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ACTIVE BIOLOGICAL MATERIALS INHIBITING TUMOR INITIATION EXTRACTED FROM MARINE ALGAE

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ABSTRACT

Ethanol extraction of seventeen algal species from 35 different samples collected from the coastal area of Abou-Qir, Egypt, South East of the Mediterranean Sea Coast, was conducted. The aim was to obtain natural products from the algal extracts which have significant biologically active algal compounds. The collected algae showed a wide variation in their occurrence during the period of collection. Thirty five extracts representing different seasonal growths of 17 marine algal species were tested for anti-tumorigenic activity. Tests performed were the toxicity bioassay using Brine shrimp *Artemia salina* and the anti-tumor bioassay against *Agrobacterium tumefaciens* on potato discs. *Codium tomentosum* (winter); *Jaina rubens* (summer) and *Padina pavonia* (winter) displayed relatively high activity.

INTRODUCTION

Marine algae vary in size from minute unicellular forms of a few microns in diameters to the large seaweeds which have many meters in length (Bold, and Wyne 1978 and Ruggieri, 1976). Marine organisms comprise over half a million species due to their usual living environment as compared with terrestrial organisms. Hopkins marine station pointed to over 5000 species of green algae known, 1500 species of brown algae almost exclusively found in marine habitats and with over 4000 species of red algae. Man has used the sea for many years as a productive source for several economically useful materials, especially to supplement his diet (Prescott, 1984). In recent years, an increasing number of marine natural products have been reported to display antimicrobial activities, and anti-tumor compounds have been isolated from sponges, tunicates, algae and other organisms (Chapman and Gellenbeck 1983). In most cases, the evaluation of the anti-cancer potential of crude extracts from different sea organisms has been carried out by *in vitro* cytotoxicity tests in malignant cell cultures (Russell, 1963). Jolles *et al.,* (1963) were the first to report the influence of degraded sulfated Laminarine (an algal extract) on tumor growth.

Isolation of cytotoxic anti-tumor substances from marine organisms has been reported in several references during the last 40 years, (Hansen, 1997; Burrows, 1991; Fadli, *et al.,* 1991 and Gonzalez *et al.,* 1982), while in recent years, hundreds of potential anti-tumor agents have been isolated from

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marine origin especially from marine algae (Adams, 1994 and Fadli, *et al.,* 1991). In the last 15 years, a new inexpensive, simple, convenient and reliable plant tumor system initiated by the application of a tumorigenic bacterium to the surface of potato discs, has been used by several researchers (Ferrigni, *et al.,* 1982 and Anand, *et al.,* 1977).

The present work aims at conducting a close study of the distribution of the seasonal algal communities along the coastal area of Abou-Qir, Egypt, South East of the Mediterranean Sea Coast and to obtained natural products from algal extracts which have significant biologically active algal compounds.

MATERIALS AND METHODS

Sampling:

Collection of algae

The algal samples were collected from an exposed rocky site near the Western edge of Abou-Qir Bay, Alexandria, Egypt. The collection was carried out seasonally, to permit the detection of any seasonal variation on the bioactivity of the collected species. The collection of samples was carried out in the first two weeks of February for Winter, May for Spring, August for Summer and November for Autumn seasons as shown in Table (1). The samples were dried at 40° C till a constant weight to determine the water content.

Identification of algal species

References used for the identification of the algal species were: Chapman and Gellenbeck 1983; Bold and Wynne, (1978) and by the herbarium specimens of late professor A.H. Nasr, (1939). Taxonomic classification of the algal species was made according to the system developed by Papenfuss, (1955) and modified by Papenfuss, (1968) and according to the system developed by Engler, (1954) and modified by Fott, (1969). The identification of specimens were checked by professor Abd-El Fattah Khalifa, Botany Department, Faculty of Science, Alexandria University.

Extraction process

Seventeen algal species including 35 different samples were prepared for ethanol extraction. Extraction from algal samples was carried out according to Ferrigni *et al.,* (1982). Twenty five grams of powdered algal sample were weighed and mixed with 62.5 ml hexane in 500 ml Erlynmeyer flasks for extraction. The process was repeated four times for the extraction of the unfiltered portion of the powdered algal sample. The final unextracted portion was treated again for 4 times using absolute ethanol instead of hexane. Ethanolic extract were preserved in vials at - 20°C.

