The Effect of Collagenic Gels with Marine Algae Extracts Mixtures in the Treatment of Recurrent Aphthous Stomatitis

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The aim of our researches was represented by the possibility of use in dentistry the natural resources offered by the Black Sea biosystem. The first objective of our research was represented by the manufacture of the collagenic gels, of the algae extracts and of the mixture, in reference with the physical and chemical properties (pH, viscosity, organoleptical properties), of each component in part and of the mixtures. The second objective of the research was the determination of the tolerability and of the healing properties of six products realized by the collagenic gel extract from marine fish skin (shark and turbot skin), combined with three marine algae (Cystoseira barbata, Ulva lactuca, and Ceramium rubrum), in the treatment of recurrent aphthous stomatitis. The trial was conducted on 186 patients (97 women and 89 men), affected by recurrent aphtous stomatitis lesions. The obtained results showed that the most beneficial in the healing action was obtained with the collagenic gel containing 10% Ceramium Rubrum. In conclusion, the resources offered by the Black Sea biosystem can be used, with good results, to reduce the healing time and the recurrence in patients suffering by aphtous stomatitis.

Keywords: marine fish and algae, mixtures, aphtous stomatitis, treatment

The aim of our researches was represented by the possibilities to use, in dental medicine, the natural resources offered by the Black Sea biosystem.

The purpose of this paper is to present a brief description of the biotechnology to obtain new pharmaceutical formulations, through incorporation into a prepared collagenic gel (obtained from fish skin), three marine algae extracts (represented by *Cystoseira barbata, Ulva lactuca, Ceramium rubrum* extracts), and to highlight our results in the treatment of aphthous stomatitis by using these mixtures.

Collagen is now the base of a dynamic industrial development of various products applied in medicine, pharmacy, cosmetics, a.s.o [1].

Although to date, 29 different types of collagen have been identified, type I collagen is the most abundant and still the best studied [2].

In the present, various methods are available for the extraction of collagen from the skin of many species of fish [3-5].

The structural modifications of the marine biomass (performed by the biotechnological processes of extraction), and the production of type I hydrolysed fibrillar collagen (by lyophilisation techniques), in order to obtain hydrogels, porous matrixes, or membranes (by free drying) and powders (by atomization process), as well as the actions of the active ingredients in the bio-cellular level are considered to be acting as nanomaterials at trans-dermal and trans-mucous levels [6-9].

It has been scientifically proved that there is a close connection between the cutaneous and mucous diseases, hereditary and environmental causes, vitamins deficiencies and infection with various microorganisms [10,11]. All these diseases can present different degrees of severity, including systemic morbidity [12-14].

The treatments with natural substances/mixtures of the soft tissues diseases in the oreo-facial area present great interest in dentistry. The anti-inflammatory ingredients obtained from natural resources, is well known, today can be used in the regenerative therapy of the affected soft tissues [15-18]. These anti-inflammatory ingredients can be obtained from natural resources such as marine algae [19,20].

In the same time, it is proved that marine algae have an anti-inflammatory action after infection with some gram negative and gram positive germs [21,22].

The recurrent aphthous stomatitis is one of the most common painful oral mucosal conditions of patients, present first in childhood or adolescence [23]. These affect 2-66% of the international population, and it affects more than 30% of the adult population and up to 37% of children of school age [24]. Despite their high prevalence, the etiopathogenesis of this disease remains unclear, and the actual revised theory is in reference with the multifactorial etiology(predisposing factors, immunological components, a.s.) [25].

