29]. The amount of reducing sugars was expressed in g/L, with a calibration curve equation $y = 0.921x$ (determination coefficient $R^2 = 0.986$).

2.6. Infrared Spectroscopy. Aqueous phase of the n-butanol extract of stems was pressed into pellets for the estimation of the infrared spectra with the scanned wave ranging from 4000 to 500 cm^{-1}. Spectra were recorded on a Perkin Elmer Universal ATR Sampling Accessory.

2.7. Polyphenol Content. Folin-Ciocalteu method based on the reduction of a phosphotungsten-phosphomolybdate complex was used to determine phenolic compounds [30]. 50 μL of Folin-Ciocalteu reagent (2 N) was added to 800 μL of extract. After 3 min of incubation at room temperature, 150 μL of sodium carbonate solution was added. The absorbance was measured at 765 nm after 2 h. Total phenolic content was expressed as gallic acid equivalents (GAE) in mg/g dry weight and using the following equation based on the calibration curve: $y = 0.0864x$, where y is the absorbance and x is the concentration (determination coefficient: $R^2 = 0.985$).

Total flavonoids content of the extracts was determined spectrophotometrically [31], using a method based on the

formation of the complex flavonoid-aluminum having a maximum absorption at 430 nm. After addition of 2 mL of 2% aluminum chloride to each extract (1 mL), the mixture was incubated for 15 min. Flavonoid content was expressed as quercetin equivalents (QE) in mg/g dry weight and following the equation based on the calibration curve: $y = 0.0301x$ (determination coefficient: $R^2 = 0.983$).

Flavonols were estimated by the aluminum chloride method [32]. Aliquots were prepared by mixing of 1 mL of extracts and 1 mL of aqueous aluminum chloride (20% in ethanol). The absorbance was determined at 425 nm against a blank. The results were expressed as quercetin equivalents (QE) in mg/g dry weight and using established equation from the calibration curve: $y = 0.035x + 0.001$ (determination coefficient: $R^2 = 0.992$).

The amount of condensed tannins (procyanidins) was measured using the modified vanillin assay for an absorbance measured at 500 nm [33]. The results were expressed as catechin equivalents (CTE) in mg/g dry weight and using the following equation based on the calibration curve: $y = 0.238x$ (determination coefficient: $R^2 = 0.998$).

2.8. Antioxidant Activity

2.8.1. Evaluation of DPPH Scavenging Activities. The radical scavenging assay of methanolic extracts was measured as equivalent of hydrogen-donating, according to DPPH method [34] with some modifications. Briefly, 1 mL of DPPH solution (10^{-4} M) was added to 1 mL of sample solution at various concentrations (0.0625–2 mg/mL) and the pH was adjusted to 7.4. The change in color from deep violet to light yellow after 20 min of incubation in dark was measured at 517 nm against a control. The inhibition percent was calculated according the equation below:

$$\text{Radical scavenging effect (\%)} = \left(1 - \frac{\text{optical absorbance of the sample}}{\text{optical absorbance of the control}}\right) \times 100. \quad (4)$$

Extract concentration providing 50% inhibition (IC_{50}) was calculated from the plot of scavenging effect (%) against the extract concentration. In the linear regression of plots, the abscissa represented the concentration of the tested plant extracts and the ordinate represented the average percent of scavenging capacity from three replicates. BHT and α-tocopherol were used as positive control.

2.8.2. Reducing Power Assay. The reducing power of the methanolic extract of *P. tomentosa* was determined according to the method previously described by [35]. This method was based on the colorimetric change of color when Fe^{3+} transforms to Fe^{2+} at 700 nm. 1 mL of extract solution at different amounts was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The obtained reaction mixture was then incubated in a water bath at 50°C for 20 min. 2.5 mL of 10% of trichloroacetic acid was added to the mixture. The upper layer solution (2.5 mL) obtained after centrifugation at 3000 was mixed to 2.5 mL of deionized water and 0.5 mL of fresh ferric chloride (0.1%). Increased absorbance of the reaction mixture indicates increased reducing power. The sample concentration providing 0.5 of absorbance (IC_{50}) was calculated by plotting absorbance against the corresponding sample concentration. BHT was used as a standard.