Toxicity bioassay

According to Meyer *et al.,* (1982) and Shinho, (1984) several naturally extracted products which had LC_{50} < 1000 µg ml^{-1} using brine shrimp bioassay were known to contain physiologically active principles. Comparing the effect of this bioassay was found to be significant in the condition that LC_{50} < 30 µg $ml^{-1.}$ Brine shrimp *Artimia salina* was used as a test organism. The extract thin films after complete ethanol evaporation were not dissolved completely in distilled or saline water, but dissolved in 100 µg dimethylsulfoxide (DMSO) as a universal solvent to dissolve the crude extracts. Algal extract (8, 12 or 16 mg) were dissolved in 2 ml DMSO and filtered through a sterile Millipore filter (0.22 µm).

Anti-tumor bioassay

These algal extracts were then tested for their biological activity as anti-tumor agents against the initiation of crown gall tumors induced by *Agrobacterium tumefaciens.* The surfaces of whole potato tubes of moderate size were disinfected by immersion in 0.5% (w/v) sodium dichloroisocyanurate for 30 min, followed by two washes in sterile water. After 20 min., the two ends of a potato were removed and a tissue cylinder of 1.5 cm diameter was taken from the remainder of the tuber with a sterile cork borer. The potato cylinder was cut into discs of 0.5 cm thickness after discarding a 1 cm length from each end. Four discs were

transferred onto the surface of sterile water agar (15 m l^{-1} agar in water) in a 9-cm Petri dish. An aliquot of 0.5 ml of dissolved extract was added to 1.5 ml sterile water, inoculated with 2 ml of a late log phase culture of *Agrobacterium tumefaciens* (10^{10} cells ml^{-1}) strain 2928, isolated from potato plant variety King Eduard which was mainly used in this study. 50 µl of this mixture was pipette onto the surface of a potato disc. Petri dishes were incubated at 25° C for 12-15 days with regular examination for gall development on the potato disc surface. After inoculation, galls were stained with Lugol's solution (1 g iodine + 2 g KI / 300 ml water) and counted with the aid of a binocular microscope, where the tumor cells lack starch.

Table (1): Active and inactive marine collection samples used in this study.

Class	Species	Season	Class	Species	Season
Active Samples			**Inactive Samples**		
Chlorophyceae	*Cladophora pellucida*	Spring	Chlorophyceae	*Ulva fasiata*	Winter
(Green algae)	*Codium tomentosum*	Winter	(Green algae)	*Ulva lactuca*	Winter
	Ulva lactuca	Summer		*Caulerpa prolifera*	Summer
	Cladophora pellucida	Winter		*Caulerpa racemosa*	Spring
				Codium tementosum	Summer
Phaeophyceae	*Colpomenia sinosa*	Spring	Phaeophyceae	*Ectocarpus siliclosus*	Spring
(Brown algae)	*Dictyota dichotoma*	Spring	(Brown algae)	*Cystoseira foeniculacea*	Winter
	Padina pavonia	Winter		*Sargussum hornschuchii*	Spring
				Colpomenia sinosa	Autumn
Rhodophyceae	*Corallina mediterranea*	Summer	Rhodophyceae	*Hypnea musciformis*	Spring
(Red algae)	*Jania rubens*	Summer	(Red algae)	*Laurencia papillosa*	Spring
	Pterocoladia capillacea	Spring		*Corallina mediterranea*	Winter
				Jania rubens	Autumn

RESULT AND DISCUSSION

Map (1 A) shows Abou-Qir area and its topography, while map (1 B) shows the different algal genera used in this study and their distribution along the collection site. Seventeen different species were obtained from thirty five algal samples, which can be categorized into three groups; green algae, brown algae and red algae. The green algae group was represented by 4 genera; *Ulva, Caulerpa, Codium* and *Cladophora*; while genera related to brown algae were *Ectocarpus, Dictyota, Padina, Colpomenia, Cytosera and Sargassum.* Meanwhile, the red algae group included 5 genera; *Pterocladia, Corallina, Jainia, Hypnea and Laurencia.*

The collected algae showed a wide variation in their occurrence during the period of collection. *Pterocladia capillacea* was found in all seasons, while *Corallina mediterranea* was found in four seasons. Other algal species were found in three different seasons, *Ulva faciata* was collected in summer, spring and autumn, while *Ulva lactuca* was collected in winter, spring and summer. In the meantime, the brown alga *Colpomenia sinosa* was collected in spring, autumn and winter, while *Jainia rubens* was sampled in winter, summer and autumn. The remaining algal species were collected in two or one season only. *Cladophora pellucida* and *Hypnea musciformis* were collected in spring and winter, while *Codium tomentosum* was collected in summer and winter. Meanwhile, *Coulerpa racemosa, Ectocarpus siliculosus, Dictyota dichotoma, Sargassum hornschuchii* and *Laurencia papillosa* were collected in spring only. In winter, *Padina pavonia* and *Cystoseria foeniculacea* were collected, while*, Caulerpa prolifera* was collected only in the summer season. Active and inactive marine collection samples are shown in Table (1).

