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EVALUATION OF THE INHIBITORY ACTIVITY OF BROWN ALGAE EXTRACTS, PADINA PAVONICA (L.), HARVESTED ON TUNISIAN COASTS

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ABSTRACT

The objective of the present work is to highlight the antibacterial activity of the four extracts of a marine algae species: *Padina pavonica*, from the coast of the Daroufa Tunisia beach. this alga has high levels of total polyphenols (51,912 ± 3,261 powder). Screening of the in vitro antibacterial activity of *Padina pavonica* extracts harvested on the Mediterranean coast of Cap Bon, Tunisia, was carried out on five strains of bacteria: *Salmonella enterica*, *Escherichia coli*, *Bacillus subtilis*, *Micrococcus luteus* and *Staphylococcus aureus*. The antimicrobial activity was evaluated by the cellulose disk diffusion method. The results revealed that the total extract of *Padina pavonica* exhibits significant activity against *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus* and *Escherichia coli* whereas no activity was recorded against *Salmonella enterica ssp.* The antibacterial activity of the Methanolic extract of the brown alga *Padina pavonica* shows that most of the bacterial strains tested are sensitive, the largest diameter of inhibition (22.4 ± 0.16 mm) vis-à-vis *Bacillus subtilis* at a concentration of 3 mg/ml. Modest antibacterial activity was noted for the same extract against the strain *Micrococcus luteus* and the lowest diameter of inhibition was detected against the strain *Salmonella enterica ssp*.

KEYWORDS: Brown algae, *Padina pavonica* (L), antioxidant activity, antibacterial activity, Tunisian coast.

1. INTRODUCTION

The marine environment and the organisms that inhabit it constitute an infinite source of active molecules with an original chemical structure.[1] Seaweed is an important source of bioactive natural substances. They have been used as food products in Asian food for centuries because they contain carotenoids, dietary fiber, proteins, essential fatty acids, vitamins and minerals.[2] These compounds are synthesized by metabolic pathways different from those observed in terrestrial environments. Among marine organisms, algae, which are most often attached to a substrate, develop chemical defenses to prevent colonization by other species, including microorganisms.[3] Algae are much less known plants than terrestrial plants and much more difficult to apprehend. They occupy a large part of the aquatic environment, in particular marine and submarine, and constitute a set of extremely diverse organisms which are very difficult to unambiguously present.[4] The use of seaweed for therapeutic purposes is far from a new phenomenon. If the active ingredients extracted from algae used in pharmacy are few, the scientific work in progress is important. Thousands of molecules have been identified. They are polysaccharides, lipids or small metabolites of a phenolic[5] or terpenic nature.[6]

Algae and their extracts are of great interest in the pharmaceutical industry.[7] Many substances obtained from marine algae such as alginate, carrageenan and agar as phycocolloids have been used for decades in medicine and pharmacy.[8] Seaweed extracts have been widely studied by several researchers.[9-10] Recently, algae have received a lot of attention for their natural antioxidant potential and most of their compounds have shown activities antibacterial.[11,12] antifungal, anti-aging, dietary, anti-inflammatory, cytotoxic, antimalarial, antiproliferative and anticancer properties.[13,14] Increasing the resistance of microorganisms to the antimicrobial agents used is due to the improper and inappropriate use of antibiotics, and this poses very serious problems. Diseases caused by microorganisms are increasingly difficult to treat with existing drugs.[15,16] Thus, scientists have turned to the search for new ways, including plants that have always been a source of bioactive compounds of natural origin.[17] The antimicrobial activity of seaweed has been demonstrated and is considered an indicator of their ability to synthesize bioactive secondary metabolites.[18] For this reason we are interested in studying the antibacterial activity of the brown alga *Padina pavonica*, harvested on the Nabeul coasts.