The recurrent minor aphthous stomatitis is the most common form of aphthous stomatitis, accounting for 80% of all cases. Aphthous ulcers are shallow ulcers in the mucosa of the mouth. They may appear anywhere in the mouth, but are frequently found on the inside of the lower lip or cheeks, or on the sides or base of the tongue. Painful and shallow, minor recurrent aphthous ulcers are smaller than 1 cm in diameter, and one or more ulcers can be present. This condition is disabling and the symptoms are noisy. The ulcers may appear whitish or yellow with a red

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base, and with a grayish layer that develops when they begin to heal. Fibrin covered ulcerations with a hyperaemic halo are typically visible on the oral mucous membrane The lesions heal without scarring within 1 to 2 weeks to months. The periodicity varies between individuals, with some having longer ulcer-free episodes and some never being free from ulcers [26]. Recurrent minor aphthous stomatitis, with 3-6 attacks per year, is restricted to the oral mucosa, and can be differentiated from complex aphthae (less than 5% of aphthosis cases), which present few unusual multiple lesions, are extremely painful, and heal slowly [27,28].

Experimental part

Materials and Methods

The first objective of our research was represented by the manufacturing operations of the collagenic gels, of the algae extracts and of the mixture, in reference with their physical and chemical properties, such as their *p*H, viscosity and organoleptical properties, of each component in part and of the mixtures.

Type I fibrillar collagen, a protein with triple-helix structure, can be obtained from the skin and cartilage tissues. The extraction of collagen from the fish skin is an easier process and has a higher yield than the extraction from mammalian skin. Also, present a relatively low risk in pathogen agents' content [29,30].

The collagen gels used in this study were extracted from shark skin *(Squalus acanthias)* and turbot skin *(Scophthalmus rhombus)* (fig. 1).

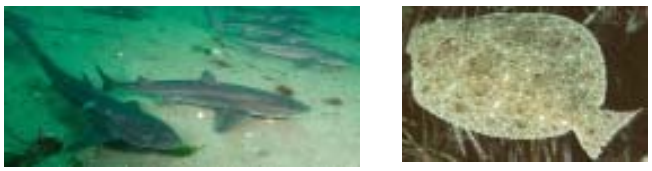

Fig. 1. The aspect in nature of the marine fishes used in our researches: Shark *(Squalus acanthias)* and turbot *(Scophthalmus rhombus)*

Our method of extracting the collagen from marine fish skin (collagen which was used ulterior to prepare pharmaceutical formulations including marine algae), consisted by the dissection of fish muscles, cutting the skin into small pieces, and the suspension, in approximately 10 volumes of 0.5M acetic acid, at a temperature of 0-5°C (because the denaturizing temperature of the collagen is low, was performed below 5°C). After soaking for a few days and stirring, the soaked pieces of skin were disintegrated using a glass homogenizer. A few more days we performed stirring, then, the suspension was filtered through a thin-spun cloth and then centrifuged at 15,000 rpm for one hour. The protein was precipitated by adding sodium chloride with a final concentration of 10%. The collagen was collected by centrifugation, re-dissolved in 0.5M acetic acid, centrifuged and precipitated by dialysis against a large volume of disodic-phosphate, then dissolved in 0.5M acetic acid, dialyzed for a long time against the same solvent, and finally, centrifuged and lyophilized. The lyophilization caused the decrease of solubility and of the emulsifying capacity. Freeze drying is an important process in sample preparation and for the preservation and storage of the pharmaceuticals. We used a Labconco Freeze-Dry systems with 12L capacity, -50° C temperature and 3/4hp refrigeration (fig. 2).

The marine algae, selected for our study for the preparation of the new pharmaceutical formulations for

Fig. 2. The aspect of FreeZone 12 Liter Console Freeze Dry System, used in our researches

the treatment of the aphtous lesions, were the extracts of seaweed *Cystoseira Barbata, Ulvae Lactuca* and *Ceramium rubrum* (fig. 3).