The eight algal extracts, exhibiting more than 20% tumor inhibition were grouped according to the (LSR) into two significant categories showing distinct differences between them. The algal species belonging to the first category as show in Tables (1 and 2) are *Jania rubens* and *Padina pavonia* have the same significant effect in inhibiting the tumor during winter, but they were insignificantly different in their activity. They were more significant than the other group including: *Pterocladia capillacea* (Summer and Spring samples). The spring sample of *Colpemenia sinosa, Dictyota dichotoma* and the summer sample of *Ulva lactuca* showed the lower inhibition percentage (32.15%). It has to be noted that, the winter sample of *Codium tomentosum* showed insignificant difference compared to the two former groups. The results presented in Table (2) showed the seasonal average yield of ethanolic algal extracts and percentage of water content.

A. tumefaciens galls on potato discs initially with each extract in 12.5% (v/v) DMSO at a concentration of 25 µg extract per disc as recommended by Ferrigni *et al.,*1982. Out of a total of 35 extracts representing 17 algal species, 11 (nine species) displayed > 20% inhibition of tumor initiation as showed by El-Masry, *et al.,* 1995.

The difference in water content in *Chlorophyceae* between the maximum value (82.4%) and the minimum value (52.22%) was 30.18%, while in *Phaeophyceae* the difference was (33.57%) and in case of *Rhodophyceae* the water content had a percentage difference equal to 35.74%. Even within seasonal samples of the same species their water content percentage showed a significant variation depending on the season of collection.

The results presented in Table (3) showed that the general range of LC_{50} was 100-1900 µg ml^{-1} for the various algae used in this study. However, the trend of their toxicity was directly proportional to the increase in the extract concentration and Table (4) showed the mean values of percentage galls inhibition and sequent trail with (F) values. Means with a common letter

(a or b) are insignificantly different from each other according to the least significant range test (LSR).

Most of the algal species (12 out of 17) collected in the spring season, which has a moderate temperature of 23° C, while *Padina pavenia* and *Cystoseria fenoculacea* were collected only in winter. *Caulerpa prolifera* was found to grow in sufficient quantity in summer, while *Ulva fasciata* and *Pterocladia capillacea* regularly appeared in spring, summer and autumn in quantities that can be easily collected. This indicates that a certain level of temperature is required for these species to grow in a massive quantity to facilitate the collection procedures.

The fresh weight used in the extraction process were significantly varied between the algal species under investigation and ranged from 49.4 gm to 191.3 gm. This variation was also noticed between samples within the species, which is due to the variation in water content of the different studied algal species.

However, the criterion to evaluate algal samples which will be relied on is that, the highest yield and the lowest water content algae which exhibited significant anti-tumor activity is the most economically favorable species recommended to be used for further advanced investigation. On the other hand, Meyer, *et al.,* (1982) stated that, the natural products extracted from plant source which show LC_{50} < 1000 g. ml^{-1} in the brine shrimp assay may contain physiologically active principles.

On that bases, algal species proved to exhibit anti-tumor activity can be arranged as follow (regarding that digits between brackets followed each species are corresponding to: the percentage of water content, fresh weight used in grams and the ethanolic yield in milligrams per gram fresh weight). *Dictyota dichotoma* (51, 52 and 8.8); summer sample to *Pterocladia capillacea* (60.3, 52 and 6.3); summer sample *to Ulva lactuca* (62.7, 66.9 and 5.3); *Padina pavonia* (60, 62 and 3.7); summer sample of *Jania rubens* (75, 101, and 4.3); *Colpomenia sinosa* (75, 103 and 3.8); spring sample of *Pterocladia capillacea* (77, 109 and 203) and winter sample of *Codmium tomentosum* (82, 142 and 102).

REFERENCES

Adams, N.M. 1994: Seaweeds of New Zealand: An Illustrated Guide. Canterbury University Press, Christchurch, New Zealand.

