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Physicochemical characterization and pharmacological evaluation of marine polyphenols from the brown algae *Padina pavonica*

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Abstract

Background and Aim: Natural products continue to be a primary resource in biomedicine and biotechnology. The marine environment is highly reserve for novel pharmaceutical and medical compounds. The aim of this work was to identify bioactive components from the brown seaweed *Padina pavonica* with specific pharmacological potential.

Methods: In the present study, we investigated the efficacy of polyphenol fraction from *Padina pavonica* for in vivo anti-inflammatory activity using the carrageen an-induced paw edema model in rats and there in vitro antioxidant activity using two methods: DPPH radical scavenging assay and ferric reducing antioxidant power (FRAP) [8].

Results: The polyphenol-rich fraction from the brown seaweed *Padina pavonica* exhibits a significant anti-inflammatory activity at the dose of 100 and 200 mg/kg and the maximum reduction of the edema was observed at the third hour with 53.49% and 58.6% of inhibition, respectively. Along with, we were interested in the investigation of the antioxidant activity. The DPPH radical-scavenging assay shows that the polyphenol fractions have an interesting cavenging activity at a low concentration (0.25 mg/mL). In addition, the Ferric reducing antioxidant power (FRAP) method reveals an antioxidant activity with IC50 = 0.4 mg/mL.

Conclusions: These findings indicate that the polyphenol fraction of *Padina pavonica* is a promising bio source of compounds with anti-inflammatory and antioxidant potential; this may be useful as a candidate for the developing of potential therapeutic products.

Keywords: Brown Seaweeds; *Padina pavonica*; Anti-Inflammatory Activity; Antioxidant Activity

Introduction

New trends in the search for active natural compounds with potential health benefits, focused on some unexplored habitats especially marine environment. Recently, a huge number of new compounds have been isolated from marine sources. The use of these metabolites steadily increased in food, cosmetics, biotechnology and pharmacy [3].

Marine brown algae have been investigated for their promising bioactive components including polyphenols, sulfated polysaccharides, sterol, pigments and peptides with beneficial bioactivities such as anti-inflammatory, anti-diabetic, anti-UV and inhibitory effect on hyaluronidase enzyme [26]. Brown algae (Phaeophyceae) synthesize unique phenolic compounds called phlorotannins, through the acetate-malonate pathway. Phlorotannins are polymers of phloroglucinol monomer units (1, 3, 5-trihydroxybenzene) of different size and composition. These predominant polyphenols may reach a high percentage of the algae dry mass (up to 15%) [18].

In the current study, we first extract polyphenols from Tunisian brown algae *Padinapavonica. Padina pavonica* belongs to the class of Phaeophyceae, the order of Dictyotales, the family of Dictyotaceae and the genus Padina [1]. Further, the antioxidant activity of the polyphenol fraction has been evaluated by two different assays. Along with, the anti-inflammatory potential was studied using the carrageen an-induced paw edema in rats.

Material and methods

Reagents and chemicals

Organic solvent and standards (Gallic acid, Phloroglucinol≥99%, Vitamin C, Quercetin and Acetylsalicylate of lysine), Carrageen an, Folin–Ciocalteu phenol reagent, free stable radicals 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Dimethoxy-benzaldehyde (DMBA) were purchased from Sigma Chemical Co (Sigma, St.Louis, MO, USA).

All the organic solvents used in extraction and antioxidant assays are of analytical grade.

Sample collection and preparation of polyphenol fraction

Padinapavonica was collected from the Mediterranean Sea in various area of the coastal region of Tabarka, Tunisia in May 2014, at a depth between 1 and 2 m. The taxonomic identification was confirmed by the National Institute of Marine Sciences and

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Technologies, Salambôo, Tunisia.400 g of finely powdered algae material of *Padina pavonica* were packed in small bags (5x10 cm) of what man filter paper No1. All bags were sealed and soaked in Ethanol in water (30% v/v) bath, steeping for 24hr at room temperature. This was repeated for three times. The 30% ethanol extract was concentrated in a rotating evaporator (Buchi, B-480) at low temperature (<40°C). The crude extract was then lyophilized to obtain powdered extract, which was stored at 2-8°C until use [15].

