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SEASONAL VARIATIONS OF THE COMPOSITION, STRUCTURAL FEATURES, AND ANTITUMOR PROPERTIES OF POLYSACCHARIDES FROM *Padina pavonica* (LEBANON) AS A FUNCTION OF COMPOSITION

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The variation of the polysaccharide composition of the brown alga Padina pavonica *from the Mediterranean Sea was studied as a function of the collection season (April–July). It was shown that the principal polysaccharide (8.0-13.3% of dry alga weight) was alginic acid. Its content did not undergo significant changes from April to July. The content of water-soluble polysaccharides (laminarans and fucoidans) was low (<0.3% of dry alga weight). Water-soluble polysaccharides of the studied alga were practically pure fucoidan. The amount of laminarans was insignificant (<0.01% of dry alga weight). The content of fucoidans increased in alga samples from May through July. It was shown that* P. pavonica *is a promising source of alginic acids, which have a high capacity for gelation, and fucoidans, which exhibit antitumor action against RPMI-7951 human melanoma cells.*

Keywords: brown algae, *Padina*, polysaccharides, laminaran, fucoidan, alginic acid, biological activity.

Brown algae were a valuable source of polysaccharides such as alginic acids [1], fucoidans [2], and laminarans [3]. These compounds exhibit a broad spectrum of biological activity and low *in vivo* toxicity. Unfortunately, their use is hampered by problems with obtaining products with standard properties because the content and structural characteristics of the polysaccharides vary depending on the habitat and collection season [4]. Furthermore, the relationship between structural features and the biological activity of the polysaccharides, especially fucoidans, is poorly studied.

The search for accessible sources of biologically active polysaccharides and the study of seasonal variations of their polysaccharide composition in order to determine the optimal collection time and to obtain preparations with standard properties are currently critical [5, 6]. This information is required for industrial harvesting of alga.

Brown algae of the genus *Padina* (Dictyotaceae) are widely distributed in the seas of Russia, Lebanon, India, Thailand, and other countries [7–9]. The properties of algal polysaccharides of this family (*Lobophora variedata*, *Dictyota menstrualis*, *Spatoglossum schroederi*, *Stoechospermum marginatum*, *Padina gymnospora*, and *P. tetrastromatica*) were studied. They were interesting as compounds with a broad spectrum of biological activity, e.g., antioxidant, anticoagulant, antithrombic, and antiviral properties [9–12]. It was also reported that polysaccharides from the brown alga *P. pavonia* (*pavonica*) exhibit anticoagulant activity [13].

The goal of our work was to study the composition and structural characteristics of polysaccharides from *P. pavonica* from the Mediterranean Sea as a function of collection time and to determine the antitumor activity of the isolated fucoidans.

Alginic acids, laminarans, and fucoidans were isolated using a modification of the published method [14] from *P. pavonica* collected on the shores of the Mediterranean Sea (Lebanon, Batroun Bay) in April–July 2009 (N 34 15.069′; E 035 39.385′). The modification consisted of selecting the sequence of separation steps (hydrophobic and anion-exchange chromatography) depending on the monosaccharide composition of the total fractions of water-soluble polysaccharides (WSPS). The first step was anion-exchange chromatography over Macro-Prep DEAE because the principal monosaccharide of total water-soluble fractions 4PpFL, 5PpFL, 6PpFL, and 7PpFL was fucose. The amount of glucose was low (<4%). Therefore, the content of laminarans in the studied samples was low.

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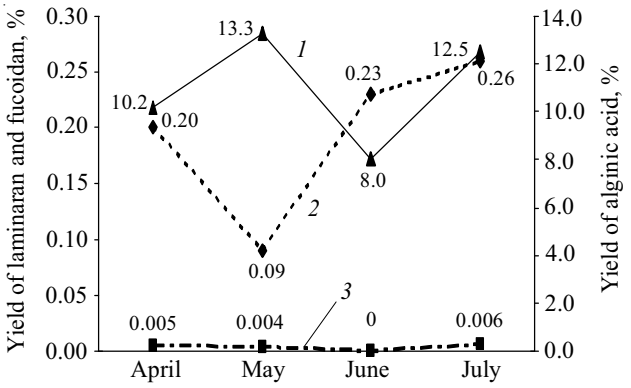

Fig. 1. Yield of polysaccharides (% of dry alga weight) from *Padina pavonica* as a function of collection month: alginic acid (*1*), fucoidan (*2*), laminaran (*3*).