Anand, V.K. and Heberleim, G. T. 1977: Crown gall tumorigenesis in potato tumor tissue. Am. J. Bot. 64: 153-158.

Bold, H.C. and Wynne, M.J. 1978: Introduction to the algae: Structure and reproduction. Englewood Cliffs, New Jersey, USA. Pp. 706.

Burrows, E.M. 1991: Seaweeds of the British Isles. Vol. 2. Chlorophyta Natural History Museum Publications, London, xi:238.

Chapman, D.J. and Gellenbeck, K.W. 1983. An historical prespective of algal biotechnology. In: "Algae and cyanobacterial biotechnology" (eds: Cresswell, R.C.; Ress, T.A.V. and Shah, V.), Longman group, U.K. pp. 1-27.

El-Masry, M.H.; Mostafa, M.H.; Ibrahim, A.M. M. and El-Naggar, M.M.A. 1995: Marine algae that display anti-tumorigenic activity against Agrobacterium tumefaciens. FEMS Microbiology Letters, 128: 151-156.

Engler, A. 1954: Syllabus der pflanzeenfamillien, 12th (ed: Gebruder, Bd.I.) Borntraeger, Berlin.

Fadli, M.; Aracil, J.M.; Jeanty, G.; Banaigs, B. and Francisco, C. 1991: Novel Meroterpnoids from *Cystoseria mediterranea*: use of the Crown-gall bioassay as a primary screen for lipophilic anti-neoplatic agents. J. Nat. Prod. 54(1): 261-264.

Ferrigni, N.R.; Putnam, J.E.; Anderson, B.; Jacobson, L.B.; Nichols, D.E.; Powell, R.G. and Smith, C.R. 1982: Modification and evaluation of the potato disc assay and anti-tumor screening of

Eupharbiaceae seeds. J. Nat. Prod., 45 (6): 679-686.

Fott, S. 1969: Studies in phycology, Academia. Prague. Pp. 304.

Gonzalez, A.G.; Darias, V and Estevez, E. 1982: Chemo-Therapeutic activity of polyhalogenated terpenes from Spanish algae. J. Planta Medica, 44: 44-46.

Jolles, B.; Remington, M. and Andrews,P.S. 1963: Effects of sulphated degraded Laminarin on experimental tumor growth Brit. J. Cancer, 17: 109-115.

Hansen, G.I. 1997: A revised check list and preliminary assessment of the macrobenthic marine algae of Oregon. Pp. 1-26. In: T. Kaye, *et al.,* (eds),Conservation and Management of Native Flora and Fungi. Oregon State University Press, Corvallis, OR.

Meyer, B.N.; Ferrigni, N.R.; Putnam, J.E.; Jaccobsen, L.B.; Nichols, D.E. and Melaughlin, J.L. 1982: Brine shrimp: A convenient general bioassay for active Plant constituents. J. Planta Medica, 45: 31-34.

Nasr, A.H. 1939: Reports on the preliminary expedition for the exploration of the Red Sea in the R.R.S. "Mabahith", Algae. Pubs. Mar. Biol. Stn. Ghardaqa, 1: 47-75.

Papenfnss, G.F. 1955: Classification of the algae. In: A century of progress in the natural sciences 1853-1953. Calif. Acd. Sci. San Francisco. 115-224.