2. EXPERIMENTAL

2.1. MATERIALS AND EQUIPMENTS

Melting points were determined in a capillary tube and are uncorrected. Thin layer chromatography (TLC) was performed on precoated silica gel plates (0.25 mm, Merck). Column chromatography was performed on Merck silica gel having size (0.063-0.200 mm). ascorbic acid, BHT, DPPH. All reagents and solvents were of reagent grade quality and were obtained from commercial suppliers. Solvents were dried and purified according to standard procedure. All other materials were purchased from Aldrich and Enamine Ltd. UV-Visible absorption spectra were recorded on Cary 2300 spectro-photometer. FT-IR: Infrared (IR) spectra were recorded on a Perkin-Elmer 65 FT-IR (ATR) instrument.

2.2. Harvest of seaweed

The seaweed Padina pavonica was harvested in June 2017 on the coast of Daroufa (36º29'N-10º49'E) at Nabeul in Tunisia. The fresh material was rinsed 3 times with fresh water, then weighed and dried for 72 hours in an oven at 40ºC. After drying, the seaweed is crushed using a blender and placed in a sterile bottle and stored in a refrigerator at a temperature of 5 ºC. Brown algae with a thallus blade or thin ribbon and often membranous.

Reign: Chromista
Division: Phaeophyta
Class: Phaeophyceae
Order: Dictyotale
Family: Dictyotaceae
Genre: Padina

Fig. 1: Photograph of seaweed specie harvested Padina pavonica.

2-2. Preparation of seaweed extracts

The total extract is obtained by percolation, adding 1 g of powder to 30 ml of methanol. The mixture obtained is then subjected to continuous stirring in the absence of light for 48 hours at room temperature, then filtration on Wattman No. 1 paper and then evaporation under reduced pressure with a rotary evaporator. five other extracts are obtained by successive extraction in petroleum ether, chloroform, ethanol and then in methanol according to the following protocol: 2 g of seaweed powder is placed in a soxhlet, then the flask is filled with 150 ml of petroleum ether, the mixture is boiled for one hour, the mass of the residue obtained after evaporation of the solvent is extracted successively with 150 ml of chloroform, with 150 ml of ethanol and then with ml of methanol. The total methanol extract were separately concentrated under reduced pressure at a temperature not exceeding 35 °C. The five extracts obtained are placed in the desiccators, before being stored in a dry place for later testing. The yield of the extract was measured and stored at -20°C.

2.3. Evaluation of antioxidant activity

2.3. 1.Pigging of the free radical DPPH (2,2-diphenyl-1-picrylhydrazyl) Principle

DPPH (2,2-Diphenyl-1-picrylhydrazyl) is a stable free radical which absorbs between 512 and 517 nm. In the presence of anti-radical compounds, the purple DPPH is reduced to 2,2 yellow diphenyl-1-picryl hydrazine.[19] The absorbance measured at 517 nm is used to calculate the percentage of inhibition of the DPPH radical, which is proportional to the antiradical power of the sample.[20]

