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Full Length Research Paper

Inhibition of growth and mycotoxins formation in moulds by marine algae *Cystoseira tamariscifolia*

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Different extracts from marine algae (*Cystoseira tamaricifolia*) were tested for their antimicrobial activities. These extracts were examined for their activities on growth of moulds and yeasts and mycotoxines formation by moulds. The Minimal Inhibitory Concentration (MIC) was determined in every case in terms of dilutions. A total inhibition (100%) was obtained with the ethanolic crude extract on all the fungal species at the concentration of 10%. The aqueous extract showed an activity only on some strains used while no activity was observed on yeasts and moulds for both methanolic and aqueous extract. The other extracts (chloroform, hexane and methanol) had no activity on the microbial growth. Mycotoxins formation in *Aspergillus flavus* was inhibited by the ethanolic extracts at the concentration of 5%.

Key words: Algae, antimicrobial, minimal inhibitory concentration, moulds.

INTRODUCTION

Marine algae are widely spread throughout the coastal lander around many continents. Almost all investigations carried out on these materials focused on the different aspects concerned with their nature and growth. Several species were reported to play a role in the preventing of biofouling phenomena, others are involved in the chemical, pharmaceutical and food industries. In Morocco, the species *Cystoseira tamaricifolia* is widespread in the Atlantic Ocean coast between Tanger and Eljadida. Little is known about this species including studies carried out on the chemical aspect (Bennamara et al., 1999; Daoudi et al., 2001).

Several works have been carried out on extracts from marine algae. Antimicrobial activities on bacteria and fungi were reported by Hellio et al (2000). Chemical characterization of the extracts from macro-algea showed the presence of diterpenes (Valls et al., 1993; Culioli et al., 2000; Daoudi et al., 2001; Culioli et al., 2001). Almost all these studies were carried out on algae of the cystoseicacaea, phoeophycea. These brown algae may have similar effect on the microorganisms' growth.

It should be more interesting to investigate the antimicrobial activities of these materials, for a possible use in food preservation. This may encourage the use of natural products for substituting chemical preservations in food systems. The present investigation focused on some aspects to promote natural products in the field of food preservation. The antimicrobial activities of crude extract from the marine algae C*yctoseira* were evaluated on bacteria, yeasts and fungi.

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MATERIAL AND METHODS

The plant was harvested from the Atlantic ocean coast in the south of Rabat (Oued Eykem). 10 kg of the fresh algae were transported to the laboratory, washed with tap water to remove salt and thereafter with distillated water. The washed plant was dried by exposing to room temperature and ground with a moulinex type mincer.

Extraction

Leaves (10 g) were mixed with 60 mL of hot water (90°C), shaken 10 min and allowed to stand for 15 min. It was then filtered on paper Whatman N°4 and allowed to cool to room temperature and stored at 4°C until use. Leaves of the algae were also extracted with methanol, hexane, ethanol and chloroform.

Growth inhibition

Leaves of algae extracted with 8 solvents including water were screened for the inhibition activities. The extracts were studied by the method of microplates. The cultures were inoculated to the medium by seeding 5 ml of each medium by 1 ml of a suspension of the culture in saline water. The medium is then filled in the wells of the microplate and different concentrations of the extract were added to the seeded medium. One well for each row was kept as control. The MIC (Minimal Inhibitory Concentration) was determined for each extract.

Assays on the whole plant

The ground dried leaves of algae (2g) were mixed with 100 mL of the medium which was autoclaved at 120°C for 20 minutes, allowed to cool to 45°C and poured into plates. The media used were PDA (Potato Dextrose Agar) for yeasts and Malt agar for moulds. The isolates were spot inoculated and incubated at 28°C for one week for moulds, 28°C for 72 hours for yeasts. A control plate of each medium was inoculated in the same way as the assays.

Microbiological procedure

The effect of the different products obtained by water, methanol and ethanol were carried out on pure cultures of yeasts moulds and bacteria including lactic acid bacteria species. All the species were taken from the culture collection of our department.

Yeasts: *Saccharomyces cerevisiae* (4 strains), *Candida albicans* (4 strains) *Debaryomyces* sp (1 strains) and *Kluyveromyces* sp (1 strains).
Moulds : *Aspergillus flavus* (3 strains), *Penicillium* sp (2 strains).