Figure 1 shows the change of polysaccharide yield (% of dry alga weight) from *P. pavonica* as a function of alga collection month.

The content of alginic acids in the studied alga was high, from 8.0 to 13.3%, and did not undergo significant changes from April to July. Alginic acids are linear polymers consisting of 1,4-bound β-D-mannuronic and α-L-guluronic acids [1]. The content of alginic acids and their structural characteristics depend on the species, season, latitude, and ecological habitat of the algae [5].

The ratio of mannuronic and guluronic acids (M/G) was calculated by comparing strengths of the corresponding resonances in the PMR spectrum and was 1.78, 1.35, 0.24, and 1.33 for 4PpA, 5PpA, 6PpA, and 7PpA, respectively. The structure of the alginic acids, in contrast with their content, depended on the collection time. In June, alginic acid enriched in guluronic acid was synthesized. The content of alginic acid in *P. tetrastromatica* (Gujarat, India) was 4.8% whereas the amount of WSPS was >6.5%. Alginic acid isolated from this alga collected in August contained about the same amount of mannuronic and guluronic acids [9].

The ability of alginic acids to gel is known to depend on the content of guluronic acid [1]. The viscosity for an aqueous solution (1%) of commercial edible sodium alginate is characteristically ≥4°E (TU 15-544-83). We determined the viscosity of aqueous solutions (1%) of alginic acids 4PpA, 5PpA, 6PpA, and 7PpA as 174 cSt (23.8°E), 215 (29.4), 1770 (241.7), and 190 (26.0), respectively. Solutions of alginic acids isolated from the studied alga samples had viscosities that were significantly greater than the standard value 4°E. A solution of the alginic acid isolated from alga collected in June had the greatest viscosity. It had a smaller M/G ratio than those of algae from other collection times. Thus, the studied alga was a promising source for obtaining alginic acids with a high ability to gel.

The content of WSPS in the studied alga samples was low (Fig. 1). The amounts of laminarans in alga collected in April, May, and July were insignificant (<0.01% of dry alga weight). Laminaran was not found in alga from the June collection. Thus, the composition of WSPS from *P. pavonica* was practically pure fucoidan. The laminaran content was <0.6% of the water-soluble fraction weight. The fucoidan contents in samples of alga collected in April, June, and July were <0.3% of the dry alga weight; in alga collected in May, <0.1% fucoidans. Alga from the April collection contained twice as much fucoidans as *P. pavonica* collected in May. The yield of fucoidans tended to increase in alga samples collected in May and July.

Laminarans are 1,3;1,6-β-D-glucans that differ in the ratio of 1,3 and 1,6 bonds and the location of the 1,6 bonds [3]. Fractions 4PpL, 5PpL, and 7PpL obtained by hydrophobic chromatography over Polykhrom-1 were practically pure glucans. The fraction of WSPS from alga collected in June contained practically no laminaran. Therefore, fraction 6PpL is missing from further structural studies. ^{13}C NMR spectra of the isolated laminarans exhibited resonances with chemical shifts 103.8 (C-1), 74.5 (C-2), 85.5 (C-3), 69.4 (C-4), 76.9 (C-5), and 62.0 (C-6) ppm that were characteristic of 1,3-bound β-D-glucopyranose units. Besides these strong resonances, additional weaker resonances with chemical shifts 104.0 (C-1), 74.7 (C-2), 77.2 (C-3), 75.8 (C-5), and 70.9 (C-6) ppm were observed. These were indicative of the presence of 1,6-bound β-D-glucopyranose units. The ratios of 1,3:1,6 bonds were calculated from PMR spectra and were 7:1, 8:1, and 5:1 for 4PpL, 5PpL, and 7PpL, respectively.

Fucoidans were purified by anion-exchange chromatography over Macro-Prep DEAE with elution by a linear gradient H_2O:NaCl (2M). Figure 2 shows the elution profiles. As a result, three fucoidan fractions were obtained from the alga sample of each collection time, i.e., 4PpF1, 4PpF2, 4PpF3, 5PpF1, 5PpF2, 5PpF3, 6PpF1, 6PpF2, 6PpF3, 7PpF1, 7PpF2, and 7PpF3. Table 1 lists the properties of these.

