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Maximizing the Antioxidant Capacity of *Padina pavonica* by Choosing the Right Drying and Extraction Methods

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Abstract: Marine algae are becoming an interesting source of biologically active compounds with a promising application as nutraceuticals, functional food ingredients, and therapeutic agents. The effect of drying (freeze-drying, oven-drying, and shade-drying) and extraction methods (shaking at room temperature, shaking in an incubator at 60 °C, ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE)) on the total phenolics content (TPC), total flavonoids content (TFC), and total tannins content (TTC), as well as antioxidant capacity of the water/ethanol extracts from *Padina pavonica* were investigated. The TPC, TFC, and TTC values of *P. pavonica* were in the range from 0.44 ± 0.03 to 4.32 ± 0.15 gallic acid equivalents in mg/g (mg GAE/g) dry algae, from 0.31 ± 0.01 to 2.87 ± 0.01 mg QE/g dry algae, and from 0.32 ± 0.02 to 10.41 ± 0.62 mg CE/g dry algae, respectively. The highest TPC was found in the freeze-dried sample in 50% ethanol, extracted by MAE (200 W, 60 °C, and 5 min). In all cases, freeze-dried samples extracted with ethanol (both 50% and 70%) had the higher antioxidant activity, while MAE as a green option reduces the extraction time without the loss of antioxidant activity in *P. pavonica*.

Keywords: *Padina pavonica*; brown algae; ultrasound-assisted extraction; microwave-assisted extraction; antioxidant activity; green extraction; phenolic compounds

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1. Introduction

Among different brown algae from the genus *Padina* (family *Dictyotaceae*, order Dictyotales), *Padina pavonica* is one of the most common species that inhabits coastal intertidal zones. This alga is growing mainly in the Mediterranean Sea and Atlantic Ocean while it is distributed in tropical and temperate seas worldwide [1–3]. Its availability makes it an interesting and suitable choice for research activities, especially considering that brown macroalgae (seaweeds) have been described as unexplored and are rich sources of compounds with different biological activities [4]. It has been reported that marine macroalgae have strong antioxidant, antimicrobial, antiviral, anti-inflammatory, antitumor, and anticancer properties [5–7]. Polyunsaturated fatty acids (comprising docosahexaenoic acid), carbohydrates, pigments, phenolic compounds (phenolic acids, flavonoids, and tannins), peptides, enzymes, lipids, vitamins, and terpenoids are commonly found in macroalgae [8]. Among them, phenolics, a group of secondary metabolites produced by algae as a response to harsh environmental conditions, are the most important bioactive compounds found in brown algae [9]. They are the primary components of algae cell walls while they also have a role as a chemical defence against herbivores, bacteria, and fouling organisms [4]. On the one hand, the biological activity of phenolic compounds from terrestrial plants has been

well studied. On the other hand, the study of compounds from marine origin, especially algal phenolics, has only recently come to focus, as these compounds show wide diversity in biological activity [4]. In algae, these phytochemicals vary from simple molecules such as phenolic acids or flavonoids to highly complex polymers called phlorotannins, a subgroup of tannins formed by polymerization of phloroglucinol units [4,10]. Compared to phlorotannins, studies on flavonoid content in algae are limited. To date, it has been reported that seaweeds are a rich source of catechins, rutin, quercetin, hesperidin, and other flavonoids [10]. Strong antioxidant properties of phenolics make them the functional ingredient for possible applications such as nutraceuticals or pharmaceuticals, as well as in functional food and cosmetic industries. In addition, there is an increased interest in natural antioxidants as a replacement for synthetic ones [11]. Studies on bioactive components from algae have intensified in the last years and most of the focus has been on the identification of new components; the preparations protocols of the material (drying, extraction) have been neglected. To identify macroalgae as a potential source of natural antioxidants requires adaptation of drying and extraction methods. Procedures that are used for terrestrial plants need to be adapted to this new matrix.

Before being subjected to extraction, the macroalgae are traditionally sun-dried, shade-dried, or oven-dried. The selection of the drying method may be a key factor that influences the phytochemical content of algae samples. It has been shown that many parameters, such as temperature, drying time, and UVA-UVB light affect the phytochemical content and antioxidant potential of algae [12]. As low temperature prevents the degradation of heat-sensitive compounds, freeze-drying, a method that is performed under vacuum, has been suggested to prevent the oxidation reactions of functional ingredients [13]. The selection of the best drying method for marine macroalgae remains to be confirmed.

Recently, researchers have investigated novel methods for assisting the extraction of phenolics from macroalgae, such as ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE). UAE and MAE are considered to be green methods since they reduce extraction time, decrease high solvent consumption, and increase the yield of targeted compounds as compared with conventional methods such as maceration or shaking [14]. Phenolic compounds have different polarities which indicate that hydro-alcoholic mixtures could be the best suitable solvent for their extraction, plus these mixtures are acceptable for food applications [15]. A hydro-alcoholic mixture has a synergistic effect, water acts as a swelling agent of the matrix, and ethanol induces the rupture of the bond between the solutes and the matrix [16]. In the last few years, the focus of studies has been on *P. pavonica*, analyzing the use of different extraction methods and different solvents to obtain bioactive compounds with specific biological activity (Table 1).

According to recent studies, it is evident that the extraction parameters, solid-to-solvent ratio, and solvent choices in the extraction methods need to be optimized and investigated to find conditions that would yield the highest amount of targeted compounds and preserve their biological activity.

Table 1. Recent studies on different extraction methods and solvents to yield particular biological compound/activity of *Padina pavonica*.

Extraction	Solvent	Targeted Compound/Activity	Reference
MAE	Ethanol and methanol 80%	Antibacterial activity, flavonoids	[17]
Extracted in centrifuge tubes in a 37 °C water bath for 2.5 h	96% Ethanol	Phenolic compounds	[18]
Stirring 12 h	Methanol and dichloromethane	Antioxidant activity, cytoprotectivepotential	[19]
Soxhlet extraction	Acetone	Pro-apoptotic activity, antioxidant activity	[9]
PLE, MAE, SFE, electroporation extraction	Petroleum ether, ethanol, ethyl acetate and water	Anti-hyaluronidase activity	[20]

Table 1. *Cont.*

Extraction	Solvent	Targeted Compound/Activity	Reference
Extracted at 100 °C for 2 h using a reflux condenser under reduced pressure	Acetone and water	Antioxidant activity, antimicrobial activity	[21]
Soxhlet extraction	Acetone	Pro-osteogenic ability, antioxidant activity	[22]
Extracted for 72 h in shaking incubators	80% Acetone, 80% ethanol, 80% methanol and water	Antioxidant activity, antidiabetic activity	[23]
Maceration at room temperature twice for 24 h	Hexane, ethyl acetate, and methanol	Antiparasitic activity, antioxidant activity	[24]
SLE (30 min)	Ethanol 50%	Antioxidant activity, anti-inflammatory activity, and antinociceptive activity	[25]
Percolation at room temperature for 2 days	95% Ethanol	Antimicrobial activity, antioxidant activity and anticancer activity	[26]

MAE, microwave-assisted extraction; PLE, pressurized liquid extraction; SFE, supercritical fluid extraction; UAE, ultrasound-assisted extraction.