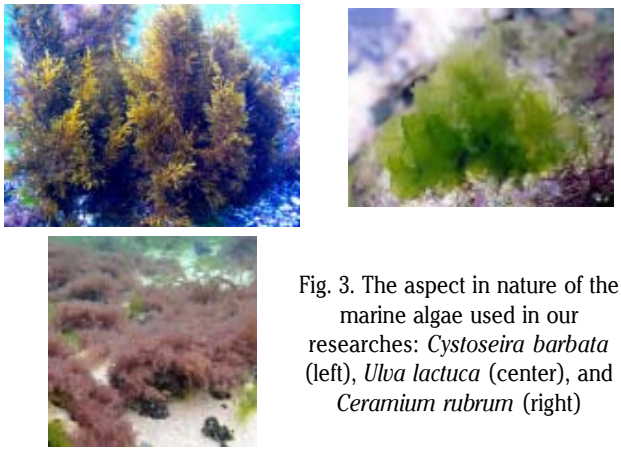

Fig. 3. The aspect in nature of the marine algae used in our researches: *Cystoseira barbata* (left), *Ulva lactuca* (center), and *Ceramium rubrum* (right)

The well-dried algae were crushed, then grounded into a mortar, until were obtained a very fine powder (to ensure a large, intimate contact surface between the plant and the solvent, in view to reducing the extraction time). The resulted powders were sieved through a 0.3 mm/mm sieve. The extraction of active ingredients was achieved by using water solution of ethyl-alcohol with a concentration of 70% by weight (g alcohol/g solution) or 74.5% by volume. Extraction lasted for 18 h at room temperature (24°C) in dark brown glass vessels, perfectly sealed (to avoid the action of light and air, as well as alcohol evaporation). The used weight ratio algae powder/hydro-alcoholic solution was of 1/4 (20 g algae for 100 g of mixture). The mixtures were stirred from time to time, in order to increase the diffusion speed of the substances contained by algae, and to reduce extraction time. The algae powders were deposited in the solution of 70% ethyl alcohol. Together with the supernatant, they formed a very clear separation interface. Measurements were made to find the concentrations of solid substances in the three hydro-alcoholic algae extracts, by evaporation in the conditioning oven, at 105°C, until constant weight was reached.

The remaining residues after dry evaporation at the mentioned temperature were redissolved entirely in the 70% solution of ethyl-alcohol for the *Ulva lactuca* alga.

The remaining residues for the *Cystoseira barbata* and *Ceramium rubrum* algae were redissolved only partially, which demonstrates that, indeed, in these cases, alterations occurred in some of the components during the process of heating up to 105°C for the evaporation of alcohol and water, and that these alterations entailed a decrease in solubility.

Table I presents some the characteristics of the three marine algae extracts: the contents in dry substance, the *p*H, and the aspect of the extracts. As can be noticed from the table, the highest concentration of components in algae for the 18 h extraction using 70% ethyl-alcohol was obtained for *Cystoseira barbata,* followed by *Ceramium*

Alga	Dry substance contents (g/100 g solution)	pH	The extract aspect
Cystoseira barbata	3,68	4,0	Brown in thick layer Yellowish-green in thin layer
Ulvae Lactuca	2,51	4,5	Dark green with brownish hues in thick layer Green in thin layer
Ceramium Rubrum	2,88	4,0	Dark green with slightly brownish hues in thick layer Green in thin layer

Table 1
THE CHARACTERISTICS OF THE HYDRO-ALCOHOLIC EXTRACTS OBTAINED FROM THE THREE ALGAE

rubrum, while the lowest concentration was that for *Ulvae lactuca.*

As far as the *p*H levels of algae extracts are concerned, they all lie in the weak acid area and virtually had the same values, within the limits of experimental errors, close to that of the initial collagen gel, which were 3.5. *p*H levels were measured by using Merck paper. It was therefore likely that introducing them into the collagen gel should not alter its pH level.

The colour of hydro-alcoholic algae extracts was determined by:

- the initial colour of the algae in fresh cut condition (which was brown for *Cystoseira barbata*, green for *Ulva lactuca*, and red *Ceramium*); they have become green in dried conditions, with a darker shade of green for the colored ones (*Cystoseira barbata* and *Ceramium rubrum*) and a lighter shade for the green alga (*Ulva lactuca*);

- the dried substances concentration (5 or 10%; the first extract presented a lighter in color, while the second concentration of extracts presented, by far, the darkest color).