TABLE 1. Properties of Fucoidans Obtained by Anion-Exchange Chromatography over Macro-Prep DEAE*

Fucoidan	Eluent [NaCl], M	Content, %**			Monosaccharide composition, mol/mol Fuc					
		total sugar	SO_3Na	polyphenols	Fuc	Gal	Man	Xyl	Rha	Glc
4PpF1	0.3–0.6	34.7	4.1	0.7	1.0	0.2	0.4	0.3	0	0.4
4PpF2	0.6–0.9	31.7	14.4	1.9	1.0	0.3	0.2	0.3	0.1	0
4PpF3	0.9–1.1	25.7	18.5	2.2	1.0	0.3	0	0.1	0.3	0
5PpF1	0.5–0.8	38.4	2.0	0.3	1.0	0.5	0.4	0.8	0.1	0.4
5PpF2	0.8–1.2	30.5	10.6	1.3	1.0	0.3	0.3	0.4	0.1	0.3
5PpF3	1.2–1.4	21.5	18.2	1.1	1.0	0.3	0	0.1	0.1	0.1
6PpF1	0.1–0.2	24.7	1.3	0	1.0	0.3	0.6	0.4	0.3	0.4
6PpF2	0.5–1.3	26.2	8.9	0.9	1.0	0.6	0.5	0.1	0.4	0.2
6PpF3	1.3–1.7	21.2	13.7	1.4	1.0	0.9	0.3	0.1	0.3	0.3
7PpF1	0.5–0.9	31.5	4.4	0.3	1.0	0.4	0.4	0.1	0.4	0.2
7PpF2	0.9–1.3	25.3	14.9	1.1	1.0	0.6	0.4	0.1	0.4	0.2
7PpF3	1.3–1.7	15.5	17.9	1.5	1.0	1.4	0.2	0.1	0.3	0.3

*Compounds of protein nature determined by the Bradford method [20] were absent in all fractions; **wt%.