Despite the diversity of marine macroalgae in the Adriatic Sea, most of them have not yet been investigated for biological activities and the reports on the total phenolic content (TPC) and antioxidant properties of macroalgae are very limited. Recently, the acetone extracts of *Dictyota dichotoma*, *P. pavonica,* and *Sargassum vulgare* from the Adriatic coast of Montenegro have shown that they have antioxidant, antimicrobial, and cytotoxic potential [27].

The aim of this study was to increase the antioxidant capacity of *P. pavonica* through the optimal drying and extraction methods. Freeze-dried, oven-dried, and shade-dried samples were extracted by four different methods (shaking at room temperature, shaking in an incubator at 60 °C, UAE, and MAE) using water and ethanol (green solvents). A large number of extracts was screened to test the highest yield of total phenolic content (TPC), total flavonoid content (TFC), and total tannins content (TTC). The antioxidant capacity was performed by multiple approaches based on ferric reducing/antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl radical scavenging ability (DPPH), and the oxygen radical absorbance capacity (ORAC).

2. Materials and Methods

2.1. Chemicals

The following chemicals that were used were analytical grade and obtained from Sigma-Aldrich: GmbH (Steinheim, Germany); Fluka (Buchs, Switzerland); Kemika (Zagreb, Croatia); Merck (Darmstadt, Germany); and Gram-Mol (Zagreb, Croatia).

2.2. Algal Material and Drying Methods

Brown macroalgae, *P. pavonica* was harvested off the coast of the island Čiovo in the Adriatic Sea in May 2020. The sea temperature was 18.3 °C and salinity was 37.4 ppt, measured using a YSI Pro2030 probe (Yellow Springs, OH, USA). Harvested algae were washed thoroughly with tap water to remove epiphytes, and then divided into groups to test the effect of different drying methods on phenolic composition and antioxidant activity. Algae were dried as follows: (i) shade-dried for approximately seven days; (ii) oven-dried in the ventilated oven for 24 h at 60 °C; and (iii) freeze-dried for 2 days using freeze-dryer (FreeZone 2.5, Labconco, Kansas City, MO, USA). All samples were ground (1 min in a high-speed grinder) after drying. A voucher specimen of tested species is deposited in the herbarium at the University Department of Marine Studies in Split, Croatia.

2.3. Extractions

The algal samples, in duplicate, were extracted with water, ethanol 50%, and ethanol 70% using four extraction methods. Based on the preliminary tests (temperature of the sample during extraction, particle size of the samples, and yield of extraction) the algae to solvent ratio were set at 1:10 for all extractions. The following extraction methods were applied: (i) shaking in the dark for 24 h at room temperature (SLE); (ii) shaking in the incubator at 60 °C for 1 and 2 h (MIX); (iii) UAE performed using an ultrasonic bath (Transsonic Tp 310H, Elma Schmidbauer GmbH, Singen, Germany) at 40 kHz frequency and 60 °C for 1 and 2 h; and (iv) MAE was performed in an advanced microwave extraction system (ETHOS X, Milestone Srl, Sorisole, Italy), for 5, 10, 15, and 30 min. The microwave power was kept constant at 200 W and the temperature was set at 60 °C. The extraction conditions used for MAE and UAE were selected during preliminary tests. After the extractions, samples were centrifuged at 5000 rpm for 8 min at room temperature. All extracts were kept in the freezer at −20 °C prior to analyses. Each extraction was done in duplicate.

2.4. Phenolic Composition

All obtained extracts were analysed for TPC, TFC, and TTC.

The TPC of *P. pavonica* extracts was determined by the Folin–Ciocalteu method [28]. Briefly, 25 µL of the extract was mixed with 1.5 mL distilled water and 125 µL Folin–Ciocalteu reagent. The solution was mixed and after one minute 375 µL 20% sodium carbonate solution and 475 µL distilled water was added. The mixture was left in the dark for 2 h at room temperature. The absorbance was read at 765 nm using a spectrophotometer (SPECORD 200 Plus, Edition 2010, Analytik Jena AG, Jena, Germany). The standard calibration (0–500 mg/L) curve was plotted using gallic acid ($y = 0.001x$, $R^2 = 0.9998$). The TPC was expressed as gallic acid equivalents in mg/g of dried algae (mg GAE/g).

The TFCs of *P. pavonica* extracts were measured using the aluminium chloride colorimetric method, previously described by Lovrić et al. [29] with slight modifications. A volume of 250 µL algae extract was mixed with 750 µL 96% ethanol, 50 µL 10% (*w/v*) aluminium chloride, 50 µL 1M sodium acetate, and 1400 µL distilled water. The mixture was kept for 30 min at room temperature before measurement. The absorbance was read at 415 nm using a spectrophotometer. The standard calibration (0–200 µg/mL) curve was plotted using quercetin ($y = 0.0068x - 0.0064$, $R^2 = 0.9989$). The TFC was expressed as mg quercetin equivalents per g of dried algae (mg QE/g).

The TTCs of *P. pavonica* extracts were determined, using the method of Zhong et al. [30] with modifications. The extract (25 µL), 150 µL 4% (*w/v*) ethanolic vanillin solution, and 25 µL 32% (*v/v*) sulfuric acid (diluted with ethanol) were mixed together in a 96-well plate. The mixture was kept for 15 min at room temperature before reading. The absorbance was measured at 500 nm using the microplate reader (Synergy HTX Multi-Mode Reader, BioTek Instruments, Inc., Winooski, VT, USA). The standard calibration (0–1000 µg/mL) curve was plotted using catechin ($y = 0.0005x + 0.0555$, $R^2 = 0.9997$). The TTC was expressed as mg catechin equivalents per g of dried algae (mg CE/g).

2.5. Antioxidant Activity

The antioxidant activity of *P. pavonica* extract was assessed by three different methods that are based on two different mechanisms of action, i.e., hydrogen atom transfer (HAT) (DPPH and ORAC) and electron transfer (ET) (FRAP)) [31].

The reducing activity was measured as FRAP, using a previously described method [32] and the results were expressed as micromoles of Trolox equivalents (µM TE).

The 2,2-diphenyl-1-picrylhydrazyl radical scavenging ability (DPPH) of extracts was measured, according to a previously described method [33] modified to 96-well microplates [34]. The decrease in the absorbance (at 517 nm) was measured after 1 h using the Synergy HTX Multi-Mode micro plate reader (BioTek Instruments, Inc., Winooski, VT,

USA). The antioxidant activity was expressed as inhibition percentages of DPPH radical (% inhibition).