The extracts from marine algae were incorporated in type I non-denatured collagen matrices.

The collagenic gel containing marine algae extracts could be obtained with optimum efficiency, having well profiled organoleptic properties. In order to establish (based on rheological measurements), whether there are interactions between the components of the three hydro-alcoholic algae extracts and the 0.6% type I fibrillar collagen gel, we prepared gels with the same collagen concentration, which contained a concentration of 5 and 10% by weight of algae extracts, respectively, solution of 70% ethyl alcohol (the dispersing medium for the collagen gels with algae extracts). We have not used an increased concentration of algae extracts, nor of ethyl-alcohol, because ethyl-alcohol can cause the viscosity decreasing of the collagen-based gel, and bigger quantity being likely to lead to its destruction, by dissociation into two phases, one rich in water, and other rich in collagen.

The gels were prepared at room temperature, by addition of appropriate amounts of distilled water and hydro-alcoholic extract of seaweed, and than we had stirred the mixtures. All gels were placed in a refrigerator at the temperature of 4°C, for maturation. They were stirred from time to time during the first four hours, after which they were left to rest for 12 hours minimum, and then subject to shearing. After maturation, the gels with neutral pH presented a higher viscosity. A specification must be made: indeed, the dilution from 1.64 to 0.6% of collagen extract with distilled water and the presence of the three algae extracts did not alter the *p*H of the initial collagen gel, so, the pH value of all included gels in the table II was of 4. We prepared gels with the same amounts of 70% ethyl alcohol hydro-alcoholic algae extracts with neutral *p*H, by adding very small amounts of very concentrated solution of sodium hydroxide, in order to not decrease the concentration (the introduced amounts were in the range of 0.03-0.04 mL, therefore the dilution can be neglected).

From organoleptical point of view, the prepared collagenic gels with algae extracts showed optimum properties to be used in the treatment of aphtous stomatitis. In the table 2 are presented the algae extracts

Fig. 4. The Aspect of Rheoviscosimeter Haake VT 550, used in our researches

concentrations, the viscosity at zero shearing speed, respectively the aspect and the colour of obtained gels.

The combined gels have been subject to the rheological measurements at 25± 0.10C, after at least 15 minutes of thermostatic treatment.

We have used in our researches, a rotation viscosimeter Haake VT 550 with coaxial cylinders, which is capable of developing a shearing speed, γ, with values between 0.6 and 3.0x104, with values between 1 and 105 Pa and τ, s-1, of measuring shearing tensions, η*, depending on the sensors system used for measuring the apparent viscosities (between 1 and 109 mPa.s.) (fig. 4).

Based on the data obtained for the shearing tensions, we have traced the rheograms (the diagrams for shearing tensions depending on the shearing speed values), out of which we have calculated the apparent viscosities (as proportions between shearing stress and speed), which have been figured in relation to the shearing speed (in order to level the dependency). The rheograms have shown the type of rheological behaviour for each gel.

Collagenic gel/ Alga extract	The algae extract's concentration, %	Viscosity at zero shearing speed, mpa.s	The aspect and the color of obtained gels
Collagenic gel without algae	5% of 70% alcohol sol.	211,020	Colourless, clear
	10% of 70% alcohol sol.	3413,81	Colourless, barely opalescent
Cystoseira barbata gel	5%	2084,97	Opalescent, yellowish green
	10%	2813,94	More opalescent, brownish yellow
Ulvae lactuca gel	5%	3248,96	Clear, barely green
	10%	2978,05	Barely opaque, barely green
Ceramium rubrum gel	5%	2380,59	Barely opalescent, yellowish green
	10%	2656,64	More opalescent, brownish green