Total phenolic content (TPC)

The total phenolic content of the polyphenol fraction was determined by the Folin-Ciocalteu method as previously described by Aroa and co-workers [14]. The absorbance was measured at 725 nm using a UV/Vis spectrophotometer (Thermo Scientific™ Evolution 201). A calibration curve of Gallic acid (0.03 to 1 mg/mL) was prepared (y = 0,0014x - 0,0152; R^2 = 0,9981) and TPC was expressed as milligram Gallic acid equivalent per gram of dry material (mg GA/g Dm). All experiments were carried out in triplicate.

Dimethoxy-Benzaldehyde assay for Phlorotannins

For the DMBA assay, the procedure followed the method developed [2]. The absorbance was determined at510 nm using a UV/Vis spectrophotometer (Thermo Scientific™ Evolution 201). Phloroglucinol (PG) was used as a standard at a concentration scale between 0.03 to 1mg/mL and a calibration curve was obtained (y = 0,4968x - 0, 5433 R^2 = 0, 9533).Results were given as microgram of phloroglucinol equivalents per gram of dry material(µg PGE/g Dm).All experiments were performed in triplicate.

Evaluation of the antioxidant activity

DPPH• radical-scavenging assay

The DPPH scavenging assay was performed using to the method described by Goulas and Manganaris [9] with slight modification. Briefly, Different concentrations (0.03-1 mg/mL) of the polyphenol fraction were prepared in distilled water. The reaction started by adding an equal volume of a 2 mM solution of DPPH in methanol, to each tested samples, positive control and blank. 30 minutes after incubation in dark, the absorbance was measured at 517 nm using a UV/Vis spectrophotometer (Thermo Scientific™ Evolution 201).All assays were performed in triplicate. The radical scavenging activity of the tested sample, expressed as percentage of inhibition of DPPH, was calculated according to the formula:

$$\% \text{ DPPH inhibition} = [(\text{AbBlank-Absample/AbBlank}) \times 100] \quad (1)$$

Where Abs Blank is the absorbance of the DPPH solution without any test compounds and the Abs sample is the absorbance of the tested samples or positive control after the reaction takes place.

Quercetin and Ascorbic acid were used as positive control, and results were expressed as half maximal inhibitory concentration (IC_{50}).

Ferric reducing antioxidant power (FRAP)

The ferric reducing power of the polyphenol fraction was studied following the method described by Oyaizu and co-workers [21]. Serial dilutions ranging from 0.03 to 1 mg/mL of the tested samples as well as the standard Gallic acid were prepared in ethanol. 200µL of each concentration were mixed with 500 µL of sodium phosphate buffer (0.2 M, pH=6.6) and 500 µL of potassium ferricyanide (1%, v/v). The mixture was incubated in water bath at 50°C for 20 minutes. Then 500 µL oftrichloro acetic acid (10%, v/v)were added. The mixtures were centrifuged at 3000 rpm for 10 min. A 500 µL of the obtained supernatant was mixed with 500 µL of distilled water and 100 µL of ferric chloride (0.1%, m/v).Samples were incubated in the dark for 30 minutes and the absorbance was measured at 700 nm using a UV/Vis spectrophotometer (Thermo Scientific™ Evolution 201).Three independent assays were performed in triplicate.

The FRAP values were expressed as half maximal inhibitory concentration (IC50). The lower the IC50, the stronger is the antioxidant activity.

Hlila Anti-Inflammatory test using carrageen an-induced paw edema model

Experimental animals

Wistar rats (150–170 g) of both sex and of approximately 6–8 weeks old were procured from the Pasteur Institute (Tunis, Tunisia). Animals were housed in polypropylene cages and maintained under standard conditions (12h light and dark cycles at an ambient temperature of 25 ± 2 °C and 45% RH). Balanced pellet diet and water were supplied ad libitum. All experimental tests were conducted in compliance with standard guidelines of the European Union regarding the Use and the Animal Care (CCE Council 86/609).