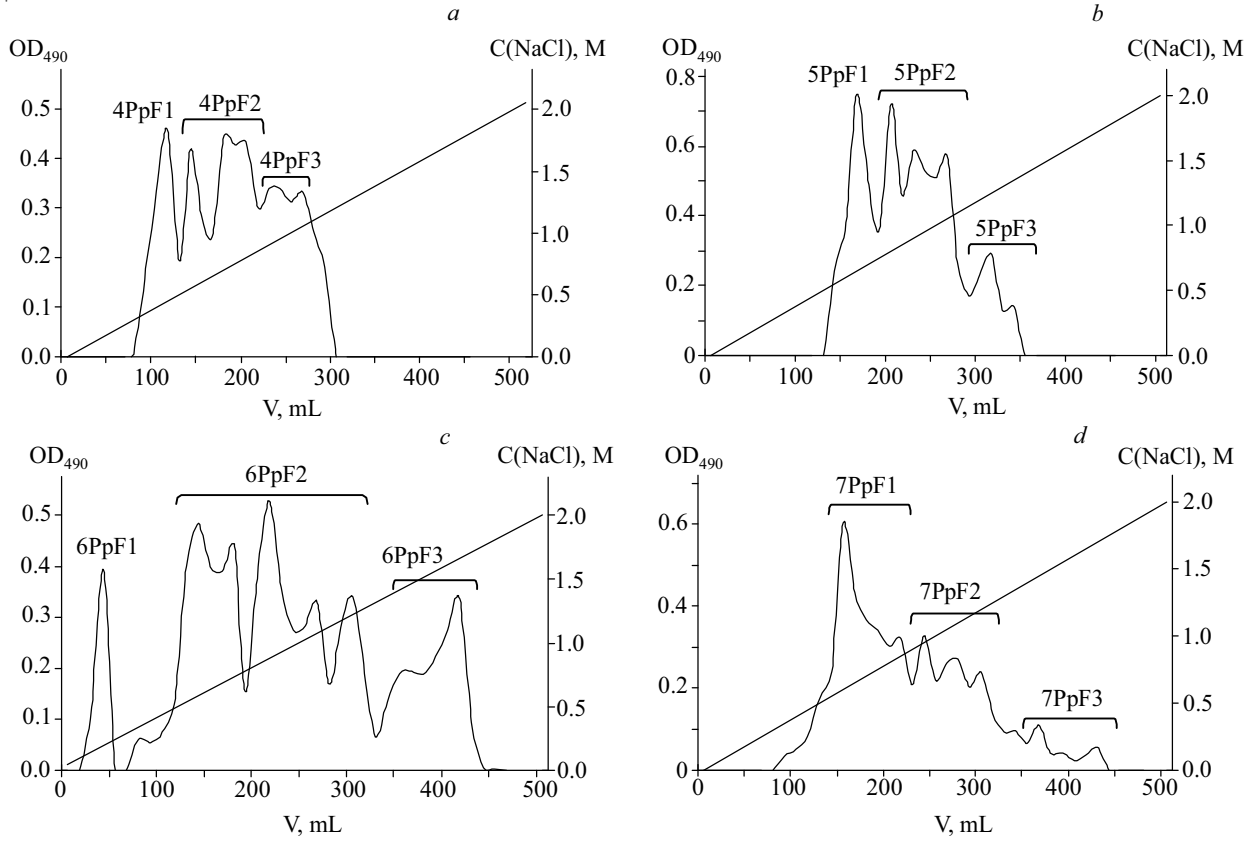

Fig. 2. Ion-exchange chromatography over Macro-Prep DEAE (Cl^--form, 2.5 × 9 cm) of fucoidans from brown alga *Padina pavonica* of various collection times: April (*a*), May (*b*), June (*c*), July (*d*).

Fucoidans are a family of sulfated fucose-containing polysaccharides. They are exceedingly variable, from polysaccharides with a high uronic acid content and low fucose and sulfate content to practically pure α-L-fucans where the principal monosaccharide unit is fucose [2, 15]. All fucoidans isolated by us were sulfated heteropolysaccharides, like analogous polysaccharides isolated from alga of the genus *Padina* [9, 13, 16].

According to the literature, fucoidans isolated from *P. pavonica* contained in various proportions fucose, xylose, glucuronic acid, galactose, and sulfates [17]. Differences in the composition and content of alga polysaccharides were related to the collection site and time. The fucoidans isolated by us were heterogeneous. The elution profiles of the fractions obtained from anion-exchange chromatography suggest that the fucoidan fractions had different charges (Fig. 2). Table 1 presents their monosaccharide compositions.

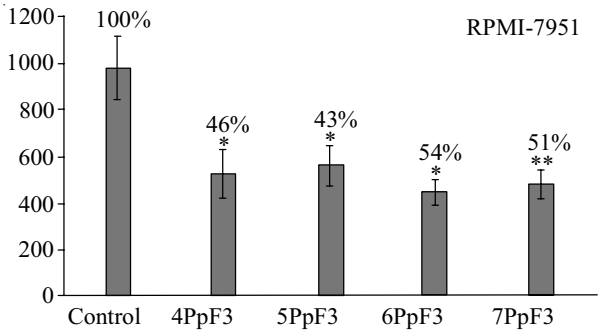

Fig. 3. Inhibiting action of fucoidans (4PpF3, 5PpF3, 6PpF3, and 7PpF3) on growth of RPMI-7951 human melanoma cell colonies.

Fractions of isolated polysaccharides obtained from anion-exchange chromatography contained fucose, galactose, mannose, xylose, rhamnose, and glucose. The contents of rhamnose and galactose increased from April to July. The contents of mannose and xylose decreased in fucoidans of all alga samples on going from the first to the third fraction. The principal monosaccharide of practically all fucoidans was fucose. The exception was high-sulfated heterogeneous fucogalactan 7PpF3. The heterogeneity of the monosaccharide composition of the isolated polysaccharides decreased and the degree of sulfation increased with increasing eluent concentration. Thus, both low-sulfated and high-sulfated fucoidans were isolated from each alga sample (April–July). The lowest extents of sulfation (1.3–4.4%) were observed for fractions 4PpF1, 5PpF1, 6PpF1, and 7PpF1, which were eluted by low concentrations of NaCl (0.1–0.8 M). The highest amounts of sulfates (13.7–18.5%) were found in the last alga fractions of each collection month, i.e., 4PpF3, 5PpF3, 6PpF3, and 7PpF3. The isolated fucoidans contained a small amount (up to 2.2%) of polyphenols. Proteins were not observed in them.