The oxygen radical absorbance capacity (ORAC) method was performed, according to a previously described method [35] modified by Burčul et al. [36]. The reaction was measured every minute during 80 min. Extracts were diluted 100-fold and the results were expressed as μM of Trolox equivalents (μM TE).

2.6. Statistical Analysis

The specific drying conditions and all extractions were replicated, while all analyses (TPC, TFC, TTC, and antioxidant assays) were performed in triplicate. The results are expressed as mean ± standard deviation. Principal component analysis (PCA) was used to determine relations among the studied variables (TPC, TFC, TTC, FRAP, DPPH, and ORAC) and the relationship between them (STATISTICA, version 13, StatSoft Inc, Tulsa, OK, USA).

3. Results and Discussion

3.1. Phenolic Composition

The results of TPC, TFC, and TTC for freeze-dried, oven-dried, and shade-dried extracts of *P. pavonica* are shown in Table 2. Freeze-dried extracts had the highest TPC as compared with other drying methods and, in some cases, freeze-dried samples had from three-fold to seven-fold higher TPC than oven-dried and shade-dried samples. The results showed evidence that freeze-drying was the best drying method for preserving TPC in algal material irrespective of the solvent used. In the comparison of oven-dried and shade-dried samples, oven-dried had a significantly higher content of TPC, with the exception of SLE in water. The application of microwaves resulted in the highest phenolics yield. The MAE yielded higher TPC with a shorter extraction time.

When comparing the three solvents in extractions of freeze-dried samples, those extracted with 50% ethanol had higher TPC than samples extracted with water and 70% ethanol. The highest TPC was in the sample extracted with MAE for 5 min in 50% ethanol (4.32 ± 0.15 mg GAE/g). Freeze-dried samples extracted with water had the lowest results for all extraction methods, but even those results were higher than TPC in the oven and shade-dried samples for the same extractions methods. When comparing the three solvents in extractions of oven-dried and shade-dried samples, those extracted with water had higher TPC than samples extracted with water-ethanol mixtures. The highest TPC among oven-dried and shade-dried samples was in extracts prepared with water using MAE for 30 and 15 min, respectively (2.09 ± 0.01 and 1.84 ± 0.46 mg GAE/g).

Overall, the TPC of *P. pavonica* ranged from 0.44 ± 0.034 to 4.32 ± 0.15 mg GAE/g. As compared to other brown algae such as algae from genus *Cystoseira* and *Fucus* where TPC was above 400 and 150 mg GAE/g dry weight, respectively [4], the TPC of *P. pavonica* was relatively low. Mannino et al. [18] also determined low TPC in oven-dried *P. pavonica*. This may be because this alga calcifies, making it a species that is hardly palatable and possibly not requiring particular high production of phenolic compounds to endure predators and the environmental challenges. Another reason could be the low sea temperature. The TPC increase was correlated with rising sea temperature [37].

Table 2. Total phenolic content (TPC), total flavonoid content (TFC), and total tannin content (TTC) of *P. pavonica* extracts (n = 6).