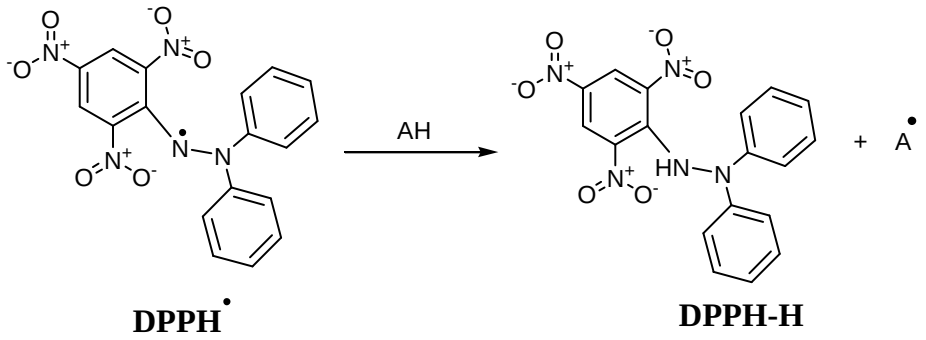

Fig.2. Free and reduced form of DPPH

The antioxidant activity of Padina pavonica extract was determined using the DPPH free radical scavenging assay according to the method described by.[21] The assay was carried out in triplicate and the mean value was recorded. Freshly prepared (0.004%w/v) methanol solution of 2,2- diphenyl-1-picrylhydrazyl (DPPH) radical was prepared and stored at 10 °C in the dark. A methanol solution of the test compound was prepared. A 40 mL aliquot of the methanol solution was added to 3 ml of DPPH solution, under light protection. Absorbance measurements were recorded immediately with a UV–visible spectrophotometer. The decrease in absorbance at 515 nm was determined continuously, with data being recorded at 1 min intervals until the absorbance stabilized (16 min). The absorbance of the DPPH radical without antioxidant (control) and the reference compound ascorbic acid were also measured. The percentage inhibition (PI) (scavenging activity) of the DPPH radical was calculated according to the formula.[22]

$$PI(\%) = \frac{AC - AT}{AC} \times 100$$

where AC = Absorbance of the control at t = 0 min and AT = absorbance of the sample + DPPH at t = 16 min

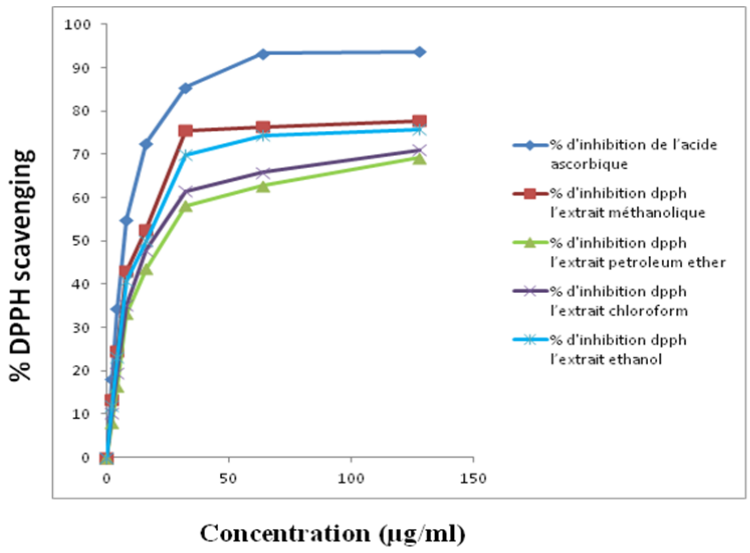

Fig.3. The scavenging activity of DPPH radicals of Padina pavonica.

Free radicals are involved in many diseases including cancer, heart disease, atherosclerosis and neurodegenerative diseases. The trapping activity of antioxidants is very useful for controlling these diseases. The DPPH test is the most commonly used method for screening for antioxidant activity and is a sensitive method for determining the antioxidant activity of

various plant extracts, fungi or algae.[23] The methanolic extract of Padina pavonica showed higher DPPH radical scavenging activity in a concentration-dependent manner (Fig.3). The maximum trapping activity (77.73%) for the methanolic extract followed by the ethanolic extract (75.77%), followed by the chloroform extract with (71.0%) and the latter was the Petroleum ether extract (69.2%) but all extracts were lower compared to the activity of the standard ascorbic acid (93.73%) at a concentration of 128 µg / ml.

2.4. Microorganisms and growth conditions

2.4.1 *Bacterial Strains*

Gram-positive bacteria

- *Micrococcus luteus* CIP5345
- *Staphylococcus aureus Subsp* CIP 4.83
- *Bacillus subtilis* CIP 5262

Gram-negative bacteria

- *Salmonella enterica ssp.* CIP 8039
- *Escherichia coli* CIP 54127

The strains were used as indicator microorganisms for antimicrobials activities tests.