The heterogeneous structure of fucoidans isolated from *P. pavonica* was confirmed by PMR spectra. Like many other fucoidans from alga, NMR spectra of the initial fucoidans were complicated and provided little information for structure analysis. Nevertheless, groups of resonances corresponding to H-1 anomeric protons (4.8–5.5 ppm) and resonances characteristic of carbohydrate ring protons H-2–H-5 (3.2–4.7 ppm) could be identified. The presence of fucose units was confirmed by strong resonances at high-frequency fields (1.1–1.4 ppm) that were typical of fucopyranoside H-6.

Polysaccharides isolated from alga are known to exhibit various biological activities such as antitumor, antiviral, anti-inflammatory, antiangiogenic, and immunostmiulating [2, 15].

According to the literature, heteropolysaccharides isolated from *P. pavonica* exhibited anticoagulant activity [13]. Fucoidans isolated from *P. gymnospora* exhibited anticoagulant [16] and antioxidant activity [18]. Sulfated fucoidan derivatives from *P. tetrastromatica* showed antiviral activity against herpes virus types 1 and 2 [9].

It seemed interesting to determine the antitumor activity of fucoidans isolated by us from *P. pavonica* and to compare it with the activities of fucoidans from *Dictyopteris polypodioides* and *Sargassum* sp. from the Mediterranean Sea that we reported earlier [14]. Treatment of RPMI-7951 cells with fucoidans from *P. pavonica* (4PpF3, 5PpF3, 6PpF3, and 7PpF3) at concentrations up to 200 μg/mL did not inhibit their growth and was not accompanied by mass lethality. Growth inhibition of RPMI-7951 cell colonies treated with fucoidans isolated from alga samples of various collection times were 46%, 43, 54, and 51 (April, May, June, and July, respectively) (Fig. 3).

We showed previously that the growth inhibition of RPMI-7951 cell colonies by fucoidans from alga of the Mediterranean Sea *D. polypodioides* (200 μg/mL) was 44%. Fucoidan from the *Sargassum* sp. exhibited weaker inhibition, 28%. *D. polypodioides* and *P. pavonica* belong to the same family Dictyotaceae and exhibit similar inhibition by fucoidans on the growth of RPMI-7951 human melanoma cell colonies. The specimens of *D. polypodioides* and *Sargassum* sp. were collected in July whereas the fucoidan with the greatest antitumor activity from *P. pavonica* was from the June collection. It is possible that fucoidans from the June specimens of *D. polypodioides* and *Sargassum* sp. had higher antitumor activity. Nevertheless, the growth inhibition of RPMI-7951 cell colonies was also high for fucoidan 7PpF3 (51%), which was isolated from the July sample of *P. pavonica*, and exceeded the analogous values for fucoidans from *D. polypodioides* (44%) and *Sargassum* sp. (28%). Thus, *P. pavonica* is the most promising for producing fucoidans of the three studied algae that are widely distributed in the Mediterranean Sea.

EXPERIMENTAL

Reagents and Materials. EtOH, acetone, NaOH, and inorganic acids and salts were commercial products (Diaem, Russia). Standards (mannose, rhamnose, maltose, glucose, galactose, xylose, bovine serum albumin) were from Sigma (USA). Chromatography resins were Polikhrom-1 (Reakhim, Russia) and Macro-Prep DEAE (Bio-Rad, USA).

Polysaccharide Extraction. Dried defatted alga *P. pavonica* (Pp, 50 g) was extracted (2×) with HCl solution (0.1 M) (1:20 ratio) for 2 h at 60°C. The extracts were neutralized and centrifuged. The supernatant was concentrated in a rotary evaporator, dialyzed against distilled water, and lyophilized to produce fractions containing WSPS 4PpFL (0.51 g), 5PpFL (0.43), 6PpFL (1.00), and 7PpFL (1.06) from *P. pavonica* samples collected in April (4), May (5), June (6), and July (7), respectively. Alga remaining after the extraction was extracted with Na_2CO_3 solution (2%, 1:30 ratio) for 2 h at 55°C. The extracts were centrifuged. The supernatant was dialyzed against distilled water. Alginic acid was precipitated from the extracts by three volumes of EtOH (96%). The precipitates were washed successively with EtOH (96%) and acetone and dried in air to produce alginic acid fractions (A) 4PpA (6.10 g), 5PpA (8.49), 6PpA (4.72), and 7PpA (7.71).