Carrageen an-Induced Paw Edema in Rats

The effect of the polyphenol extract on carrageen an-induced paw edema in rat model was evaluated as reported before [15]. The animals were divided into four groups of 6 rats each (n=6). The control group received the vehicle intraperitoneally (2.5 mL/kg of saline solution, *i.p.*). Animals of the control group received the reference drug (Acetyl salicylate of lysine (ASL), 300 mg/kg *i.p.*). The same route with the polyphenol fraction of *Padina pavonica* (EtOH-PAD, 100 and 200 mg/kg) pretreated the test groups. 30 minutes after administration of different substances, each animal received a sub-cutaneous injection of 0.05 mL of freshly prepared carrageen an suspension (1%, w/v in 0.9% saline solution) into the sub-plantar side of the left hind paw. The paw volume was measured using Plethysmometer (model 7150, UgoBasile, Italy) before carrageen an injection (V_0) and 1, 2, 3, 4 and 5 h after injection (V_T).

The percentages of inhibition were then calculated as follows:

$$\%inhibitionedema = \frac{(V_t - V0)Control - (V_t - V0)treated}{(V_t - V0)Control} \times 100 \quad (2)$$

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Where V_t: is the mean edema volume obtained for each group at the indicated t time, after carrageen an injection and Vo: is the mean edema volume obtained for each group at t0before the phlogistic stimulus injection.

Statistical analysis

All experimental results were expressed as mean ± SEM. Data were analyzed by two-way analysis of variance (ANOVA) followed by the post hoc Turkey's test or Fisher's LSD test as appropriate for multiple comparisons. The p values less than 0.05 ($p< 0.05$) were considered statistically significant. Graph Pad Prism (version 6.0 software, CA, USA) was used for all statistical analysis.

Results and Discussion

Extraction yield

The extraction of polyphenols from brown algae involves the use of one or several organic solvent, which can be used alone or in combination with water. In most cases, alcoholic solvents have been the most preferred for the extraction of bioactive compounds from marine algae [27]. However, the extraction method have shown a marked influence on the yield of different preserving compounds [20]. In the actual study, a solution of 30% ethanol was used to extract polyphenols from *Padinapavonica* and a total of 23.2 g was obtained with a yield ratio of 5.42 % of dry material.Similar finding have been reported for the brown algae *Cystoseiracompressa* [15] .Another Tunisian team working in polyphenols from the *Padina pavonica* reported a yield of 9% for the acetonic extract [11].

Phenolic content

Phenolic compounds, including phenols and flavonoids, are secondary metabolites that have an important role in the maintenance of the human body. Phlorotannins and fucoxanthin have been reported to be the major compounds in seaweed extracts [23].

Brown seaweed generally contains higher amounts of polyphenols in comparison with red and green algae [27]. The total phenolic content (TPC) was estimated using Folin-ciocalteu method. As results, the amount of phenolic compound quantified in a *pavonica* was 123.33 mg GAE/ g dry material. Chiboub and co-workers [5] reported that the TPC of the brown algae *Padina pavonica* is closely related to the solvent used and the EtOAc -fraction had significantly higher content of phenolic component (17.4 mgGAE/g of dried algae).A similar study performed with three species belonging to brown algae revealed high values of TPC ranged from 3 to 10% [29]. In addition, algae of the same genus (*Padinaantillarum*) contained 24.30 mg GAE/g of phenolic compounds [19].

Phlorotannins content

Phlorotannins derived from brown seaweeds are polymers of phloroglucinol often extracted in aqueous organic mixture and are generally quantified using colorimetric assays such as DMBA-assay [25]. In general, phlorotannin content varies from 1 to 10% of the algal dry mass [6]. In this study, the phlorotannins content in *Padina pavonica* was determined using the DMBA-assay with a phloroglucinol. *Padina pavonica* collected from the Tunisian coast in July was analyzed earlier and it was reported that it exhibited much more phlorotannins with 56.68 µg PGE/g of the dried algae [2]. This variation confirms the influence of different factors in phlorotannin levels: higher content of phlorotannin seems to be influenced mainly by higher temperatures and light exposure, the solvent used and the extraction method.

The antioxidant activity

Phlorotannins derived from brown seaweed have shown protective effect against free endogenous radicals responsible for oxidation damage and were reported as potent antioxidant agent [13]. Earlierreports on the antioxidant potential of brown algae used different in vitro method. Herein, the ethanolic extract (30%, v/v) of *Padina pavonica* revealed good ferric reducing antioxidant power (FRAP) and DPPH• scavenging activity. EtOH-PAD showed a remarkable dose-dependent free radical scavenging activity and antioxidant effect towards both assay performed [28].