2.9. Antifungal Activity. *Fusarium oxysporum f. sp. lycopersici* was grown on plates of Potato Dextrose Agar (PDA) at 30°C for approximately 1 week to be well sporulated before testing. The inoculum was prepared by scraping the superficial mycelium and directly suspending the fungal material in water. The resulting suspension was then filtered through sterile gauze to obtain a homogeneous suspension of small hyphae. The CFU (Colony Forming Unit) of the inoculums suspension of *Fusarium* was approximately 0.4 × 104 to 5 × 104 CFU/mL. Tests were carried out by the microdilution method [36]. The microdilution test was performed by using sterile and multiwell microplates (96 U-shaped wells). The two times extracts concentrations were dispensed into the wells of rows 1 to 10 of the microdilution plates in 100 μL volumes diluted with RPMI 1640 medium. After 24, 48, and 72 hours of incubation, the MIC_0, MIC_1, MIC_2, and MIC_3 were noticed for each concentration of the designed extract. The MIC (Minimal Inhibitory Concentration) was defined as the lowest extract concentration that substantially inhibits visually growth as detected when testing the antifungal agent. In addition to the extracts obtained by different solvents, we prepared aqueous extracts for each organ of the plant.

2.10. Statistical Analyses. Independent samples of each item were analyzed in triplicate and data were presented as mean ± SD (standard deviations) and the confidence limits were set at $p < 0.05$ for all values.

3. Results and Discussion

3.1. Morphological Characterizations. Opposite leaves having dimensions of 14.25 to 46 mm of length and 20 to 52.7 mm of width present petioles of 13 to 25 mm length. Branching stems are cylindrical, climbing, and pubescent with small gray hairs along their length. Roots are pivotal and fairly rigid. They are deep and have bristly and short root-hair. The fruits are elongated follicles of 24.5 × 60 mm. They are in two halves follicles assembled together, rarely found in the form of three or four, and each of them contains 31 to 50 seeds. Seeds are ovoid, flattened, and compressed against each other. They are 4 × 9.2 mm and weigh 0.8 to 1.1 mg. At maturity, they are surmounted at their point by a tuft of white hairs of 3 to 4 cm being detached by the wind. The fragrant flowers grouped into false-umbels. The calyx is glabrous and purple, while the corolla is whitish-yellow with five linear divisions which are acute, reflected, and rolled at their margins.

3.2. Proximate Analysis and Nutrients Composition. The study of the nutritional content (Table 1), for instance, the moisture content presented in the different parts of the plant, could reflect its ability to resist the environmental conditions

Table 1: Nutritional values of different organs of *Pergularia tomentosa*. DW: dry weight of plant. Cal: calorie. The values are the mean of three determinations ± standard error.

Parameters	Roots	Stems	Leaves	Fruits
Moisture (%)	11.12 ± 0.56	13.02 ± 0.47	15.31 ± 0.79	23.1 ± 0.85
Ash (%)	6.72 ± 0.75	8.55 ± 0.42	19.44 ± 0.51	15.73 ± 0.68
Protein (%)	3.67 ± 0.13	5.14 ± 0.2	5.72 ± 0.26	6.89 ± 0.33
Lipids (%)	2.51 ± 0.25	3.53 ± 0.18	4.63 ± 0.19	5.46 ± 0.26
Carbohydrates (%)	76.58 ± 0.52	70.62 ± 0.38	55.82 ± 0.71	49.93 ± 0.49
Total energy (kcal/100 g)	343.59 ± 0.3	334.81 ± 0.25	287.83 ± 0.39	276.42 ± 0.36
pH	7.23 ± 0.13	6.41 ± 0.09	7.86 ± 0.07	6.47 ± 0.23
Titratable acidity (% citric acid)	0.031 ± 0.004	0.127 ± 0.02	0.029 ± 0.006	0.08 ± 0.007
Total sugars (g DW/L)	20.35 ± 0.15	22.12 ± 0.09	15.37 ± 0.11	19.98 ± 0.12
Reducing sugars (g DW/L)	1.56 ± 0.13	0.74 ± 0.12	0.27 ± 0.07	1.55 ± 0.09