Yeasts

The amount of the extract to be tested was added to 0.5 mL of the liquid medium in microplate wells, which were inoculated and incubated at the temperatures mentioned above. Controls for each concentration of the extracts were made.

Moulds

Sterile malt extract solutions (2%) were filled to the microplate wells. The extracts to be tested were added aseptically and the wells were inoculated with one drop of a spore suspension in a saline water-Tween 80 solutions and incubated at 22°C for 7 days.

Mycotoxins inhibition in moulds

The extracts were added to the medium YES (yeast extract sucrose) at different concentrations in Erlenmeyer flasks, which were inoculated with one strain of *A. flavus* (mycotoxin producing). The flasks were wrapped with aluminum paper to prevent light and placed on a shaker. One control without the extracts was carried out in the same conditions as the assays. After one week incubation at 28°C the medium was filtered (Watman N° 4) to remove the mycelium and the filtrate was extracted as described bellow.

Aflatoxin extraction and quantification

Aflatoxin B_1 (AFB_1) and Aflatoxin G_1 (AFG_1) were extracted and purified according to the method described in the “Official Journal of European Communities N° L102/2-L102/18” and reported by Frayssinet and Fremy (1991). AFB_1 and AFG_1 were determined using a UV spectrum (Shimadzu UV 160A) as reported by Smiley and Draughon (2000). Absorbance values were measured at 360 nm. Standards of AFB_1 and AFG_1 from *A. flavus* (Promochem, France) were prepared by suspending in benzene/acetonitrile (90/10, v/v) to make 10 µg/ml stock solutions. This concentration of the stocks was calculated by recording the UV spectrum (Frayssinet and Fremy, 1991). The two solutions were stored at -20°C in the dark.

RESULTS

Growth inhibition

The unexpected observation that all the extracts other than ethanol had not shown an effect on the microbial growth can be tentatively explained by the lack of antimicrobial principles in the algae as well as by the low concentration of the antimicrobial compounds if they can occur in the algae. The second argument is more tangible. Some authors (Bennamara et al., 1999) had demonstrated an antimicrobial compound called methoxybifurcaremore but the authors had used a concentration procedure and purification of the pure compounds.

The antimicrobial activities of the different extracts on yeasts are reported in Table 1. Results showed the inhibition of all species by the ethanolic extract. 100% inhibition activity was obtained by ethanolic extracts. This may indicate that some of the chemical compounds are more soluble in ethanol than other solvents.

A weak inhibition was also observed with the methanolic extract for all strains. This may complement the explanation of the hypothesis developed for the ethanolic extract, which may suggest that the partial inhibition is due to a partial extraction of the active compounds, which are more extractable, by ethanol than by methanol. This is only broadly studied on the crude extract. Since this study is only on the crude extract, the antifungal activity ought to be well characterized.

Table 1. Antimicrobial activities test of the algae extract on yeasts.

Organism	Extracts (10%)							
	Methanolic		Hexanic		Ethanolic		*Water*	
	A	C	A	C	A	C	A	C
Saccharomyces cereviceae	±	+	+	+	-	+	+	+
Saccharomyces cereviceae	±	+	+	+	-	+	+	+
Saccharomyces cereviceae	±	+	+	+	-	+	+	+
Saccharomyces cereviceae	±	+	+	+	-	+	+	+
Kluveromyces	±	+	+	+	-	+	+	+
Debaryomyces	±	+	+	+	-	+	+	+
Pichia	±	+	+	+	-	+	+	+
Rhodotorula	±	+	+	+	-	+	+	+
Candida albicans	±	+	+	+	-	+	+	+
Candida albicans	±	+	+	+	-	+	+	+
Candida albicans	±	+	+	+	-	+	+	+
Candida albicans	±	+	+	+	-	+	+	+

+ : Growth, **± :** Low growth, **- :** Inhibition A: Assay C: Control

Table 2 shows the antifungal activity of the different extracts on moulds. The same results as for yeasts were observed. Therefore, the ethanolic extract could inhibit all the strains used in the study. A partial inhibition by the methanolic extract was also observed for mould strains. The antifungal activity of the crude ethanolic extract and/or the methanolic extract is demonstrated.