Anion-exchange Chromatography. Fractions containing WSPS (4PpFL, 5PpFL, 6PpFL, 7PpFL) were dissolved in HCl (0.04 M) and placed on a Macro-Prep DEAE column (8 × 2.5 cm). Neutral polysaccharides were eluted by HCl (0.04 M); charged WSPS, by a linear gradient H_2O:NaCl (2M). The fractions were dialyzed against distilled H_2O, evaporated in a rotary evaporator, and dried (lyophilization) to produce fucoidan fractions (F) 4PpF1 (42.3 mg), 4PpF2 (67.9), 4PpF3 (7.0), 5PpF1 (12.9), 5PpF2 (33.1), 5PpF3 (13.0), 6PpF1 (7.7), 6PpF2 (107.7), 6PpF3 (20.8), 7PpF1 (33.2), 7PpF2 (76.3), 7PpF3 (54.7).

Hydrophobic Chromatography over Polikhrom-1 (polytetrafluoroethylene). Fractions containing neutral polysaccharides were placed on a Polikhrom-1 column (7 × 0.7 cm) and rinsed successively with H_2O and aqueous EtOH (5%). Laminarans were eluted by aqueous EtOH (15%) (until the eluent gave a negative reaction for total carbohydrates), evaporated in a rotary evaporator, and lyophilized to produce laminaran fractions (L) 4PpL (2.7 mg), 5PpL (2.5), and 7PpL (2.9).

The content of carbohydrates was determined colorimetrically using the reaction with phenol and H_2SO_4 [19] and glucose and fucose as standards; **the content of proteins**, by the Bradford method [20] using bovine serum albumin as a standard; **the content of polyphenols**, colorimetrically by the Folin–Ciocalteau method [21] using phloroglucinol as a standard.

Acid Hydrolysis of Polysaccharides. A sample (5 mg) was dissolved in TFA (0.5 mL, 2 N). Hydrolysis was carried out at 100°C for 6 h. Samples were neutralized by aqueous NH_4OH and evaporated to dryness.

The monosaccharide composition of polysaccharides after hydrolysis was determined on a Biotronik IC-5000 carbohydrate analyzer (Germany) using a Shim-pack ISA-07/S2504 column (0.4 × 25 cm) and elution by potassium borate buffer at flow rate 0.6 mL/min. Bicinchoninate was used for detection. The integrating system was Shimadzu C-R2 AX. The standards were maltose, rhamnose, mannose, fucose, galactose, xylose, and glucose.

Sulfate content in polysaccharides was determined turbidimetrically [22].

Kinematic viscosity was measured using a thermostatted capillary viscosimeter with the corresponding capillary constant at 25°C.

PMR and ^{13}C NMR spectra were taken from D_2O solutions of compounds on a Bruker DPX-500 NMR spectrometer at 30°C using acetone and MeOH as internal standards.

Cultivation of Cells. RPMI-7951 human melanoma cells were cultivated in MEM growth medium and FBS (10%) with added penicillin (100 U/L) and streptomycin (100 μg/L) in a Sanyo MCO-18AIC incubator (Japan) at 37°C and 5% CO_2.

Determination of Fucoidan Cytotoxicities. RPMI-7951 human melanoma cells (2×10^4/mL) were seeded into 96-well plates, cultivated in RPMI-1640 (200 μL, 10%) in a CO_2-incubator at 37°C for 24 h, treated with fucoidans at various concentrations (10, 50, 100, and 200 μg/mL), incubated for 24 h, treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoline bromide (MTS reagent, 15 μL), and incubated for 4 h (37°C, 5% CO_2). Optical density was measured on a Bio-Tek Instruments spectrophotometer (USA) at 490/630 nm ($A_{490/630}$).

Formation of Cancer Cell Colonies (Soft Agar Method). The action of brown alga fucoidans on the formation and growth of RPMI-7951 human melanoma cell colonies was determined by the soft agar method.

Statistical data treatment was performed using the Student *t*-criterion at a given confidence probability of 95% (SigmaPlot 2000, version 6, SPSS Inc., USA).

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