Table 2: Extract yield (%) from *Pergularia tomentosa* organs with different solvents. The values are the mean of three determinations ± standard error.

	Hexane	Chloroform	Ethyl acetate	n-Butanol	Water	Methanol
Roots	1.96 ± 0.03	3.4 ± 0.07	0.48 ± 0.2	3.64 ± 0.05	14.56 ± 0.51	17.23 ± 0.33
Stems	1.62 ± 0.01	0.72 ± 0.1	0.68 ± 0.06	6.51 ± 0.25	18.2 ± 0.29	25.11 ± 0.7
Leaves	4.34 ± 0.12	3.71 ± 0.02	0.98 ± 0.09	9.16 ± 0.34	22.71 ± 0.62	28.6 ± 0.58
Fruits	4.32 ± 0.08	1.02 ± 0.18	1.7 ± 0.13	8.66 ± 0.98	13.2 ± 0.34	27.55 ± 0.84

and to adapt to drought as, by the continuous personal observations of *Pergularia tomentosa* in its ecological habitat, it was found that it remains practically all the year green and maintains the health aspect. The percentages in ash content in leaves and fruits could be involved in important nutritional mineral elements [37]. The content of carbohydrate in roots was higher in comparison with the peel of *Picralima nitida* (37.7 ± 0.18%) [38] and so lower than the seeds of *Saba florida* (79.56 ± 0.033%) [39]. Calculated total energy in that *P. tomentosa* may encourage its use as a source of feed and fodder for animals and/or of possible human use in addition to its medicinal properties. Titratable acidity, especially low in leaves and roots, can be responsible for the proliferation inhibition of the microbial flora in the plant organism. The increased pH and the decrease in titratable acidity that occurs with a degree of maturity can be affected by the loss of citric acid. The wealth of total and reducing sugars in stems and roots allows the maintaining of the turgor and the cytosolic volume and the preservation of the membrane integrity of dried organs [40]. The different variations registered may depend on ecological factors, geographic distribution, plant age, cultivation climatic conditions, and vegetative cycle as well as the adopted mechanism of adaptation to the new condition of *P. tomentosa*.

3.3. Extraction Yield. The obtained yield of each sample relative to the dry weight as well the methods of extraction was shown in Table 2. The results showed that there is a significant variation in the extraction yield between solvents for each plant organ. Concerning the increasing gradient of polarity, the n-butanol extract seems to have the more important extraction yield followed by the hexane extract of leaves and fruits and the chloroform extracts of roots and stems. However, extraction yields of aqueous and methanolic samples were higher in comparison with those of fractional extraction. It seems that the extraction yield depends on the solubility degree of the compounds in the used solvent during the extraction manipulation. The extraction at room temperature and with continuous agitation may lead to extract the maximum of bioactives compounds and prevent their degradation. A certain temperature degree can inactivate bioactives compounds and decrease their extraction yield in the used solvent.

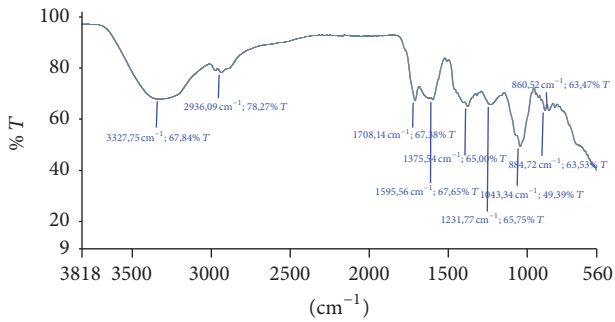

Figure 2: Infrared (IR) spectra of the aqueous phase of the n-butanolic extract of stems of *Pergularia tomentosa*.