Table 2. Antimicrobial activities test of the algae extract on moulds.

Organism	**Extracts**					
	Methanolic		Hexanic		Ethanolic	
	A	C	A	C	A	C
Penicillium	±	+	+	+	-	+
Penicillium	±	+	+	+	-	+
Penicillium	±	+	+	+	-	+
Aspergillus flavus	±	+	+	+	-	+
Aspergillus flavus	±	+	+	+	-	+
Aspergillus flavus	±	+	+	+	-	+

+ : Growth, **± :** Low growth, **- :** Inhibition A: Assay C: Control

The antifungal activity was also studied in liquid media. The strains were inoculated in liquid medium containing different concentrations of the ethanolic extract to determine the minimal inhibitory concentration. Results reported in Table 3 showed an inhibition activity of the ethanolic extract at the concentration of 2.5 %. All species were inhibited at this concentration except one *C. albicans* strain. All inhibited by 5% of the ethanolic extract. Moulds were more resistant than yeasts and the MIC was around 7.5 % (Table 4).

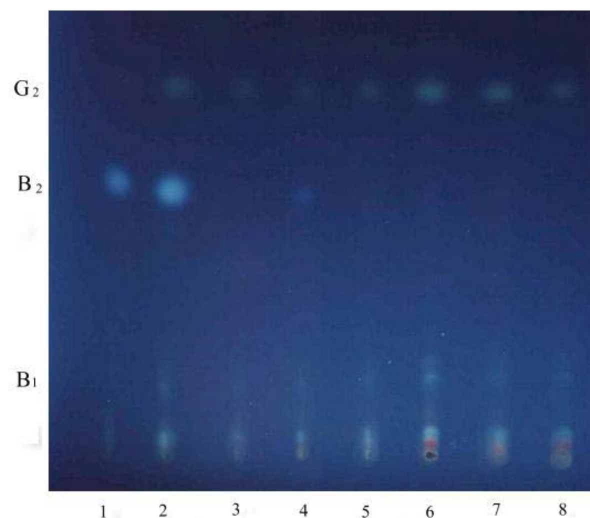

Figure 1. Mycotoxins B_1, B_2 and G_2 from cultures of *Aspergillus flavus* in presence of the different concentrations of the ethanolic extract 1 (control), 2 (control ethanol), 3 (1% ethanolic extract), 4 (2.5% ethanolic extract), 5 (5% ethanolic extract), 6, 7 and 8 (7.5% ethanolic extract).

Mycotoxins inhibition in moulds

The culture of *A. flavus* in the presence of the two extracts, methanolic and ethanolic, has shown a total inhibition of of aflatoxins by the ethanolic extract as it was revealed by the thin layer chromatography (TLC) in Figure 1. It should be emphasized here that while

Table 3. Minimal inhibitory concentrations of ethanol extract on yeasts growth.

Organism	Extracts (% concentrations)							
	1	1.5	2	2.5	5	7.5	10	15
Saccharomyces cerevicea	+	+	±	-	-	-	-	-
Saccharomyces ceroviceae	+	+	±	-	-	-	-	-
Saccharomyces ceroviceae	+	+	±	-	-	-	-	-
Saccharomyces cereviceae	+	+	±	-	-	-	-	-
Kluyveryomyces sp	+	+	±	±	-	-	-	-
Debaryomyces sp	+	+	±	±	-	-	-	-
Pichia sp	+	+	±	±	-	-	-	-
Rhodotorula sp	+	+	±	-	-	-	-	-
Candida albicans	+	+	±	+	-	-	-	-
Candida albicans	+	+	±	+	-	-	-	-
Candida albicans	+	+	±	+	-	-	-	-
Candida albicans	+	+	±	+	-	-	-	-
Candida albicans	+	+	±	+	-	-	-	-

+: Growth, **± :** Low growth, **- :** Inhibition

Table 4. Minimal inhibitory concentrations of ethanol extract on moulds.