The bacterial strains used as indicator microorganisms were cultured Overnight in Tryptone-Soy Broth (TSB) under aerobic conditions and constant shaking (200 rpm) at 30° C for *Micrococcus luteus* CIP5345, *Bacillus subtilis* s CIP 5262, and at 37 ° C for *Staphylococcus aureus Subsp* CIP4.83, *Salmonella enterica ssp.* CIP 8039 and *Escherichia coli* CIP54127, then 1: 100 diluted in LB medium and incubated for 5 h under constant agility (200 rpm) at the appropriate temperature.

2.4.2. Dissemination method of Agar Well

The agar disk diffusion method was used to determine the effect of an antimicrobial effect, it is also referred to as an agar dilution technique for evaluating the antimicrobial activities of seaweed extracts at different concentrations in DMSO ranging from 1mg/ml to 5 mg / ml and 20µl were added to the discs. The synthetic compounds can diffuse into medium agar "LB" agar for bacterial strains test. Sterile discs were placed in each of the plates which were uniformly sown on the surface using sterile glass beads or buffers and a bacterial suspension of 10^8 CFU / ml. Petri dishes are incubated at the appropriate temperature for each strain of

bacteria. After 24 hours of incubation, the resulting inhibition zones (including the diameter of the disc) will be uniformly circular because there will be a confluent growth curve. The antimicrobial activities were tested by measuring the inhibition diameters in millimeters.

2.5. Statistical analysis

Statistical study carried out by the statistical software SPSS V 19.0. All experiments were performed in triplicate, the results are expressed as mean ± SD. The results are analyzed using the one-way variance "ANOVA" and the multiple comparisons between the groups were used using the Newman-Keuls multiple comparison test at the 5% threshold and $P < 0.05$ was considered statistically significant that $P < 0.01$ was very significant.

3. RESULTS AND DISCUSSION

3.1. Yield in crude extract

The extraction of secondary compounds after 48 hours of maceration from the brown alga Padina pavonica enabled us to calculate the yield of the extract which is expressed as a percentage per 100 g of ground dry algae. The average of the results obtained after different extractions is evaluated at (10.2%).

According to Michel et al. (2012)[24], the yield of extractions by solvents of increasing polarity depends on the nature of the solvent used and the chemical properties of the molecules to be extracted.

As well, the extraction method (maceration, decoction, infusion) also plays an important role in the determination of the yield as well as the chemical composition of the prepared extracts.[25]

3.2. Determination of phenolic compounds

The results obtained from the different assays are expressed in mg of gallic acid (mg EAG) for total phenols and total tannins and in mg equivalent of quercitin (mg EQ) for flavonoids per mg of extract, using the equations linear regression of calibration curves (Fig. 4 and 5). The results are shown in Table 01.

