SEASONAL VARIATION OF ANTIBACTERIAL ACTIVITY OF THE BROWN ALGA *PADINA PAVONICA* (L) THIVY COLLECTED FROM NORTHERN COAST OF TUNISIA

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ملخص

التقلبات الموسمية للنشاط المضاد للجراثيم للطحلب البني بادينا بافونيكا من الساحل الشمالي لتونس : تم جمع الطحلب البني *Padina pavonica*، شهريا من الساحل الشمالي لتونس (رأس زبيب)،من شهر جويلية 2006 إلى غاية جوان 2007 و ذلك لتقييم التغيرات في نشاط المضاد البكتيري للمستخلص الخام لهذا الطحلب. و قد تم اختبار المستخلص باستعمال المواد المذيبة : ثاني كلوروميتان و ثاني كلوروميتان /ميتانول على مجموعة من البكتيريا المتلفة و مجموعة البكتيريا المعزولة من الطحلب نفسه. و قد أثبتت النتائج وجود اختلاف كبير في نشاط المضاد البكتيري للمستخلص مع المذيب العضوي المستعمل، حيث أن المستخلص الطحلبي بثاني كلوروميتان /ميتانول كان الأكثر فاعلية. كما أثبتنا وجود تباين موسمي لنشاط المضاد البكتيري حيث أن المستخلص الطحلبي لفصل الصيف كان الأكثر فاعلية على البكتيريا غير أن المستخلصات الطحلبية لفترة أكتوبر ونوفمبر وديسمبر لم تظهر أي نشاط ضد كافة البكتيريا المختبرة . كما أثبتنا أن مجموعة المستخلصات ذات الفاعلية على البكتيريا المتلفة ليس لها أي فاعلية أو ذات فاعلية ضعيفة جدا على البكتيريا الطحلبية. تؤكد هذه النتائج إمكانية استخدام الطحلب البني *Padina pavonica* كمصدر للمركبات المضادة للبكتيريا ويعتبر فصل الصيف أفضل فترة لجمع الطحلب لهذا الغرض.

الكلمات المفاتيح: الطحلب البني، بادينا بافونيكا، نشاط المضاد البكتيري، التباين الموسمي، البكتيريا المتلفة ، البكتيريا اللاصقة

RÉSUMÉ

variabilite saisonniere de l'activite antibacterienne de l'algue brune *padina pavonica* (l) thivy recoltee de la cote nord de la tunisie : L'algue brune *Padina pavonica* a été récoltée durant une période d'une année (Juillet 2006 à Juin 2007), dans la côte nord de la Tunisie (Cap Zebib) en vue d'évaluer l'activité antibactérienne d'extraits bruts de l'algue et leur variabilité saisonnière. Les extraits d'algue ont été préparés en utilisant le Dichlorométhane et le Dichlorométhane/Méthanol et testés périodiquement (tous les mois) sur une gamme de bactéries pathogènes et des bactéries associées isolées de l'algue. Une variation significative de l'activité antibactérienne liée au solvant d'extraction utilisé a été notée. Ainsi, les extraits au Dichloromethane/Méthanol sont plus actifs. Aussi nous avons noté la présence d'une variabilité saisonnière de l'activité antibactérienne. Ce sont les extraits d'algue estivaux qui ont montré le plus large spectre d'activité contre le panel de bactéries pathogènes testées. Les extraits d'algues collectées au printemps sont actifs en particulier sur les bactéries à Gram+ alors qu'aucune activité n'a été observée pour les algues collectées en Octobre, Novembre et Décembre. Aussi, les extraits d'algue sont inactifs ou à faible activité sur les bactéries associées et isolées de *Padina*. Il en découle de cette étude la possibilité d'utiliser *Padina pavonica* comme source de composés antibactériens notamment lorsque récoltée en période estivale.

Mots clés : *Padina pavonica*, Activité antibactérienne, Variabilité saisonnière, Bactéries pathogènes, Bactéries-associées.

ABSTRACT

Variations in antibacterial activities of the brown alga *Padina pavonica* collected from the Northern coast of Tunisia: Cap Zebib, were evaluated monthly during one year period (from July 2006 to June 2007). Dichloromethane and dichloromethane/methanol crude extracts of the alga were screened against a panel of pathogenic bacteria and *Padina* surface-associated bacteria. Crude extracts of the alga collected in summer showed the largest spectrum of activity against both Gram+ and Gram- pathogens whereas extracts collected in spring exhibited a varying amount of activity especially against Gram+ bacteria whereas and those collected in winter did not exhibit any activity against tested bacteria. From the two organic solvents used for alga extraction, the most suitable was the dichloromethane /methanol with better effectiveness on bacteria. Effect of the alga extracts, were very weak against associated bacteria. This selective toxicity may be important for theses epibionts since, it should maintains a chemical defense against microbial pathogens. The present results highlight the possible use of *Padina pavonica* as source of antibacterial compounds mainly provided in warm seasons from the alga.