3.4. Infrared Spectra. Only the n-butanol extract (IV) of stems represents two phases: aqueous and organic. The first phase was analyzed by IR.

In IR spectra (Figure 2), the bands at 3327.45 cm^{-1} and 2936.09 cm^{-1} correspond to the hydroxyl group of alcohols.

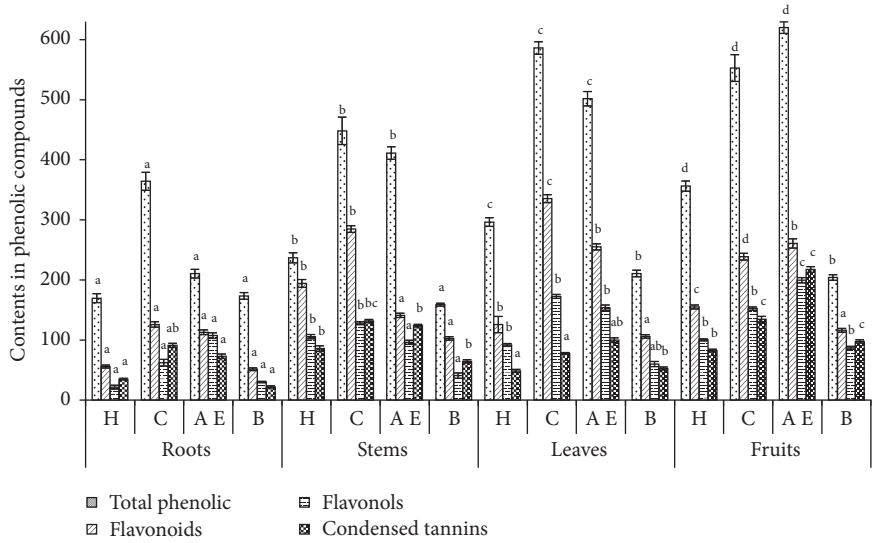

Figure 3: Amounts of total phenolic (mg Eq GAE/g DW), flavonoids (mg Eq QE/g DW), flavonols (mg Eq QE/g DW), and condensed tannins (mg Eq CTE/100 g DW) of different extracts of *Pergularia tomentosa*. H: hexane; C: chloroform; A E: ethyl acetate; B: n-butanol; mg Eq GAE/g DW: mg of gallic acid equivalents per g of dry weight; mg Eq QE/g DW: mg of quercetin equivalent (QE) per g of dry weight; mg Eq CTE/100 g DW: mg catechin equivalents (CTE) per g dry weight. The values are the mean of three determinations ± standard error. The different lowercase letters represent significant differences between samples versus different solvents by Student's t-test ($p < 0.05$). Values for the same compound and the same solvent, not sharing a common letter (a, b, c, or d), differ significantly at $p < 0.05$.

The peak around 1708.14 cm^{-1} was due to stretching vibrations of C=O of esters and ketones which were abundant practically in the same region (1680–1740 cm^{-1}) [41]. While the band at 1598.56 cm^{-1}, of low intensity, indicates the vibrations of the C=C aromatic compounds, the band at 1375.54 cm^{-1} confirmed the presence of the aliphatic chain CH_3. The more intense absorptions at 1043.34 and 1231.77 cm^{-1} could be attributed to stretching vibrations of C-OH side groups and the C-O-C glycosidic band vibration. Peaks located at 884.72 and 860.52 cm^{-1} indicate, respectively, β-configuration and α-configuration of the sugar units [42].