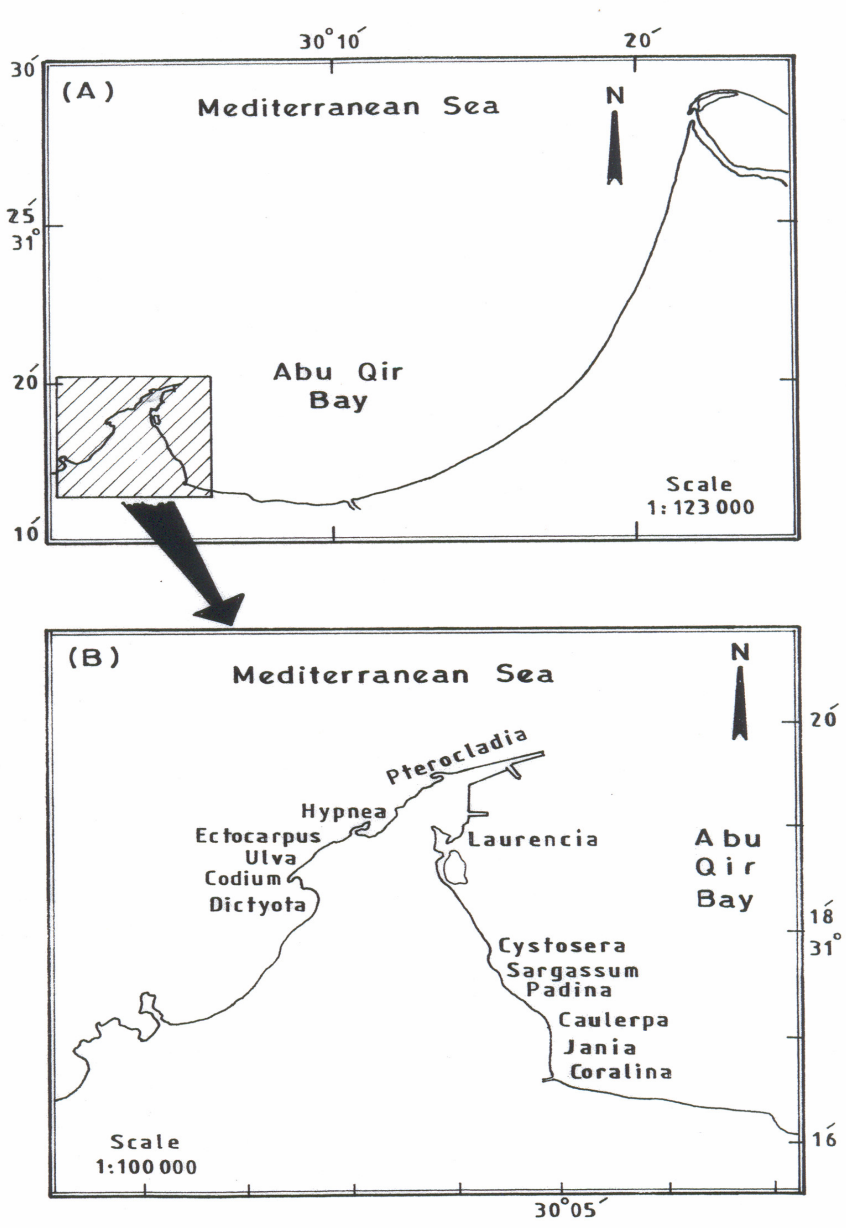
Papenfuss, G.F. 1968: A history catalogue and biolography of Red Sea Benthic Algae. Isreal, J. Bot., 17: 1-118.

Prescott, G.w. 1984: Economics of algae. In: "The algae: A Review". Otto koeltz Science publishers D-629 Koenig-Stein, W. Germany.

Rugggieri, G.D. 1976: Drugs from the sea. Science, 194: 491-497.

Russell, F.E. 1963: Advances of marine biology. Academic press, New York. 3: 255-256.

Shinho, K. 1984: Anti-tumor glycol proteins from *Chlorella* and other species. Jpn. Kokai. Tokky. Koho. JP., 1986, 105: 30034t.



Map (1): Abu Qir area (A) and the distribution of algal samples along the collection site (B).

Table (2): Seasonal average yield of ethanolic algal extracts and percentage of water content ethanolic extract