The radical scavenging activity of EtOH-PAD was tested using an ethanolic solution of the stable free radical DPPH•. Fig. 1 & Fig. 2 showed that the radical-scavenging activity of EtOH-PAD, of

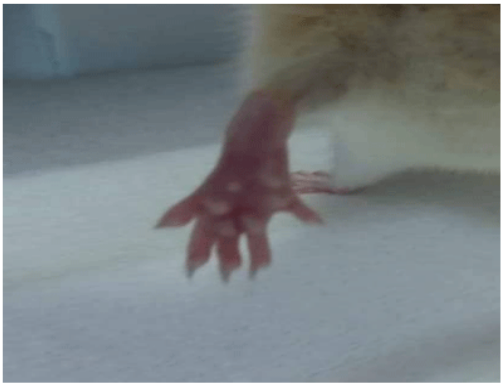

Figure 1: Representative morphology image of the carrageenan-induced paw edema in rats.

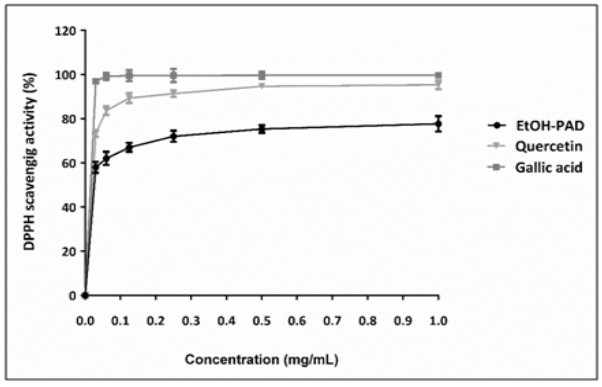

Figure 2: DPPH radical radical-scavenging activity of the polyphenol fraction of Padina pavonica, in comparison to Quercetin and Gallic acid.

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Quercetin and Gallic acid increased in a dose-dependent manner. Interestingly, the 30% ethanolic extract exhibited a strong antioxidant activity at a low concentration (0.25 mg/mL) with an inhibition percent of 72%. The maximum reduction percentage was reached at 1 mg/mL (77.6%) with an IC50 value of 0.026 mg/mL (Table 1). However, standards Quercetin and Gallic acid showed the most important antioxidant activity with 95.33% and 99.66% of inhibition at 1 mg/mL, respectively (fig. 3).In another study, Pinteus and co-workers [22] reported lower values of IC50 value in the acetonic extract of *P.pavonica* (0.338 mg/mL) which can be explained by the difference of the collection season and the geographic location.

Table 1: IC_{50} values of DPPH• radical-scavenging activity and FRAP assay of the polyphenol fraction of *Padina pavonica*, in comparison with the standard Quercetin and Gallic acid.

Sample	DPPH, IC_{50} (mg/mL)	FRAP, IC_{50} (mg/mL)
EtOH-PAD	0.26	0.4
Quercetin	0.02	-
Gallic acid	0.015	0.033

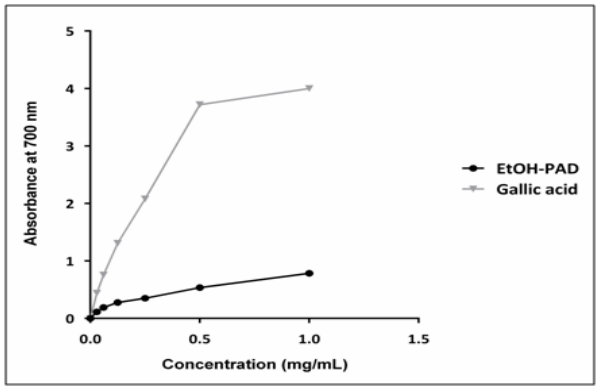

Figure 3: Reductive potential of the polyphenol fraction of *Padina pavonica* and the standard Gallic acid using spectrophotometric detection of Fe^{+3}-Fe^{+2} transformations (FRAP assay).