3.5. Analysis of Phenolic Compounds. The phenolic compounds were commonly involved in the prevention of many cancers and in the defense against microbial invasion [4]. The total phenolic, flavonoid, flavonol, procyanidins, and gallotannin content (Figure 3) show differences in amount depending on solvents polarities. The highest amount was found in ethyl acetate extracts of fruits and in chloroform extracts of leaves followed by chloroform-fruits and ethyl acetate-leaves.

For the four organs, the important values of different phenolic compounds were unregistered for both chloroform and ethyl acetate. Moreover, high solubility of phenols in polar solvents provides a high concentration of these compounds in the extracts [43]. These amounts were higher than the concentration registered in the leaves of *Convolvulus arvensis* (244.6 ± 2.9 mg GAE/g DW) [44]. The synthesis of polyphenols may likely be influenced by environmental conditions (temperature, sunlight exposure, dryness, and salinity), genetic difference, also to the time of collection, and the suitable method of storage.

Flavonoids have antiviral, antitumoral, and anti-inflammatory properties [4]. Extracts of chloroform-leaves and chloroform-stems contain the high amounts of flavonoids (Figure 3). The important content of flavonol was found in acetate ethyl-fruits extract and chloroform-leaves extract whereas hexane and n-butanol extracts have the lowest amounts. These results were in accordance with the literature and they depend on the polarity of solvents used in the extraction [45]. Procyanidins present a defense against attacks from predators such as insects and herbivorous [46]. Fruits and stems showed the highest levels of condensed tannins.

3.6. Antioxidant Activity. Antioxidant activity can depend on many factors such as the lipid composition, the antioxidant concentration, and the kind of plant. The antioxidant capacity of *Pergularia tomentosa* samples was reported to be highly dependent on the composition of these extracts and on the manipulation conditions during in vitro tests. Following DPPH method, the activity was evaluated by determining the IC_{50} value, corresponding to the concentration of the extract that was able to inhibit 50% of the free radicals. Under the assay conditions (Figure 4), among the four extracts, the most potent antioxidant activity was detected in leaves extract. The powerful antioxidant activity of leaves and fruits extract can be attributed mainly to the phenolic content, due to their hydroxyl groups, and/or to flavonoids which react with the DPPH radical by hydrogen atom donation to free radicals [47], while a highly positive correlation between total phenolic content and antioxidant activity was established in case of many plant species [48, 49].

The assessment of antioxidant activity by reducing iron represents the ability of a substance to transfer an electron or

Table 3: Minimal Inhibitory Concentration (MIC) of *Pergularia tomentosa* extracts against *Fusarium oxysporum f. sp. lycopersici*.

Extract	Concentration (mg/mL)							
	2	1	0.5	0.25	0.125	0.063	0.032	0.016
n-Butanol, fruits				MIC_0		MIC_1	MIC_3	
Ethyl acetate, fruits	MIC_2	MIC_3						
Water, stems	MIC_0	MIC_2	MIC_3					
Water, leaves	MIC_3							

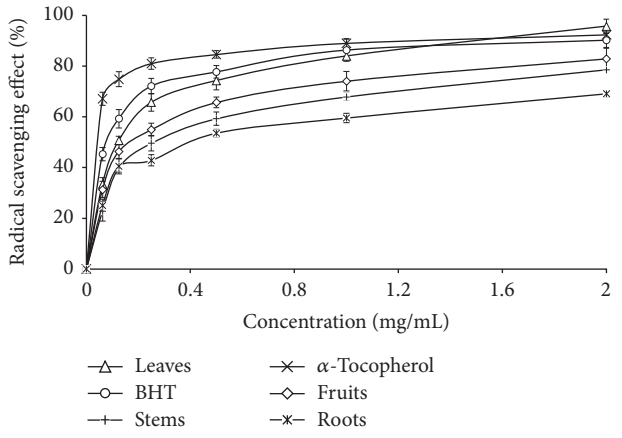

Figure 4: Radical scavenging effect (%) on DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals of *Pergularia tomentosa*'s organs. The values are the mean of three determinations ± standard error.