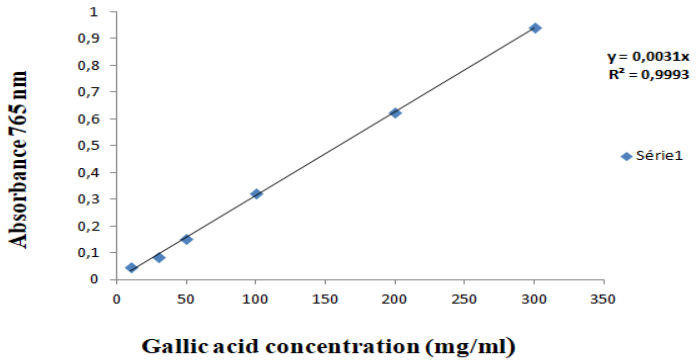

Fig.4. Gallic acid calibration curve for the determination of total phenols

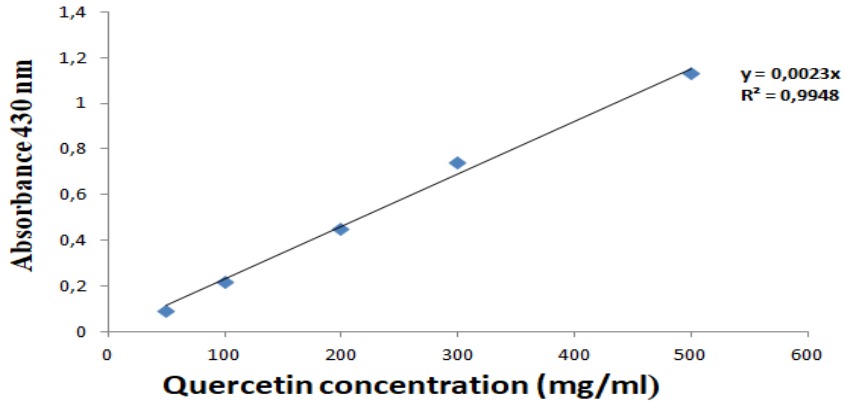

Fig.5. Quercetin calibration curve for flavonoid assay

Tabel 01: Total phenols, total tannins and flavonoid content of the crude extract of Padina pavonica.

	Total phenols (mg EAG/mg extract)	Total tannins (mg EAG/mg extract)	Flavonoids (mg EQ/mg extract)
Raw extract	51,912 ± 3,261	20,274 ± 1,97	0,823 ± 0,064

The results are expressed as the mean value ± standard deviation of three repetitions. The determination of the polyphenols was carried out using the Folin-Ciocalteu reagent. Despite the sensitivity and simplicity of this method which is widely used, it is not specific to polyphenols. Indeed, the reagent can react with proteins, sugars, ascorbic acid and sulfur compounds, which can influence the results obtained.[26]

As far as our study is concerned, the analysis of phenolic compounds shows that the methanolic extract obtained from the brown alga Padina pavonica has a high total phenols content of (50.966 ± 3,261 mg EAG / mg extracted). According to the literature, the

methanolic extracts are the richest in phenolic compounds, thus the methanol remains the best solvent to extract these compounds, this affinity is supported by several works.[27]

This is due to the ability of methanol to inhibit the action of polyphenol oxidase which causes the oxidation of polyphenols in plant tissues.[28]

The total tannin content recorded in this study is (20,274 mg EAG / mg extract). This result must be taken with moderation, because the method of determination used can influence the result obtained. Moreover, it is reported that in marine algae, there is a specific class of polyphenols called phlorotanins, the latter have a less complex structure than that of terrestrial tannins and are represented by polymers of phloroglucinol (1,3,5-trihydroxybenzene). They can constitute up to 15% of the dry weight of brown algae.

The flavonoid content recorded in this study is low (0.823 mg EQ / mg extract). This result is in agreement with other works. According to the literature, there is little work on the content of flavonoids in marine algae[29,30], it is reported that brown algae contain levels ranging from (20.72 to 32.89 mg / g DM).

3.3. Antimicrobial activity

The antibacterial activities of *Padina pavonica* methanolic extract were determined using well-diffusion method. All the investigated algal extracts showed antibacterial activities (Tables 3).

The antibacterial activity of *Padina pavonica* revealed that the highest activities; 22.40 ±0.16 mm and 20.70 ± 0.44 mm were obtained against *Bacillus subtilis* by Methanolic extract and ethanolic extract respectivly (Table 3). The extract of *Padina pavonica* revealed significant antibacterial activity against *Staphylococcus aureus* (17.3 ±0.5 mm; 3 mg/ml), with raw extract *Micrococcus luteus* (15.5 ±0.28 mm; 3 mg/ml), *Bacillus subtilis* (22.4 ± 0.16 mm; 3 mg/ml) with methanolic extract, *Salmonella enterica ssp.* (11.5 ± 0.16 mm; 3mg/ml) petrolum ether extract and *Escherchia coli* The highest antibacterial activity obtained by methanolic extract at 3mg/ml (12.6 ± 0.16 mm; 3mg/ml).