Keywords: *Antibacterial activity, Padina pavonica, Algal extracts, Pathogenic bacteria, Epibiotic bacteria.*

INTRODUCTION

In marine ecosystems, several organisms produce bioactive metabolites in response to ecological pressures such as competition for space, feeding and reproduction (König *et al.,* 1994; Shanmughapriya *et al.* 2008). Within these groups, macroalgae were listed containing broad range of biological activities with rich pharmacological potential (Ballesteros *et al.,* 1992; Bhosale *et al.,* 2002; Rodrıguez-Bernaldo de Quiros 2010). Extracts of marine algae were reported to exhibit antibacterial activity (Sachithananthan and Sivapalan, 1975; Mahasneh *et al.,* 1995; Siddhanta *et al.,* 1997; Paul and Puglisi, 2004). Secondary metabolites with antibacterial properties in algae may function as active defense mechanisms against epiphyte in marine environment, and to maintain the capacity to recover and regenerate rapidly after predator or abrasion damage (Pesando, 1990; Vlachos *et al.* 1997).

Earlier studies revealed intraspecific variability of antibacterial activity within the same algal specie due to variation in ecology, growth or sexual maturity (Pratt *et al.,* 1951; Chesters and Stott, 1956; Burkholder *et al.,* 1960; Pesando, 1990). Recently, influence of geographical zone, sampling season, algal generation and sample preparation methods on antimicrobial activity were highlighted (Salvador *et al.,* 2007). Nevertheless, such studies are still scarce.

Padina pavonica is a brown alga belonging to Phaeophyceae class, growing abundantly in the Mediterranean Sea. This alga is mainly abundant from June to September.

Previous studies reported the biological potentialities of *P. pavonica* (Ktari *et al.,* 2001; Shanmugam and Mody, 2000 and Kamenarska *et al.,* 2002). Chemical composition of this alga was studied by Kanias *et al.* (1992) and Kamenarska *et al.* (2002). Pigments were investigated in *P. pavonica* by Khafaji (1986) and Cowan (1999). *P. pavonica* collected from Tunisian coast was investigated for its cytotoxic activity (Ktari *et al.,* 1999), allelopathic and antifungal potentialities by Omezzine *et al.* (2009), abiotic and biotic factors were carried out for *P. pavonica* collected from Cap Zebib by Ben Said *et al.* (2002) and the natural biomass available was evaluated by Ksouri *et al.* (2008). The purpose of the present study was to evaluate efficacy of the brown alga *P. pavonica* collected from the rocky shore of Cap Zebib for producing antibacterial activity against pathogenic and surface-associated bacteria and study the influence of seasonal variations on antibacterial substances production.

MATERIALS AND METHODS

Sampling

Algal material was collected in shallow water: the rocky shore of Cap Zebib from northern coast of Tunisia (N 37° 16.2', E 10° 3.6') from July 2006 to June 2007. Once harvested, seaweeds were placed on ice for transportation to the laboratory. Previous to analysis, samples were washed twice with sterile seawater and fresh water to remove salts, epiphytes, sand particles and other matter, and dried in shade in air stream. Once dried, they were stored in dark until extraction. Voucher specimens were conserved monthly in formol 3%.

Preparation of seaweeds extracts

About 20g of dried and powdered alga were extracted consecutively with two organic solvents with increasing polarity: dichloromethane (D) and dichloromethane/methanol (D/M) (1:1 v/v). Each extraction was carried out three times by maceration for 24 h at room temperature. These extracts were pooled, filtered, and concentrated under reduced pressure in a rotary evaporator apparatus. Extracts were stored at -20°C until use.

Test strains and growth conditions

In vitro antibacterial susceptibility tests were performed using a panel of strains including fish pathogens: *Aeromonas hydrophila, Pseudomonas cepacia, Vibrio anguillarum, Vibrio tapetis* CECT 4600, *and Aeromonas salmonicida*, clinical pathogens: *Salmonella typhymurium, Streptococcus sp, Staphylococcus aureus, Vibrio alginoliticus,* reference human pathogens: *Escherichia coli* O126 :B16, *Escherichia coli* ATCC 25922, *Pseudomonas fluorescens* AH2, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Eenterococcus feacalis* ATCC 29212 and eight *P. pavonica* surface-associated bacteria (P1-P8). Sample of each bacterium was grown in T. soy broth (TSB) at appropriate temperature for human and fish pathogens and at 20°C for alga-associated bacteria and maintained on T. soy agar plates (TSA) at 4°C.