Table 2
THE CHARACTERISTICS OF THE PREPARED COLLAGENIC GELS AND OF THE MIXTURES

Fig. 5. The aspect of the collagenic gels with marine algae extract used in our researches

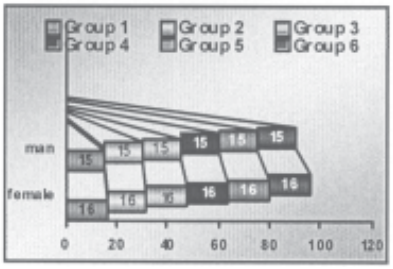

Fig. 6. The distribution of patients in groups, after gender and the used gel

Type of gel	Objective / Subjective		Assessment / no. of patients				
			1	2	3	4	5
Collagenic gel with alga *Cystoseira Barbata* 5% 31 patients	Evolution	Good	5	11	15	21	28
		Unsatisfactory	26	20	16	9	3
	Pain	Absent	3	8	13	17	27
		Present	28	23	18	14	4
Collagenic gel with alga *Cystoseira Barbata* 10% 31 patients	Evolution	Good	7	12	19	23	29
		Unsatisfactory	24	19	11	8	2
	Pain	Absent	5	10	14	19	28
		Present	26	21	17	12	3
Collagenic gel with alga *Ulvae Lactuca* 5% 31 patients	Evolution	Good	4	11	17	21	26
		Unsatisfactory	27	20	14	10	5
	Pain	Absent	2	6	12	20	24
		Present	29	25	19	11	7
Collagenic gel with alga *Ulvae Lactuca* 10% 31 patients	Evolution	Good	6	15	21	24	28
		Unsatisfactory	25	16	10	7	3
	Pain	Absent	4	8	14	23	26
		Present	27	23	17	8	5
Collagenic gel with alga *Ceramium Rubrum* 5% 31 patients	Evolution	Good	8	14	23	25	31
		Unsatisfactory	23	17	8	6	-
	Pain	Absent	6	12	15	21	30
		Present	25	19	16	10	1
Collagenic gel with alga *Ceramium Rubrum* 10% 31 patients	Evolution	Good	11	17	24	28	31
		Unsatisfactory	20	14	7	3	-
	Pain	Absent	8	12	17	26	31
		Present	23	19	14	5	-

Table 3
THE EVOLUTION OF THE SYMPTOMATOLOGY

Because the collagenic gels had a pseudoplastic behaviour, in order to determine their dynamic viscosity, the curve levels of their apparent viscosity has been performed, dependent on the shearing speed (by performing the logarithm of both the variable, the function and the extrapolation of the line at zero value of the shearing speed logarithm). In this way, we obtained the so-called viscosity at zero shearing speed, η_o. The zero value of the shearing speed logarithm represents the 1 value of the shearing speed. At the shearing speed of 1 s^{-1} is low enough to consider that all systems have a Newtonian behaviour (the viscosity at zero shearing speed is considered the absolute viscosity of the fluid).

We mention that, for all samples realized for our studies, the quantity of used collagen was 0.6%.

The second objective of the research was the determination of the tolerability and of the healing properties of the six products, realized by the collagenic gel extract from marine fish skin (shark and turbot), combined with the marine algae extracts (Cystoseira barbata 5 and 10% concentration, Ulva Lactuca 5 and 10% concentration, Ceramium rubrum 5 and 10% concentration). All these products were used in the treatment of affected the oral cavity mucosa by aphthous stomatitis. Practically, we determined the required number of applications and the needed time of healing, for every gel in part, and the remission period.