Extraction Method *	Solvent	Time	FD **	OD	SD	FD	OD	SD	FD	OD	SD
			TPC (mg GAE/g)			TFC (mg QE/g)			TTC (mg CE/g)		
MIX (60 °C)	H_2O	1 h	1.85 ± 0.11	1.79 ± 0.07	1.57 ± 0.03	1.06 ± 0.08	1.59 ± 0.04	1.64 ± 0.11	8.07 ± 0.79	10.02 ± 0.51	6.77 ± 0.52
		2 h	1.94 ± 0.05	1.74 ± 0.01	1.56 ± 0.03	1.01 ± 0.04	2.01 ± 0.08	1.68 ± 0.07	9.02 ± 0.77	9.79 ± 0.85	8.67 ± 0.35
	EtOH 50%	1 h	4.25 ± 0.12	1.29 ± 0.04	0.79 ± 0.06	0.81 ± 0.02	0.71 ± 0.05	0.69 ± 0.01	4.64 ± 0.46	4.06 ± 0.35	3.02 ± 0.28
		2 h	3.90 ± 0.12	1.01 ± 0.04	0.65 ± 0.03	0.80 ± 0.05	0.62 ± 0.04	0.67 ± 0.00	3.25 ± 0.38	1.95 ± 0.12	1.63 ± 0.16
	EtOH 70%	1 h	3.84 ± 0.09	1.01 ± 0.03	0.71 ± 0.04	2.79 ± 0.07	2.21 ± 0.11	1.92 ± 0.08	7.43 ± 0.27	3.00 ± 0.24	2.18 ± 0.09
		2 h	3.48 ± 0.19	0.89 ± 0.01	0.66 ± 0.05	2.21 ± 0.08	2.00 ± 0.06	2.07 ± 0.10	1.88 ± 0.02	2.86 ± 0.05	1.30 ± 0.08
SLE (room temperature)	H_2O	24 h	1.51 ± 0.11	1.31 ± 0.06	1.48 ± 0.05	0.84 ± 0.02	0.49 ± 0.02	0.36 ± 0.02	8.14 ± 0.64	6.23 ± 0.05	3.92 ± 0.35
	EtOH 50%	24 h	3.41 ± 0.12	0.63 ± 0.02	0.49 ± 0.09	0.84 ± 0.02	0.31 ± 0.01	0.43 ± 0.03	2.27 ± 0.10	2.30 ± 0.22	2.22 ± 0.19
	EtOH 70%	24 h	3.31 ± 0.13	0.53 ± 0.06	0.44 ± 0.03	2.22 ± 0.09	0.73 ± 0.05	1.15 ± 0.06	2.19 ± 0.14	1.44 ± 0.06	1.53 ± 0.04
UAE (60 °C)	H_2O	1 h	1.72 ± 0.03	1.62 ± 0.12	1.43 ± 0.03	0.63 ± 0.02	0.32 ± 0.01	0.45 ± 0.01	5.34 ± 0.17	7.05 ± 0.46	5.96 ± 0.26
		2 h	1.70 ± 0.02	1.59 ± 0.12	1.31 ± 0.08	0.78 ± 0.01	0.38 ± 0.02	0.61 ± 0.02	10.41 ± 0.62	7.67 ± 0.03	6.16 ± 0.27
	EtOH 50%	1 h	3.94 ± 0.08	0.96 ± 0.08	0.64 ± 0.08	0.89 ± 0.04	0.48 ± 0.03	0.63 ± 0.02	2.90 ± 0.10	0.62 ± 0.03	0.94 ± 0.14
		2 h	3.97 ± 0.12	0.92 ± 0.00	0.77 ± 0.09	0.92 ± 0.01	0.50 ± 0.04	0.65 ± 0.04	3.63 ± 0.05	1.16 ± 0.08	2.03 ± 0.19
	EtOH 70%	1 h	3.51 ± 0.09	0.82 ± 0.07	0.66 ± 0.06	2.83 ± 0.11	1.31 ± 0.01	1.83 ± 0.08	2.62 ± 0.20	2.12 ± 0.13	2.08 ± 0.07
		2 h	3.45 ± 0.05	1.04 ± 0.05	0.61 ± 0.01	2.75 ± 0.02	2.25 ± 0.12	1.57 ± 0.05	2.41 ± 0.23	2.46 ± 0.08	2.07 ± 0.16
MAE (200 W, 60 °C)	H_2O	5 min	2.06 ± 0.01	1.56 ± 0.04	1.38 ± 0.05	0.51 ± 0.02	0.68 ± 0.02	0.47 ± 0.02	9.03 ± 0.79	0.32 ± 0.02	5.20 ± 0.41
		10 min	1.88 ± 0.19	1.95 ± 0.01	1.77 ± 0.07	0.52 ± 0.02	0.66 ± 0.01	0.54 ± 0.05	7.77 ± 0.45	3.45 ± 0.17	5.48 ± 0.46
		15 min	1.90 ± 0.05	1.90 ± 0.18	1.84 ± 0.05	0.50 ± 0.03	0.68 ± 0.01	0.61 ± 0.04	6.25 ± 0.44	5.60 ± 0.19	3.12 ± 0.22
		30 min	2.11 ± 0.08	2.09 ± 0.01	1.75 ± 0.12	0.62 ± 0.03	0.69 ± 0.02	0.61 ± 0.02	5.86 ± 0.30	6.82 ± 0.64	5.14 ± 0.48
	EtOH 50%	5 min	4.32 ± 0.15	1.41 ± 0.05	1.01 ± 0.06	0.91 ± 0.04	1.06 ± 0.05	0.77 ± 0.04	2.19 ± 0.13	1.09 ± 0.08	1.06 ± 0.10
		10 min	4.32 ± 0.13	1.46 ± 0.07	1.13 ± 0.05	1.48 ± 0.03	0.77 ± 0.05	0.82 ± 0.03	1.89 ± 0.18	0.92 ± 0.06	0.94 ± 0.09
		15 min	3.79 ± 0.07	1.51 ± 0.06	1.06 ± 0.05	1.12 ± 0.04	0.81 ± 0.02	0.77 ± 0.03	2.79 ± 0.13	1.38 ± 0.04	1.40 ± 0.06
		30 min	4.10 ± 0.13	1.64 ± 0.03	1.19 ± 0.05	1.27 ± 0.07	0.92 ± 0.06	0.86 ± 0.00	3.13 ± 0.26	0.93 ± 0.08	1.49 ± 0.13
	EtOH 70%	5 min	3.28 ± 0.02	1.12 ± 0.05	0.82 ± 0.05	2.87 ± 0.01	1.84 ± 0.01	1.79 ± 0.05	3.51 ± 0.16	1.56 ± 0.06	1.76 ± 0.08
		10 min	3.48 ± 0.06	1.17 ± 0.10	0.87 ± 0.08	2.09 ± 0.12	1.82 ± 0.12	1.71 ± 0.05	2.45 ± 0.22	1.14 ± 0.11	1.22 ± 0.11
		15 min	3.46 ± 0.01	1.14 ± 0.05	0.89 ± 0.03	2.16 ± 0.08	1.82 ± 0.13	1.86 ± 0.02	3.30 ± 0.32	1.44 ± 0.04	1.86 ± 0.13
		30 min	2.98 ± 0.04	1.33 ± 0.05	0.95 ± 0.04	1.76 ± 0.03	2.11 ± 0.11	1.97 ± 0.12	3.51 ± 0.24	2.29 ± 0.19	1.87 ± 0.15

* MIX, shaking in the incubator; SLE, shaking in the dark; UAE, ultrasound-assisted extraction; MAE, microwave-assisted extraction; ** FD, freeze-dried; OD, oven-dried; SD, shade-dried.

The freeze-dried samples had the highest TFC and TTC, with some exceptions. Regarding the used solvents, 70% ethanol yielded higher TFC amounts than water and 50% ethanol for all drying and extraction methods. Comparing UAE and MAE, samples extracted with 70% ethanol had two- or three-fold higher TFC than samples extracted with water and 50% ethanol. However, water appears to be the best solvent for extracting tannins among tested solvents. Water extracts prepared using UAE and MAE had TTC more than three-fold higher than those of water-ethanol mixtures for all drying methods. The freeze-dried sample extracted with water using UAE (for 2 h) and MAE (for 5 min) had the highest TTC. These results showed that the two green, novel extraction methods yielded higher TFC and TTC amounts, and it was evident that MAE yielded more TFC in a shorter extraction time.

Magnusson et al. [38] reported a 70% increase in the yield of extracted polyphenols in brown macroalgae (*Carpophyllum flexuosum*) when using MAE (water/biomass solvent ratio of 1:30, 160 °C, and 3 min) as compared with solid-liquid extraction. Yuan et al. [39] reported that the TPC and antioxidant potential of four brown algae increased when using MAE at 110 °C for 15 min as compared with conventional extraction at room temperature for 4 h.

The effect of temperature was not a specific aim of this study; however, all extractions, except SLE, were performed at 60 °C to preserve phenolic compounds susceptible to thermal degradation [4]. The results showed a difference between two similar extraction methods (SLE and MIX), performed at two different temperatures (24 and 60 °C). The higher temperature increased TPC and shortened extraction time 24 times. This could be because a higher temperature increased intermolecular interactions within the solvent which increased the solubility of the compounds [40]. In some studies, authors describe that high temperatures (>60 °C) resulted in a higher yield of TPC from brown algae [39,41,42]. Although the TPC increases (probably due to hydrolysis of complex phlorotannins into simpler compounds), the application of high temperatures brings to question the susceptibility of phenolic compounds to thermal degradation [4].

Treating the samples for 1 and 2 h at the same temperature using UAE resulted in no differences among the TPCs of the extracts. The reason for this could be that the yield of TPC extracted by UAE increases with time and it happens in two phases. The first phase is the "washing" phase in which soluble components on the surfaces of the matrix are being dissolved, it lasts for the first 10–20 min, and up to 90% of the total phenolic content is recovered. In the second phase, i.e., the "slow extraction" phase, diffusion is responsible for mass transfer of the solute from the matrix into the solvent and it can last from 60 to 100 min [15,16]. Apparently, in this experiment, most of the mass transfer occurred in the first 60 min. Some authors have reported that ultrasound frequency does not influence TPC significantly. In fact, Ummat et al. [43] confirmed that UAE frequency of 35 and 130 kHz yielded statistically similar TPC from algae. For this reason, the effect of ultrasound frequency was not tested, and 40 kHz was used. Although studies on flavonoid content in algae are limited, few reports have shown evidence of *P. pavonica* extracts being rich in flavonoids. Alghazeer et al. [17] prepared flavonoids rich extract (70.08 mg/g rutin equivalent) from *P. pavonica* using MAE and demonstrated its potential antibacterial activity, while Bernardini et al. [9] reported high levels of TFC in air-dried *P. pavonica* extracted with acetone using Soxhlet extraction.