Isolation of alga-associated bacteria

Seaweed surfaces were washed three times with autoclaved seawater, so that only bacteria with a strong affinity for the host were sampled (Burgess, 2003). Epibiotic bacteria were then removed from the seaweed surface by vortexing 10g of the alga in 90 ml autoclaved seawater for 6 min; bacteria were isolated by serial dilution up to 10^{-3} using autoclaved seawater. A volume of 100 µl from each dilution was spread-plated in triplicate on Marine agar (MA). The plates were incubated at 20°C for at least 7days (Lemos *et al.,* 1985). Morphologically distinct bacterial colonies were selected and isolated in pure representative colonies. The strains were stored at −80°C Marine broth medium supplemented with 20% glycerol.

Antibacterial test

The antibacterial assays were evaluated by using standard paper disc method (Casida 1986). Briefly, 500 µg of each crude extract dissolved in appropriate solvent (10 µl) was applied to sterile filter paper discs

(6 mm). After solvent evaporation, discs were placed on TSA plates, inoculated with 18 h cultured of the tested pathogen (10^6 bacteria /ml) in TSB. As control, a disc loaded with solvent was simultaneously prepared. Plates were incubated overnight at appropriate temperature. The diameter (mm) of growth inhibition halo was measured after 24h incubation. Assays were carried out in triplicate.

RESULTS

Extracts of *P. pavonica* harvested monthly in Cap Zebib, were tested for their antibacterial activities. Results on the antibacterial screening assays are summarized respectively in table I and II.

Table I: Seasonal variation of antibacterial activity of *Padina pavonica* crude extracts (500µg/disc) against pathogenic bacteria (inhibition zone was measured with paper disc)

	Dichloromethane										Dichloromethane/Methanol									
Test strains	Jul	Aug	Sep	Oct	Nov	Dec	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Mar	Apr	May	Jun
E.coli O126B16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E.coli ATCC 25922	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
V. tapetis CECT4600	+	++	+	-	-	-	-	-	-	+	++	++	+++	-	-	-	-	-	-	+
V. anguillarum	+	++	++	-	-	-	-	+	-	-	++	++	++	-	-	-	-	+	-	-
V. alginoliticus	+	+	+	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-
Ps. cepacia	++	++	++	-	-	-	-	-	-	+	++	++	+++	-	-	-	-	-	-	+
Ps. fluorescens AH2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ps. Aeruginosa ATCC 27853	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. salmonicida	++	++	+	-	-	-	-	+	-	+	++	++	++	-	-	-	-	+	+	+
A. hydrophila	-	-	++	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
S. typhymurium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Streptococcus.sp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. aureus	+	+	+	-	-	-	+	+	+	+	+	++	++	-	-	-	+	+	+	+
S. aureus ATCC 25923	+	+	+	-	-	-	+	+	+	+	+	+	++	-	-	-	+	++	+	+
E. feacalis ATCC 29212	+	+	+	-	-	-	+	+	+	+	+++	+	+++	-	-	-	+	++	++	++

Inhibition zones > 20mm were declared to strong (+++), from 12 to 20mm as moderate (++) and from 7 to 12 as weak activities (+), no activity (-).

Table II: Antibacterial activity of active extracts (500µg/disc) against *Padina pavonica*-associated bacteria. (Inhibition zone was measured with paper disc)

Padina Isolates		CH2Cl2				CH2Cl2/CH3OH			
		Jun	Jul	Aug	Sep	Jun	Jul	Aug	Sep
Gram(-) Isolates	P1	-	-	-	-	-	-	-	-
	P2	-	-	-	-	-	-	-	-
	P3	-	-	-	-	-	-	-	-
	P4	-	-	-	-	-	-	-	-
	P5	-	-	-	-	-	-	-	-
Gram(+) Isolates	P6	+	+	-	+	+	+	-	+
	P7	-	-	-	+	-	-	-	+
	P8	-	-	-	+	-	-	-	+

Inhibition zones from 7 to 12mm declared as weak activities (+), < 7mm no activity (-).