Table 2. Antibacterial activity of and standard antibiotics.

	Inhibition zone (mm)	
	Spiramycin	Streptomycin
Bacterial strains/concentration µg/ml	**500**	**500**
Micrococcus luteus	26,33±1,25**d**	36,17±0,7 **b**
Staphylococcus aureus Subsp	18,17±0.57 **e**	36,00±0.5 **b**
Bacillus subtilis	24,83±0.76 c	37,33±1.25 **a**
Salmonella enterica ssp.	23,67±0.76 d	31,17±1.04 **b**
Escherichia coli	18,67±0.76d	29,33±1.04 **b**

The values (averages of three repetitions) followed by the same lowercase letter horizontally or the same capital letter vertically are not significantly different according to the Newman-Keuls multiple comparison test at the 5% threshold.

Table 3: Inhibition diameter obtained for the different extracts of the alga *Padina pavonica* vis-à-vis the different strains used.

	Inhibition zone (mm)														
	raw extract			Methanol			Ethanol			DEE			Chloroforme		
Strains/ concentration(mg/ml)	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Micrococcus luteus	6,8 ±0,16g BC	10,2±0,16f	15,5±0,28e	7,5±0,16d	9,3±0,44c	14,1±0,16b	6,5±0,44a	7,9±0,28e	10,3±0,5c	ND	ND	ND	6,8±0,16f	9,4±0,16d	12,3±0,16b
Staphylococcus aureus Subsp	9,3±0,33g A	14,7±0,33f	17,3±0,5 e	8,0±0,33 f	11,3±0,28b	16,2±0,57e	7,2±0,33f	9,8±0,16 f	13,2±0,28 c	6,5±0,16 f	8,1±0,28b	10,4±0,33d	6,9±0,33 f	8,3±0,5 e	11,9±0,33 b
Bacillus subtilis	8,8±0,16 g A	14,5±0,16 f	18,4±0,28e	10,6±0,16 d	15,2±0,44d	22,4±0,16b	8,1±0,16f	12,9±0,16 f	20,7±0,44c	7,0±0,33f	9,6±0,16f	13,5±0,28b	7,9±0,33 f	11,5±0,16f	17,8±0,26 b
Salmonella enterica ssp.	ND	ND	ND	6,4±0,57 d	8,3±0,16 a	10,7±0,16d	ND	ND	ND	6,9±0,44d	9,7±0,28e	11,5±0,16b	ND	ND	ND
Escherichia coli	7,5±0,16 g C	9,1±0,33 b	11,3±0,5c	7,7±0,33d	10,8±0,44a	12,6±0,16c	6,5±0,28e	8,3±0,28e	11,1±0,16c	ND	ND	ND	ND	ND	ND

The values (averages of three repetitions) followed by the same lowercase letter horizontally or the same capital letter vertically are not significantly different according to the Newman-Keuls multiple comparison test at the 5% threshold.

The results of the antibacterial tests show that the extract of *Padina Pavonica* harvested during the month of June 2017 has an antibacterial activity. This activity is shown against gram-negative bacteria (*Escherichia coli* CIP 54127, *Salmonella enterica subsp.*) and Gram-positive bacteria (*Bacillus subtilis* CIP 5262, *Micrococcus luteus* CIP5345, *Staphylococcus aureus Subsp* CIP 4.83). In contrast, activity is absent against gram-positive bacterium (*Micrococcus luteus*), with petroleum ether extract (Table 03).

The inhibition of *Padina Pavonica* extracts remains considerably lower compared to that of commercial antibiotics used as standards in this study (Streptomycin, spiramicin) (Table 2 & 3).