3.2. Antioxidant Activity

The antioxidant activity was determined using the FRAP, DPPH, and ORAC assays and the results are shown in Figures 1–3. The FRAP values ranged from 98.46 ± 3.53 to 537.95 ± 9.11 μM TE. The freeze-dried samples prepared with 50% and 70% ethanol had the highest FRAP values for all extraction methods. When water was used as a solvent, freeze-dried samples had lower FRAP than the oven and shade-dried ones. Freeze-drying appears to be the best drying method for obtaining extract with high reducing activity. The highest FRAP was found in freeze-dried samples prepared with 70% ethanol, using

MAE for 30 min (537.95 ± 9.11 µM TE) and using UAE for 1 h (461.28 ± 18.97 µM TE). The highest FRAP result among oven-dried samples was also found in the sample extracted for 30 min in 70% ethanol using MAE.

The free antioxidant ability of *P. pavonica* extracts against DPPH radical ranged from 2.33 ± 1.34% to 62.88 ± 3.13%. The highest free radical scavenging activity was obtained for freeze-dried samples in all extraction methods, with the exceptions of two water extracts (Figure 2). When comparing solvents used for extraction, the results showed that water/ethanol mixtures were more suitable for maintaining high DPPH scavenging activity of investigated algal samples, especially 50% ethanol. In all ethanol extracts, oven-dried and shade-dried samples had significantly lower inhibition capacity than samples obtained by freeze-drying. Novel extraction methods, UAE and MAE, gave high DPPH scavenging activity with inhibition over 50%. Freeze-dried samples prepared in 50% ethanol using UAE for 1 h and MAE for 5 min had 59.42 ± 1.30% and 54.57 ± 1.97% of inhibition, respectively. Water extraction gave better antioxidant capacity in oven-dried and shade-dried samples. As for FRAP, water extracts of freeze-dried samples had lower scavenging activity. Among all water extracts of oven-dried and shade-dried samples, MAE gave the highest DPPH of 41.11 ± 0.83% and 32.99 ± 5.35%, respectively.

The ability of *P. pavonica* extracts to scavenge peroxyl radicals was measured with ORAC assay. While peroxyl radicals are the predominant free radicals found in biological systems, ORAC values are considered to be biologically relevant [44]. The results obtained by the ORAC method ranged from 8.57 ± 0.37 to 71.37 ± 1.97 µM TE. The freeze-dried samples had two- to three-fold higher ORAC values than oven-dried and shade-dried samples when they were extracted with water-ethanol mixtures. Similar to the DPPH method, when water alone was used as a solvent, ORAC values in oven-dried and shade-dried samples were slightly higher than in freeze-dried samples. Among them, samples prepared using MAE for 15 min gave the highest ORAC values for oven-dried (36.40 ± 0.52 µM TE) and shade-dried (35.11 ± 0.73 µM TE) samples. The results suggested that freeze-drying in combination with ethanol as a solvent is the best method for preserving the antioxidant activity of *P. pavonica*. The highest ORAC value was found in a freeze-dried sample extracted with 50% ethanol during MIX for 1 h. However, a similar ORAC value (69.68 ± 1.69 µM TE) was found for freeze-dried sample extracted with MAE for 10 min in 50% ethanol. Using this green extraction method, time was reduced six-fold without losing the oxygen radical absorbance capacity of the alga samples.

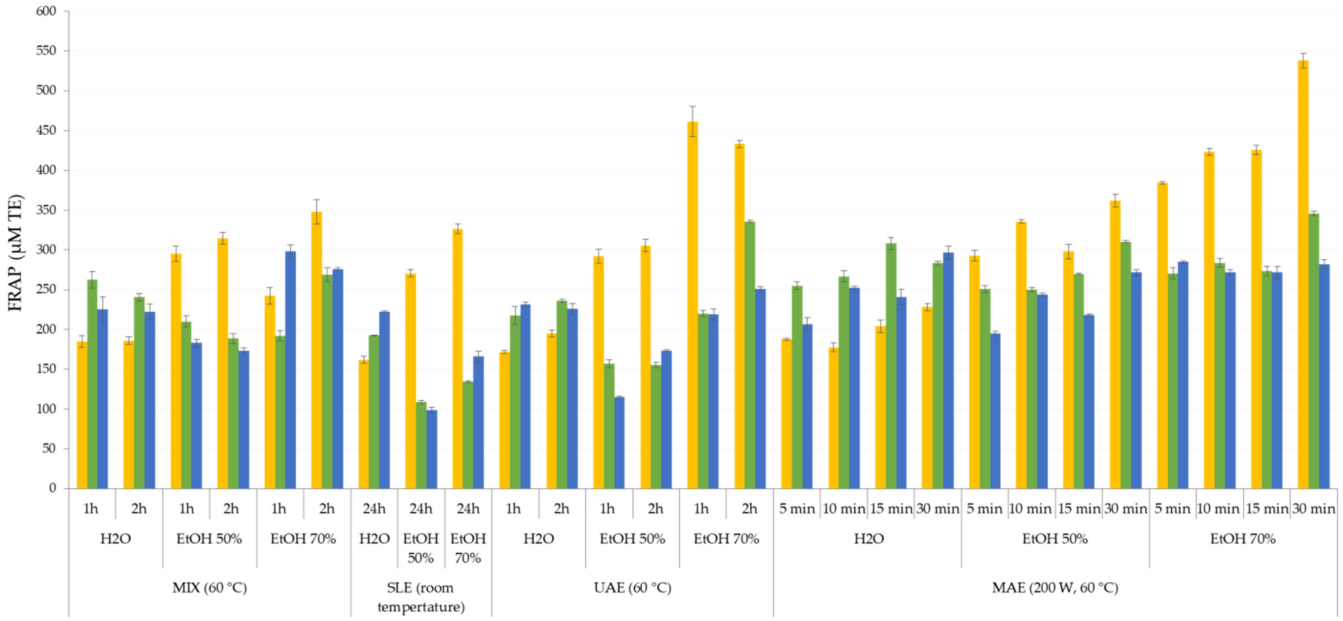

Figure 1. Ferric reducing/antioxidant power (FRAP) of freeze-dried (yellow), oven-dried (green), and shade-dried (blue) extracts of *P. pavonica* (n = 6).