Class	Species	Season	Water	Fresh	Yield	Color
			Content %	Wt. gm	Fresh wt. mg.g-1	
Chlorophyceae	*Ulva fasciata*	Spring	52.22	52.2	3.5	Blackish green
		Summer	60.31	63	3.7	Dark green
		Autumn	71.56	87.9	2.1	Dark green
	Ulva lactuca	Winter	70.91	85.9	5.7	Very dark green
		Spring	62.65	66.9	5.3	Yellowish green
		Summer	65.15	71.7	2.9	Light green
	Caulerpa prolifera	Summer	60.38	63.1	5.6	Dark green
	Caulerpa racemosa	Spring	80.15	125.9	3.5	Dark green
	Codium tomentosum	Summer	79.3	120.8	3.6	Dark green
		Winter	82.4	142	1.2	Blackish green
	Cladophora pellucida	Spring	53.5	53.8	1.6	Brownish green
		Winter	62.1	66	11.4	Dark green
Phaeophyceae	*Ectocarpus siliculosus*	Spring	49.91	49.9	12.5	Yellowish green
	Dictyota dichotomma	Spring	51.98	52.1	8.8	Dark green
	Padina pavonia	Winter	60.15	62.7	3.7	Dark green
	Colpomenia sinosa	Spring	75.49	102	3.8	Dark green
		Autumn	70.13	83.7	3.3	Yellowish brown
		Winter	79.24	120.4	2.6	Yellowish brown
	Cystoseria foeniculacea	Winter	65.89	73.3	7.3	Dark green
	Sargassum hornschuhii	Spring	45.67	46	5	Yellowish green
Rhodophyceae	*Pterocladia capillacea*	Winter	86.93	191.3	2.3	Yellowish brown
		Spring	77.19	109.6	1.9	Yellowish green
		Summer	60.25	62.9	6.3	Yellowish green
		Autumn	78.16	114.6	1.5	Dark green
	Corallina mediterranea	Winter	62.24	66.2	4.1	Dark green
		Spring	51.19	51.2	4	Yellowish green
		Summer	58.85	60.8	4	Yellowish green
	Jania rubens	Winter	55.7	56.4	3.5	Yellowish green
		Summer	75.28	101.6	4.3	Dark green
		Autumn	71.78	88.6	2.2	Dark green
	Hypnea musciformis	Spring	55.82	56.6	6.8	Yellowish green
		Winter	67.91	77.9	3.2	Brownish green
	Laurencia papillosa	Spring	83.17	148.4	1.6	Yellowish green

Fresh wt.: This fresh weight corresponds to the 25 gm dry weight used in the extraction process.

Table (3): Brine shrimp toxicity bioassay resulted using ethanolic extracts of algal species

mortality percentage after 24 hrs						
Class	**Species**	**Season**	**400** μ g. ml^{-1}	**800** μ g. ml^{-1}	**1000** μ g. ml^{-1}	**LC_{50}** μ g. ml^{-1}
Chlorophyceae						
	Ulva fasciata	Spring	30	66	91	560
		Summer	30	40	57	800
		Autumn	24	37	70	680
	Ulva lactuca	Winter	14	37	61	820
		Spring	24	57	77	620
		Summer	27	37	57	820
	Caulerpa prolifera	Summer	47	54	74	450
	Caulerpa racemosa	Spring	14	37	51	1000
	Codium tomentosum	Summer	30	40	60	840
		Winter	24	57	100	720
	Cladophora pellucida	Spring	20	23	62	920
		Winter	22	24	50	1000
Phaeophyceae	*Ectocarpus siliculosus*	Spring	25	42	54	900
	Dictyota dichotomma	Spring	77	77	87	100
	Padina pavonia	Winter	40	47	55	800
	Colpomenia sinosa	Spring	14	37	54	900
		Autumn	40	29	52	960
		Winter	45	81	91	560
	Cystoseria foeniculacea	Winter	40	71	81	470
	Sargassum hornschuhii	Spring	10	64	81	450
Rhodophyceae	*Pterocladia capillacea*	Winter	57	81	94	360
		Spring	40	50	67	560
		Summer	24	50	61	800
		Autumn	24	54	64	740
	Corallina mediterranea	Winter	64	81	91	400
		Spring	30	54	64	680
		Summer	15	30	41	1200
	Jania rubens	Winter	30	71	91	500
		Summer	27	31	51	960
		Autumn	40	54	90	500
	Hypnea musciformis	Spring	57	74	81	350
		Winter	47	64	84	480
	Laurencia papillosa	Spring	10	30	33	1900

Table (4): Mean values of percentage galls inhibition and sequent trail with (F) values. Means with a common letter (a or b) are insignificantly different from each other according to the least significant range test (LSR).

			Mean value of % of galls inhibition	
Class	**Species**	**Season**	**Exp. (1)**	**Exp. (2)**
Chlorophyceae				
	Ulva lactuca	Summer	63.8a	32.1 b
	Codium tomentosum	Winter	73.7 a	51.9 a, b
	Cladophora pellucida	Spring	72.8 a	------
Phaeophyceae	*Dictyota dichotomma*	Spring	64.1 a	37.2 b
	Padina pavonia	Winter	57.4 a	64.8 a
	Colpomenia sinosa	Spring	70.5 a	31.4 b
Rhodophyceae	*Pterocladia capillacea*	Spring	60.6 a	42.9 b
		Summer	73.7 a	43.8 b
		Autumn	63.8 a	------
	Corallina mediterranea	Summer	34.9	----
	Jania rubens	Summer	59.3 a	67.1 a

(F) Values: 2.71 and 3.55, (P) < 0.01.