According to the results presented in Table 1 the extracts of the alga *Padina Pavonica* has no activity against *Escherchia coli* with chloroform and petroleum ether, and a weak zone of inhibition against *Escherichia coli* in the presence of the dry extract, ethanolic and methanolic. This result indicates a moderate sensitivity of this bacterium to the extract. In addition, we notice a sensitivity of *Escherichia coli* towards the antibiotic used as standard.

According to the results presented in tableau 3 we notice that the dry extract of *Padina pavonia* presents an absence of zone of inhibition tell the stump *Bacillus subtilis* with the studied concentrations, on the other hand we notice that the methanolic extract has a rather considerable antibacterial effect against the stump tested with a zone of inhibition of 22,4 mm in 3 mg / ml, followed by the methanolic extract then the extract in the chloroform or we obtained a zone of inhibition of 17,8 in 3 mg / ml and in last position it was the extract of the petroleum ether or we obtained the low activity against *Bacillus subtilis*.

Both antibiotics: Streptomycin, spiramicin exert a greater inhibitory effect on *Bacillus subtilis* unlike extracts with which the strain is less sensitive.

It is found that the methanolic extract and the crude extract have no inhibitory effect on the strain tested Salmonella enterica ssp., No inhibition zone was observed with the conentrations studied. Both antibiotics have a considerable inhibitory effect against *Salmonella enterica ssp.*, This bacterium is potentially resistant to the cloroform extract or has no inhibition zone. The best activities observed against this strain are with methanolic extract and petroleum ether, respectively 10.7 and 11.5 mm.

The algae are the subject of numerous studies throughout the world nevertheless, the data presented in this modest study constitute the first results of the brown seaweed *Padina pavonica* of this Tunisian coast.

Kayalvizhi and al. (2012)[31] tested the antibacterial activity of the brown alga *Padina boergesenii* with a maceration of three days using acetone as extraction solvent, they obtained weak zones of inhibition (7mm) vis-à-vis *P. aeruginosa* and *E. coli* comparing to our results where a higher inhibitory zone is recorded for *Stapylococcus aureus Subsp.* (16.2mm) and comparable activity against E. coli. or an area of 12.6 mm was detected with the methanol extract, while for Kayalvizhi and al. (2012)[31] where he found 10 mm as zone of inhibition. Omar and al. (2012)[32] observed a 20mm inhibition zone with respect to *B. subtilis* and

24mm against MRSA for the methanolic extract of *Padina pavonica*, these zones of inhibition are superior to those we obtained with all our extracts test except the extract of methanol or our results are slightly higher with 22.4 mm at 3mg / ml.

4. CONCLUSION

The present work is moving towards a valorization channel very little exploited in Tunisia. It aims to study the natural potential of marine green algae, which is an interesting and very promising source of biologically active substances.

The research undertaken includes a phytochemical study and an evaluation of the antioxidant and antimicrobial activities of the crude extract of the brown alga *Padina pavonica* collected on the Tunisian coast.

The results obtained after different extractions enabled us to calculate the yield of crude extract evaluated at (10.2%). The quantitative determination of the main classes of secondary metabolites resulted in a high total phenol content of (51.912 ± 3.261 mg EAG / mg extract), a mean total tannin content of (20.274 mg EAG / mg extract) and a total low in flavonoids (0.823 mg EQ / mg extract).

Several studies conducted by Sarah Saleh Abdu-llah Al-Saif and al. (2013)[33] showed chloroform extract activity of brown algae on one or more bacteria. Nevertheless, these results are difficult to compare since the methods used are different. The choice of the extraction protocol and more particularly that of the solvent is very important.[34] In addition to other factors such as the algal species, the bacterial strain, seaweed conditioning[35], harvest season[36] can also influence the results of antibacterial tests. In addition, it would be interesting to be able to conduct a monthly antibacterial screening to determine at what time of year the harvest of the seaweed is the most advantageous and secondly to achieve the fractionation, purification and characterization of active compounds with a view to promoting these natural compounds.

5. ACKNOWLEDGMENTS

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