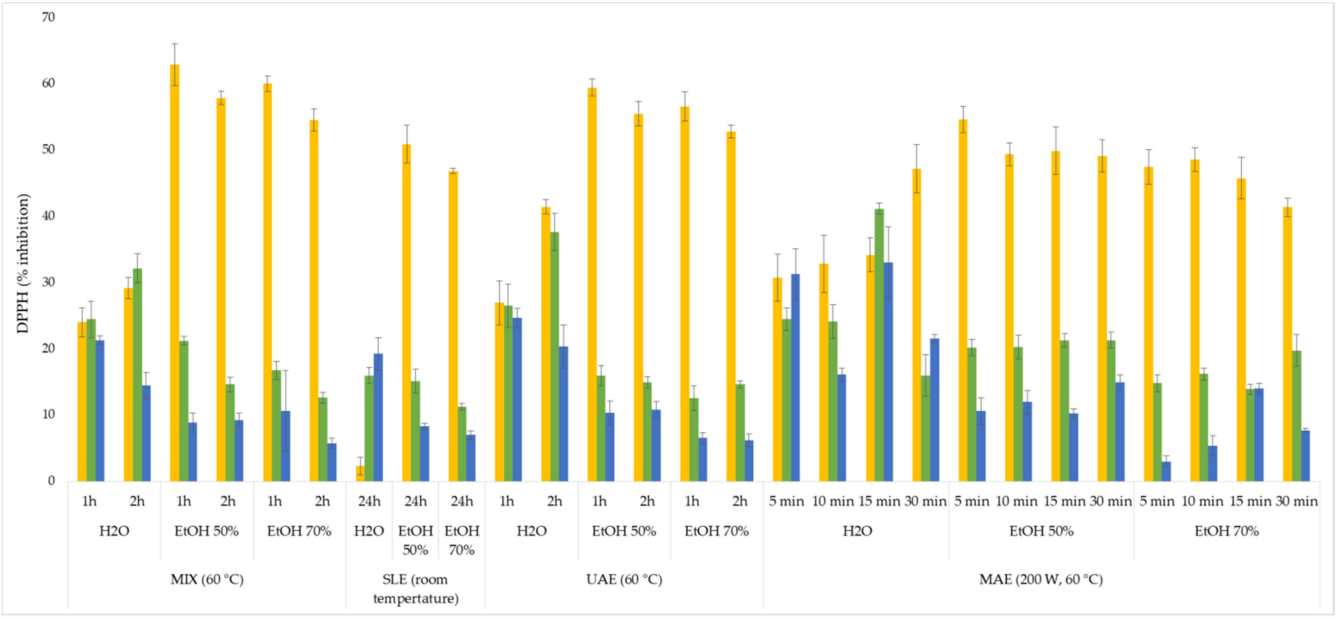

Figure 2. The 2,2-diphenyl-1-picrylhydrazyl radical scavenging ability (DPPH) of freeze-dried (yellow), oven-dried (green) and shade-dried (blue) extracts of *P. pavonica* (n = 6).

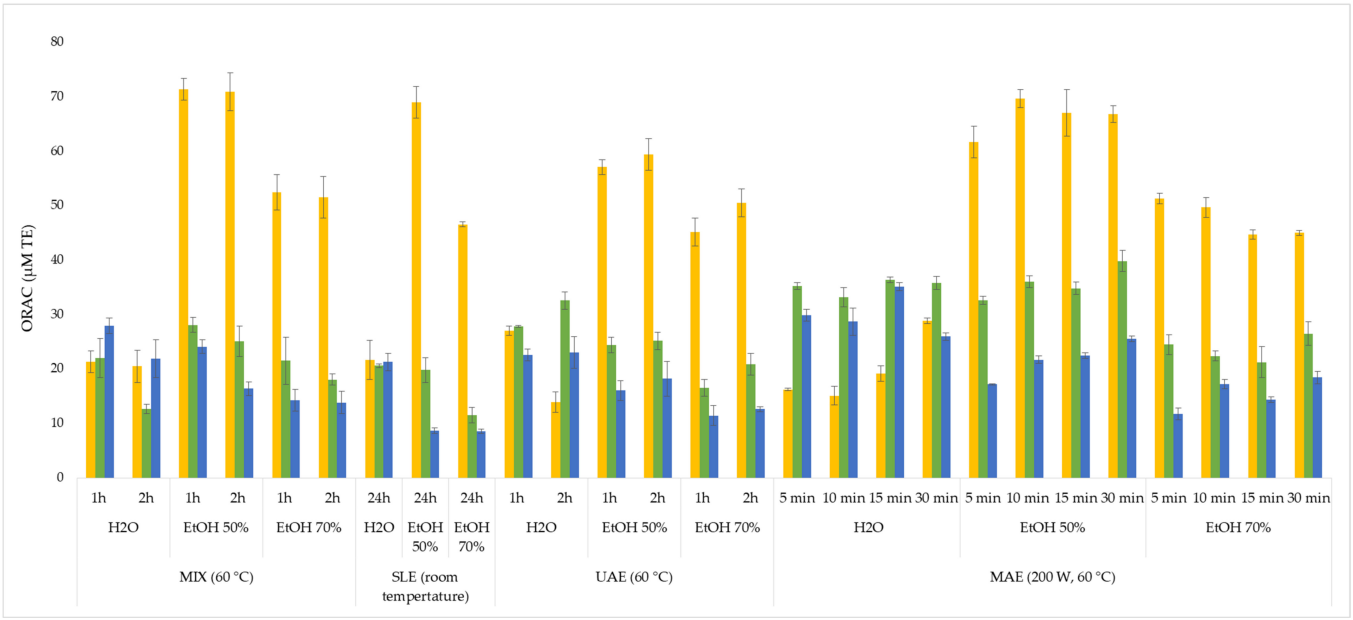

Figure 3. Oxygen radical absorbance capacity (ORAC) of freeze-dried (yellow), oven-dried (green), and shade-dried (blue) extracts of *P. pavonica* (n = 6).

To understand the effect of drying and extraction methods on the TPC, TFC, TTC, and antioxidation assays from *P. pavonica*, all the obtained data were submitted to PCA. The first two factors (Figures 4 and 5) described 79.04% of the initial data variability. Parameters that have the highest values of factor coordinates for the PC1, with the highest variable contributions, based on correlations were DPPH and ORAC. The PC2 was characterized by TPC, TFC, and TTC. The position of the samples in the multivariate space was arranged in two main areas, based on the strong influence of DPPH, ORAC, and FRAP (Factor 1) and total phenolic content (Factor 2), pointing out the clear separation among the samples. The extracts with the highest antioxidant activity were grouped on the left side while those with lower activity were positioned on the right part of the multivariate space.