The trial was conducted on 186 patients (97 women and 89 men), affected by aphtous stomatitis lesions. The injuries due to canker sores were treated with collagenic gel containing:

- *Cystoseira barbata* 5%, the first group of patients (31 patients, 16 female and 15 man);
- *Cystoseira barbata* 10%, the patients belong to the second group (31 patients, 16 female and 15 man);
- *Ulva lactuca* 5%, the third group of patients (31 patients, 16 female and 15 man);
- *Ulva lactuca* 10%, the fourth group of patients (31 patients, 16 female and 15 man);
- *Ceramium rubrum* 5%, the patients of group five (31 patients, 16 female and 15 man);
- *Ceramium rubrum* 10%, the sixth group of patients (31 patients, 16 female and 15 man).

The distribution of the patients in groups, after gender and after the used gels, is presented in figure 6.

The application of the gels was performed on the affected area four times daily, for 7 days.

The evolution of the clinical symptomatology, after the first application of gels, was performed daily in the first three days, in the 5th day, and 7th day, in order to ascertain the healing process, both objectively (the healing stage of aphtous lesions and the remission period of the disease), and subjectively (the degree and the time of disappearance of the pain). All the patients were followed for 1 year and 6 months.

The objective assessments consisted by recording the decreasing level of the dimension and of the redness (erythema) of aphtous lesion and the remission period of disease. For the subjective assessments, the patients were asked to relate the presence/absence of pain on tasting, speaking, and eating/chewing.

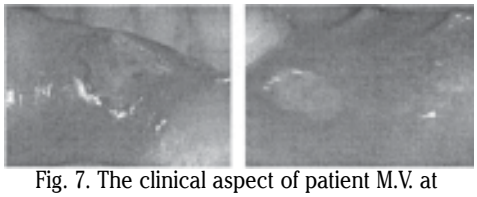

Fig. 7. The clinical aspect of patient M.V. at presentation (left) and after 3 days of treatment (right) with collagenic gel with *Ceramium rubrum 10%*

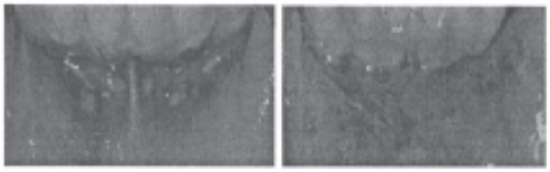

Fig. 8. The clinical aspect of patient S.A. at presentation (left) and after 5 days of treatment (right) with collagenic gel with *Ceramium rubrum 5%*

Results and discussions

The evolution of the clinical symptomatology in the patients with aphtous stomatitis treated with the collagenic gels with seaweed extracts is visualized in the table 3.

The results of our researches showed that the most beneficial and the quickest action was obtained by using the collagenic gel containing *Ceramium Rubrum* 10%. In decrees order, the beneficial results were obtained with collagenic gels containing *Ceramium Rubrum* 5%, *Cystoseira Barbata* 10%, *Cystoseira Barbata* 5%, *Ulvae Lactuca* 10% and *Ulvae Lactuca* 5%.

Also we emphasize that the aphtous stomatitis recurrence in patients treated with the collagenic gel containing 10% *Ceramium rubrum* extract, was more reduced than in patients treated with the other gels (fig. 7 and 8).

There are visible the dimensional and the profundity differences of aphtous lesion.

The amount of b-carotene and retinol in the alga *Ceramium rubrum* is higher than in other two types of marine algae used in our researches (*Cystoseira barbata* and *Ulva Lactuca*), and this is probably the reason that the mixture of collagenic gel containing the alga *Ceramium Rubrum* 10% induced the best beneficial effect on aphtous stomatitis lesions.

The extracted collagens from marine fish skin form viscous gels, which are very convenient for applications on the affected surfaces by aphtous stomatitis.

In dentistry, collagenic gels can be successfully used as bioresorbable membranes and matrices, due to their potential in incorporation of the active ingredients that are released gradually.

The thermal stability of collagen at elevated temperatures, in the range 170–200°C, is high enough to allow the processing of mixtures with thermoplastic polymers in order to obtain new materials based on collagen-thermoplastic mixtures [1].