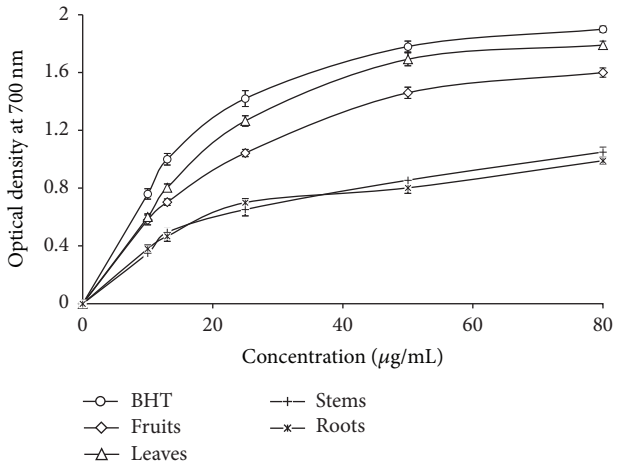

Figure 5: Reducing power of methanolic extracts of *Pergularia tomentosa*, as measured by changes in optical density at 700 nm. The values are the mean of three determinations ± standard error.

a hydrogen atom from another substance and an antioxidant ability to reduce the oxidized intermediates during the peroxidation processes [50]. Figure 5 shows the plot of reducing power of *P. tomentosa* methanolic extracts with the concentration range of 10–80 μg/mL and comparing with BHT as a reference antioxidant. Ferric reducing antioxidant power was proportional to the increase of the concentrations of extracts. Leaves and fruits seem very potent. Roots representing the highest IC_{50} imply the lowest reducing power. The reducing activity of fruits and leaves was attributed to the presence of phenolic compounds that may act by donating the electrons and reacting with free radicals to convert them to more stable products and terminate radical chain reaction [51]. A positive correlation was established between antioxidant activities and reducing power with a determination coefficient $R^2 = 0.8812$. However, the reducing power of an extract can be used as a significant indicator of its antioxidant activity [52]. Another positive correlation was established. The extracts with the highest levels of phenolic compounds also showed the highest antioxidant activity which is consistent with the literature [53].

3.7. Antifungal Activity. *Fusarium oxysporum f. sp. lycopersici* was responsible for the most important and widespread disease of the tomato fields by causing root-rot and vascular diseases on the plants [54]. After 72 h, the observation of the wells reveals that only aqueous extracts of stems and leaves, n-butanol extract of fruits, and ethyl acetate extract of fruits showed positive results (Table 3). In the case of ethyl acetate extract of fruits, the total inhibition action is higher than 2 mg/mL and 75% of *Fusarium* can grow in the presence of 1 mg/mL of *P. tomentosa* extract. Fruits extracted with n-butanol constitute the most potent effective fungicide with a minimum concentration of 0.25 mg/mL. Aqueous extract of stems has a total inhibitory value of the fungi growth at a concentration higher than or equal to 20 mg/mL. Aqueous extract of leaves shows an inhibition of 25% of the fungal growth at 20 mg/mL. Those four extracts could be used as a cheaper natural source to have minimal environmental impact and danger to consumers of tomato in contrast to synthetic pesticides. Our results were considered promoted compared with *Acorus calamus* which showed the highest antifungal activity at 1000 mg/mL [55]. The differences in the inhibitory effect of various plant extracts may be due to qualitative and quantitative differences in the present antifungal compounds and to the used solvent for extraction. Fruits and stems seemed to contain important metabolites resistant to the fungal growth.

The mechanism of action against pathogens can be explained by the fact of production of an enzymatic inhibition by phenols through compound oxidation and synthesis protein inhibition in the cell by tannins [56]. A synergistic interaction between extract and antimicrobial agents was recorded by Adwan et al. [57]. A survey against our studied *Fusarium* attributed the antifungal activity to the presence of some compounds, such as ethyl iso-allocholate; 7,8-epoxylanostan-11-ol; and 3-acetoxy [58].

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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