The freeze-dried samples extracted with water/ethanol mixtures (both 50 and 70%) are grouped as extracts with high antioxidant activity. Water extracts of all samples grouped on the upper right side, while water-ethanol mixture extracts of oven-dried and shade-dried samples remained in the lower part of the graph. Amorim et al. [13] investigated the effect of freeze-drying, oven-drying, and silica-drying on the antioxidant properties of four Brazilian macroalgae (*Gracilariopsis tenuifrons*, *Pterocladiella capillacea*, *Sargassum stenophyllum*, and *Ulva fasciata*) and showed that freeze-drying had the lowest alteration of antioxidant potential in tested algae. However, Ling et al. [12] studied the effect of different drying methods (oven-drying, sun-drying, hang-drying, sauna-drying, shade-drying, and freeze-drying) on the phytochemical content and antioxidant activity of red macroalgae *Kappaphycus alvarezii*. The algae were extracted by shaking in 80% methanol (1:10 ratio) for 2 h at room temperature. They found that oven-drying at 40 °C provided the highest values of phytochemical content and displayed better scavenging and reducing ability. In their study freeze-drying yielded low TPC and TFC, and lower antioxidant activity (FRAP and DPPH).

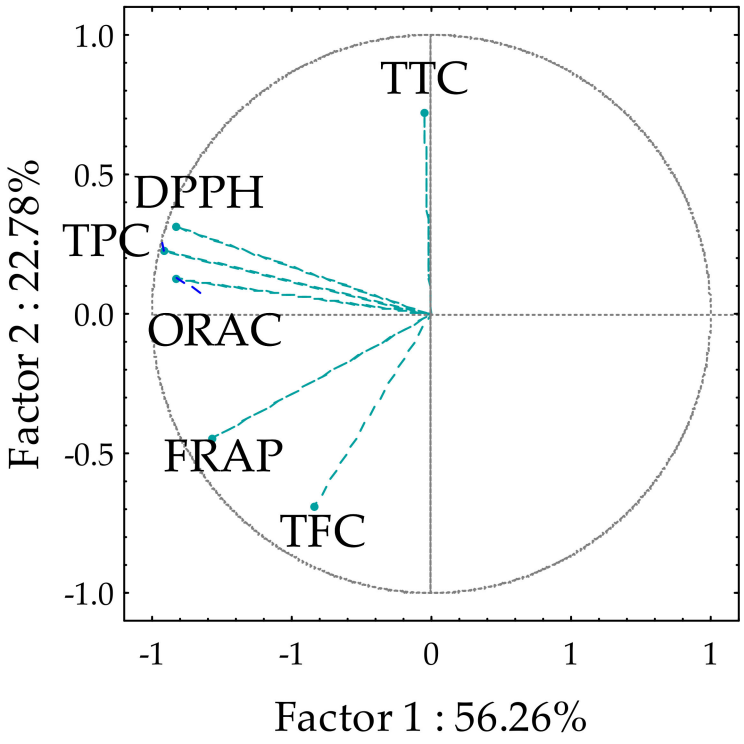

Figure 4. Correlation plots on the TPC, TFC, TTC, and antioxidation assays for *P. pavonica* according to principal component analyses (PCAs) describing all data.

The comparison of results with other studies is difficult in many ways. The TPC of an alga is influenced by environmental factors such as sea temperature, sampling location, salinity, size, age, and reproductive status, which makes the comparison even between the samples of the same species difficult. In many studies, these data are rarely reported. In addition, different standard compounds are used for the expression of the results, and results are reported on the base of wet or dry algae weight, milliliters of extracts, or even dry extracts. In general, in studies on algae, researchers have focused on finding new compounds and their bioactive properties rather than optimizing drying and extraction method which could enhance the activity of the extracts. Bernardini et al. [9] measured the TPC, TFC, and TTC in air-dried *P. pavonica* samples extracted with acetone in a Soxhlet extractor, and obtained 27.0, 54.8, and 54.3 mg per g of extract, respectively. These values correspond to 0.81, 1.64, and 1.63 mg/g dry material. They also reported a FRAP value of 25.6 ± 0.2 µmol of Fe^{2+}/100 mg of extract. The authors aimed to show the anticancer

properties of *P. pavonica* extracts against osteosarcoma cell lines. The TPC, TFC, TTC, and FRAP values are lower in comparison to our results. However, the authors used different drying and extraction methods, and the alga was sampled in the coastal area of Moorea. Hlila et al. [21] reported the TPC of 90.61 and 57.34 mg CE/g in *P. pavonica* acetone and water extract, respectively. Acetone extracts demonstrated a DPPH of 72.92% of inhibition and antimicrobial activity against eight microbial strains. The acetone was not always selected as the best solution. Ismail et al. [23] investigated four extraction solvents (80% acetone, ethanol, methanol, and water) on six different shade-dried seaweeds using shaking for 72 h. The results of TPC and DPPH *for P. pavonica* were the highest in the water extracts, 3.32 mg GAE/g dry weight and antioxidant capacity was 55.2%, respectively. They found more than two-fold higher TPC in water extract than in acetone, ethanol, and methanol extracts. Their results showed a correlation between TPC and antioxidant activity. The TPC of shade-dried *P. pavonica* extracts in our study were lower than reported by Ismail et al., however, freeze-dried water-ethanol extracts had higher TPC for all extraction methods. Another study tested methanol and dichloromethane as suitable solvents for TPC extraction and antioxidant activity of brown algae [19]. *P. pavonica* had TPC of 44.61 mg GAE/g dry extract, and DPPH IC_{50} of 338.8 µg/mL. In addition, ORAC values were 1543.6 µmol TE/g of dry extract. Abdelhamid et al. [25] tested three Mediterranean brown macroalgae (*Cystoseira sedoides*, *P. pavonica,* and *Cladostephus spongeosis*) for phenolic content and antioxidant, anti-inflammatory, and antinociceptive potential. Solid-liquid extraction was performed with 50% ethanol while shaking for 30 min at 50 °C. The extracts were purified in three steps (by using petroleum ether, dichloromethane, and ethyl acetate) to increase the phlorotannin content. *P. pavonica* had the phenolic content of 7.06 ± 2.52 mg PGE/g dry algae and the DPPH IC_{50} of 91.78 ± 1.98 µg/mL. Dang et al. [45] compared phenolic content and antioxidant activities among six freeze-dried brown algae extracted with 70% ethanol using UAE (30 °C, 60′, and 150 W). Collected from Bateau Bay (Australia), *Padina sp.* had high TPC (124.65 ± 0.78 mg GAE/g extract), TFC (20.74 ± 0.49 mg CAE/g extract), and TTC (56.17 ± 0.22 mg CAE/g extract) content.

P. pavonica has also been reported for other biological activities, but studies have shown that biological activities vary with season. Ismail-ben Ali et al. [46] reported seasonal variation of antibacterial activity of the *P. pavonica* collected from the northern coast of Tunisia, with the highest activity in July, August, and September which could be correlated with higher sea temperature. However, the authors reported no data on algae composition. In general, reports on seasonal effect on phytochemical content and their biological potential in brown algae are limited. A recent study showed that different green extractions (pressurized liquid extraction (PLE), MAE, supercritical fluid extraction (SFE), and electrochemical/electroporation extraction) could be used to obtain anti-hyaluronidase water extracts from *P. pavonica* with possible application in cosmetic anti-age products [20].