The marine algae *Cystoseira barbata, Ulvae lactuca, Ceramium rubrum* and the collagen from *Squalus acanthias* marine fish skin are valuable to turn to good account for natural marine resources by clean biotechnology [31].

The marine algae extracts contain many active principles with therapeutic importance. The study of [33] reveals the antioxidant capacity of some marine seaweed extracts, like the extracts of green algae *Ulva lactuca*, brown algae like *Cystoseira barbata* and red algae *Ceramium rubrum* with a concentrations of 20 mg dry vegetal product/ml solvent. Also, the paper presents the correlation regarding the anti-oxidative capacity and the heavy metals content (Zn, Cu, Pb and Cd) of the marine algae extracts [32].

The species *Cystoseira barbata* present high antioxidant activity, that could be used in the therapy of degenerative diseases [33].

After the researches of Dubber D. and Tilmann H., methanol extracts of *Ceramium rubrum* at 10 mg dry weight per mL, and hexane extracts of *Laminaria digitata* at 31 mg dry weight per mL, evoked strong antibacterial activities and inhibited almost all tested bacteria. The assays revealed different susceptibilities of the bacterial phyla under investigation to algal extracts. Gram-positive marine Bacillaceae were generally more susceptible than Gram-negative marine Vibrionaceae [34].

The red alga *Ceramium rubrum* is well-known as an agar source, and can be used to obtain chlorophyll, green pigment being an useful therapeutic agent [35].

The vitamin B12 content of *Ceramium rubrum* seaweed is high [36].

Some of these species of seaweed are known to have in their thallus considerable quantities of vitamins, microelements or substances with antimicrobial action [37].

Antioxidant activity of marine algae may arise from pigments such as chlorophylls and carotenoids, vitamins and vitamin precursors including α-tocopherol, β-carotene, niacin, thiamine and ascorbic acid, phenolics such as polyphenolics and hydroquinones and flavonoids, phospholipids particularly phosphatidylcholine, terpenoids, peptides, and other antioxidative substances, which directly or indirectly contribute to the inhibition or suppression of oxidation processes [38].

Because of their high content of polyunsaturated fatty acids, proteins, vitamins and minerals, seaweeds have been used for a long time as health promoting supplements for human and animal food. Phycocolloids produced by seaweeds, such as agar, carrageenan and alginates are widely used as gel-forming substances for many purposes in medicine, microbiology and pharmacy, and as laxatives, haemostyptics, antacidics, etc. In recent years, a broad spectrum of bioactive secondary metabolites with, for example, cytostatic (e.g. halomon), antiviral (e.g. sulphated polysaccharides), antibiotic (halogenated terpenes) or other interesting properties have been found [39].

It is obvious that the sea can provide plenty of reaches and research must be further done in order to know and use them [40].

The treatment strategies in aphthous stomatitis must be directed toward providing symptomatic relief by reducing pain, increasing the duration of ulcer-free periods, and accelerating ulcer healing [41].

Worldwide there is a high interest in dental medicine professionals in the prevention and treatment of soft tissue affections of the orofacial system, as well as in the discovery of new pharmaceutical formulations accessible to public, with impact on such diseases [42].

Conclusions

The collagenic gels extracted from shark and turbot skin presented plastic behavior, allowing their use in the development of various pharmaceutical formulations.

The gels with marine algae extracts presented good organoleptic properties and the application the gels were facile.

The reduction of the aphtous stomatitis symptomatology has been observed from the second assessment in all patients, so, all collagenic gels with marine algae extracts have been beneficial.

The therapeutic effect of the collagenic gel containing 10% *Ceramium rubrum* extract was quicker than of the other products.

The recurrence of aphtous stomatitis disease in the patients treated with collagenic gel containing 10% *Ceramium rubrum* extract was more reduced than in patients treated with the other gels.

The offered resources by the Black Sea biosystem can be used, with good results, to reduce the healing time and the recurrence of aphtous stomatitis.

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