The FRAP assay was employed to evaluate the ability of EtOH-PAD extract and Gallic acid likewise to reduce Fe+3into Fe^{+2} at concentration ranged from 0.03 to 1 mg/mL. Fig. 3 showed that both polyphenol fraction and Gallic acid exerted a dose-dependent reducing effect. Gallic acid used as standard had the highest reducing power with an IC50value of 0.033 mg/mL followed by the polyphenol fraction (IC_{50} = 0.4 mg/mL).It is worth noting that the antioxidant effect as measured by the FRAP assay is important to evaluate the contribution of different compounds (phenolic and non-phenolic) present in the extract which may have a synergistic interaction that could enhance its potential [10]. This fact suggests that the nature of compounds is more important than the total phenolic contents of extracts from brown seaweeds [4].

Effect of EtOH-PAD on Carrageenan-Induced Rat Paw Edema

Anti-inflammatory activity of the polyphenol fraction of the brown algae Padinapavonica was assessed by carrageen an-induced edema in rat model. Carrageenan-induced inflammation assay is one of the most commonly used assays to assess anti-inflammatory activity of natural products [17]. The percentage of edema inhibition by the polyphenol fraction of Padinapavonica with two different dosesis shown in table 2. All treated groups showed a statistically significant inhibition of inflammatory activity in comparison to control group ($p<0.05$). Intraperitoneal administration of EtOH-PAD reduced the edema volume during the entire period of observation when compared to the carrageen an-treated group (control). In addition, EtOH-PAD dose-dependently reduce the paw edema with a maximum effect three hours following the sub plantar injection of carrageen an. As shown in Table 2, a significant inhibition of edema was obtained at the dose of 100 and 200mg/kg by reducing the edema by 53.48 % and 586%, respectively. This effect was higher than that of the reference drug LSA with an inhibition of edema that does not exceeded 45.73 %.Several studies have also reported that organic fractions purified from the brown algae *Cystoseiracompressa* exhibited anti-inflammatory activity [15].

Table 2: Effect of the intraperitoneal administration of polyphenol fraction from *Padina pavonica* on carrageenan-induced paw edema in rats in comparison with the reference drug Acetyl salicylate of lysine (ASL)

Sample	Dose(mg/kg)	Edema volume (10^{-2}mL)			Edema inhibition (%)		
		1 h	3 h	5 h	1 h	3 h	5 h
Control	-	36.5±1.2	64.5±1.3	75±2	-	-	-
ASL	300	24.5±1.8*	35±1.5**	39±1.4**	32.87	45.73	48
EtOH-PAD	100	19±1.6**	30±2.4**	38.5±1.3**	47.94	53.48	48.66
	200	16.5±2.5**	27±1.8**	31±1.6**	54.79	58.6	58.53

Data are expressed as mean ±SD; n=6 animals. * $p<0.01$, ** $p<0.001$ when compared to the carrageenan treated group

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Carrageenan is a phlogogen agent that can produce an acute inflammation resulting from the sequential action of several mediators mainly Histamine, serotonin, kin in and prostaglandins [7]. Acute inflammation is also accompanied by an early release of pro-inflammatory cytokine in the hind paw which such as TNF-α and IL-6. IL-6 in turn promotes the release of the cyclooxygenase involved in the arachidonic acid (AA)cascade and AA is converted into prostaglandins PGE2 [16]. Basing on earlier reports, the anti-inflammatory effect of brown seaweeds may be due to the inhibition of inflammatory mediator's (NO, iNOS, COX-2, and PGE2) production in a dose-dependent manner [24]. This interesting effect observed with *P.pavonica* could be due to the high amount of antioxidant compounds present in the ethanolic extract.

Conclusion

The present study aimed to valorize natural resources present in the coastline of Tunisia. The polyphenol fraction of *Padina pavonica* exhibited a significant anti-inflammatory activity associated with antioxidant potential. These activities are likely related to the presence active substances including phenolic and non-phenolic compounds acting synergistically. Further investigations will focus on other valuable pharmacological effect and on their molecular mechanism in order to be exploited as active ingredients for preparation of nutraceutical, cosmeceutical and pharmaceutical products.

Acknowledgments

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