In this study the chemical analyses of the phenolic profile were not done, however it would be useful to determine the specific compounds responsible for the antioxidant activity. Besides, other molecules, such as pigments, carbohydrates, and proteins are also present in the extract [4] and they can contribute to antioxidant activity.

1, 28, 55 MIX 1h H_2O
2, 29, 56 MIX 2h H_2O
3, 30, 57 MIX 1h EtOH 50
4, 31, 58 MIX 2h EtOH 50
5, 32, 59 MIX 1h EtOH 70
6, 33, 60 MIX 2h EtOH 70
7, 34, 61 SLE H_2O
8, 35, 62 SLE EtOH 50
9, 36, 63 SLE EtOH 70
10, 37, 64 UAE 1h H_2O
11, 38, 65 UAE 2h H_2O
12, 39, 66 UAE 1h EtOH 50
13, 40, 67 UAE 2h EtOH 50
14, 41, 68 UAE 1h EtOH 70
15, 42, 69 UAE 2h EtOH 70
16, 43, 70 MAE 5′ H_2O
17, 44, 71 MAE 10′ H_2O
18, 45, 72 MAE 15′ H_2O
19, 46, 73 MAE 30′ H_2O
20, 47, 74 MAE 5′ EtOH 50
21, 48, 75 MAE 10′ EtOH 50
22, 49, 76 MAE 15′ EtOH 50
23, 50, 77 MAE 30′ EtOH 50
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27, 54, 81 MAE 30′ EtOH 70

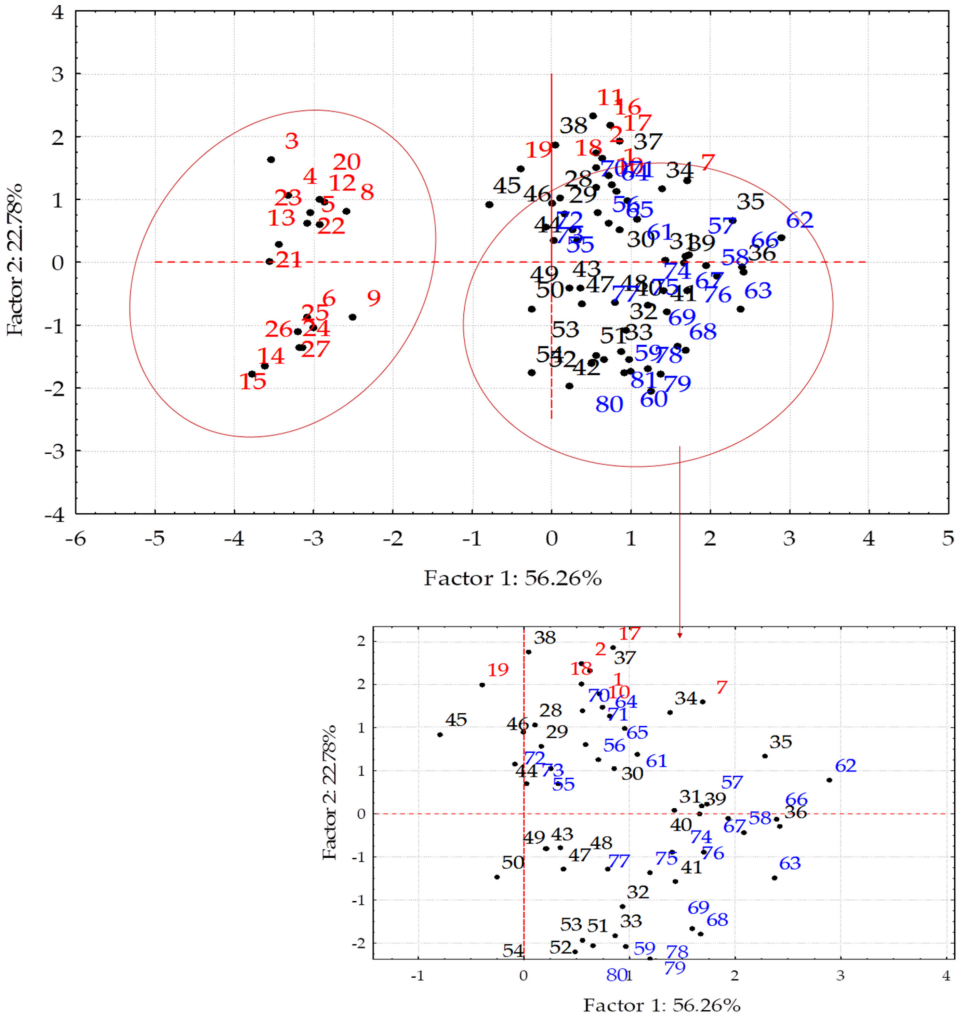

Figure 5. PCA for the antioxidant activity (Factor 1) and TTC (Factor 2) for freeze-dried (red, from 1–27), oven-dried (black, from 28–54), and shade-dried (blue, from 55–81) extracts of *P. pavonica* (lower graph is enlarged part of the main graph).

4. Conclusions

The results of this study contribute to the characterisation of brown algae as a matrix that can yield components with antioxidant potential. The novel, green methods (UAE and MAE) have shown more efficiency than the conventional SLE and MIX.

The TPC *of P. pavonica* was in the range from 0.44 ± 0.03 to 4.32 ± 0.15 mg GAE/g and in high correlation to DPPH and ORAC. The best method for achieving high TPC was freeze-drying while the best extraction method was MAE. In all cases, freeze-drying and extraction with ethanol (both 50% and 70%) maximized the TPC and antioxidant activity. It is an energy consuming method but based on PCA it is obvious that freeze-dried samples and water/ethanol mixtures are the best choices to obtain high reducing activity. MAE as a green option might reduce the extraction time and solvent consumption without loss in the activity of *P. pavonica* extracts.

The results obtained from this study show that *P. pavonica* should have good antioxidant activity despite relatively low TPC, but further analyses should be performed to identify the phenolic profile of the extracts, seasonal effect on phytochemical content, as well as their biological potential.

Author Contributions: Conceptualization, V.Š., G.T., and F.Ö.; methodology, D.S. and V.Š.; formal analysis, M.Č. and D.S.; data curation, M.Č., D.S., and V.Š.; writing—original draft preparation, M.Č., D.S., and V.Š.; writing—review and editing, V.Š., G.T., and F.Ö.; supervision, V.Š.; project administration, G.T., and V.Š.; funding acquisition, V.Š. All authors have read and agreed to the published version of the manuscript.

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