Strains (*S. aureus, S. aureus* ATCC 25923 and *E. feacalis*) were strongly inhibited by both alga extracts collected in summer and spring seasons, while strains (*V. tapetis, V. anguillarum, V. alginoliticus, P. cepacia and A. salmonicida*) were only inhibited by the alga extracts collected in summer, probably due to the production of different compounds at different periods of *P. pavonica* growth. A weak inhibition is revealed for *A. hydrophila* by the D and D/M extracts of alga collected in September. *E. coli* O126 :B16, *E. coli* ATCC 25922, *Ps. fluorescens* AH2, *Ps. aeruginosa* ATCC 27853, *S. typhymurium and Streptococcus sp.* strains were resistant toward all the tested extracts. Antibacterial products of *P. pavonica* seem to be selective for some kinds of bacteria.

The strongest antibacterial activities were obtained by D/M extracts for samples collected in warm seasons, especially in September, against both $Gram^+$ and $Gram^-$ bacteria exhibiting an inhibition halo of 21 to 35 mm diameter. In contrast algal extracts of cold seasons did not exhibit any activity against all tested pathogens. During warm season, *P. pavonica* seems to produce more actives compounds than in winter season. These results revealed variable toxicities related to the tested bacterial strains, depending of the harvesting season and the solvent used for extraction.

D and D/M extracts of the alga collected in warm periods, with strong activity against pathogen bacteria were also tested for their activity against *Padina*-associated bacteria (Table II). The results revealed no activities against the $Gram^-$ *Padina*-associated bacteria. Indeed, *P. pavonica* extracts harvested in September, exhibited weak activity against the growing of the *Padina*-isolates identified as *Bacillus pumilus, Brevibacterium sp.* and *Staphylococcus sp* (unpublished data). Moreover, June and July extracts showed an inhibitory activity only against the P6 isolate identified as *Brevibacterium sp*. Algal extracts of June, July and August were inactive against P7 and P8 isolates identified as *Staphylococcus sp* and *Bacillus pumilus* respectively.

This selective toxicity may be important in enabling certain bacteria to live in close association with their host (*P. pavonica*) while it maintains a chemical defense against microbial pathogenesis.

DISCUSSION

The main objective of this study is to assess ability of *P. pavonica* collected from Northern coast of Tunisia to produce antibacterial secondary metabolites and to determine the most suitable period for production of these metabolites.

According to our observation, *P. pavonica* was abundant during the summer, spring and autumn periods and in lower quantity in winter, the maximum biomass was recorded during summer. Previously, Ben Said *et al.* (2002) reported that a warm temperature is required for its massive growth. Nevertheless, Ibrahim *et al.* (2005), observed *P. pavonica*, growing in winter along the Egyptian coast reflecting differences with present findings, certainly due to other biotic and abiotic factors.

Our results herein show that *P. pavonica* compounds had large antibacterial activity against $Gram^+$ pathogens and less important against $Gram^-$. In similar study, González Del Val *et al.* (2001) reported close findings when screened extracts from 44 species of seaweed from Spain, for production of antibacterial compounds against a panel of $Gram^+$ and $Gram^-$ bacteria.

All extracts of *P. pavonica*, did not display any toxicity against *E. coli* O126 :B16. This result was previously highlighted by Khaleafa *et al*., (1975) and Kamenarska *et al.,* (2002) confirming the resistance of this particular bacterium to several marine organism extracts.

In addition, our results revealed seasonal variation of antibacterial activities, with higher activity observed for summer samples which might be due to rapid growth associated with fast proliferation of the alga and photosynthetic activity. Previous studies (Ktari *et al*., 1999, Marti *et al*., 2004, Ibrahim *et al*., 2005) reported variable cytotoxic and antitumoral activities depending on location and/or season.

Else, geographic location seems to be an important factor in antibacterial activity variation for algae since in previous study of Ktari *et al.* (2001), *P. pavonica* collected in spring from the eastern coast of Tunisia weren't actives against $Gram^+$ pathogen *S. aureus* and $Gram^-$ pathogen *V. Anguillarum*. In contrary, in the present study, extracts of *P. pavonica* collected in spring from Northern coast were active against these two pathogens. These results were reliable with those of Tariq (1991), Martí *et al.* (2004), Maréchal *et al*. (2004) and Salvador *et al*. (2007), who highlighted the seasonal, physical and biological factors influence on alga toxicity. Few studies concerned the antibacterial properties of seaweeds on their associated bacteria. The weak activities obtained for these epibionts confirm their affinity with their host.

CONCLUSION

Results obtained showed that *Padina pavonica* collected from Cap Zebib (northern coast of Tunisia) should be considered as eventual source for antibacterial activity, worthy of further investigations. Interestingly, Dichloromethane/Methanol extracts demonstrated large activity against both $Gram^+$ and $Gram^-$ human and fish pathogens and warm season seems to be the suitable period for *Padina pavonica* harvest for antibacterial compounds extraction.

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