	Concentrations (%)						
Organism	1%	1.5%	2%	2.5%	5%	7.5%	10%
Aspergillus flavus	+	+	+	+	+	+	-
Aspergillus flavus	+	+	+	+	+	+	-
Aspergillus flavus	+	+	+	+	+	+	-
Penicillium	+	+	+	+	+	+	-
Penicillium	+	+	±	±	-	-	-
Penicillium	±	±	-	-	-	-	-

+: Growth, **±:** Low growth, **- :** Inhibition

ethanolic concentration of 1% did not inhibited growth, aflatoxins production was, however, terminated.

In figure 1, spot N° 3 may indicate a low concentration of the aflatoxin in the medium as revealed by the TLC. This concentration is compared to the spot N° 4 which may correspond to the culture in presence of ethanol. It is assumed that ethanol stimulates the aflatoxins formation. Spot N° 5 is the control with distilled water. The methanolic extract had been revealed as inhibitory, it was applied at different concentrations to study more deeply and also to confirm the effect of this extract on the production of aflatoxins. Figure 2 showed results relative to the different concentrations of the ethanolic extract. It could be seen that the concentration of 1 is inhibitory.

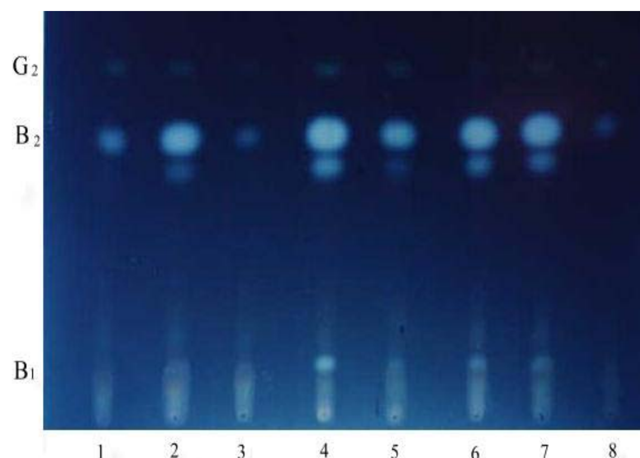

Figure 2. Mycotoxins B_1, B_2 and G_2 from cultures of *Aspergillus flavus* in presence of ethanolic and methanolic extracts of the algae detected by thin layer chromatography: 1 (1% ethanolic extract), 2 (2.5% ethanolic extract), 3 (5% ethanolic extract), 4 (control ethanol), 5 (control), 6 (1% methanolic extrcat), 7 (5% methanolic extract) 8 is a duplicate of 3.

DISCUSSION

This investigation focused on the effects of extracts from the brown algae, *C. tamariscifolia* on moulds and yeasts.

Four solvents; haxane, methanol, ethanol, and water, were used for extracting, but only the ethanolic extract have an effect on yeasts and moulds. Some works concerning the antimicrobial activity of marine algae showed some activities on bacteria (Hellio et al., 2000). The extracts studied were obtained from retail and various algae were studied by the authors. Bennamara et al. (1999) tried a purified compound from brown algae, methoxybifurcarenone, on bacteria and moulds and they found an antimicrobial activity on moulds only. The purified component is a minor component of the fatty components in the algae.

Several works on marine algae has been carried out to determine the active components in the extracts of the most abundant algae species. Culioli et al. (2000) reported the identification of geranylgeraniol derived diterpens from the brown algae. Four novel diterpens from the brown algae collected from the Moroccan Atlantic coast have been identified (Culioli et al., 2001). These compounds could be among the active principles in the brown algae but their effect on the microbial growth and mycotoxin formation have not been adequately studied. Daoudi et al. (2001) have also isolated acyclic diterpens and sterols from the genera *Bifurcaria* and *Bifurcariopsis*. Furthermore, Bennamara et al. (1999) isolated a meroditerpenoid from the brown algae *Cystoseira tamariscofolia* which was identified as methoxybifurcarenone. These authors demonstrated the antifungal activity of the compound on *Botrytis cinerea, Fusarium oxysporum* and *Verticillicum alboactum*, which are not involved in food hazards.

The antifungal activity demonstrated in our investigation would suggest a very adapted compound, which may have a strong inhibition activity on moulds and yeasts. The active substance(s) in the extracts should be isolated and studied for a possible use in food preservation and/or therapeutic to inhibit or delay fungal growth.

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