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New Formulation with Marine Algae from Black Sea

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Abstract

Seaweed is a natural treasure that can be intensely evaluated for therapeutic purposes. During the past years, it became obvious that the ecosystem presents **a marine algae excedent**, which should be utilized in one way or another. Marine algae have been intensely studied. The superior exploitation of the marine biomass represents a highly important resource for the pharmaceutical industry, supplying raw material for the extraction of bioactive substances (vitamins, sterols, and aminoacids) and various other substances, the purity of which is strongly connected to the state of the marine ecosystem. In present work the extracts from marine algae are incorporated in type I non-denatured fibrillar collagen matrixes for obtain new pharmaceutical product. In order to obtain therapeutic effects at nanostructure level, it is important to know the rheological characteristics of the relevant mixtures of collagen gels and extracts from marine algae selected for use. In this survey we have studied mixtures made of non-denatured fibrillar collagen hydro-gels where different concentrations of marine algae have been incorporated.

Keywords: marine biomass, fibrillar collagen, marine algae, seaweed, bioactive substances

1. Introduction

The first data regarding the use of algae dates back to the year 2500 B.C, while in China they are recorded in literature as early as the 8-6th centuries B.C. In Japan, the product Kombu, which is prepared from *Laminaria (angusta, japonica, religiosa)* and *Alaria* species, is consumed. However, only beginning with the 1670s have they been grown a large-scale. Antique Greeks used algae as a remedy against intestinal worms [1]. In Western countries, algae are not yet accepted as normal daily nutrient, maybe due to their less than appealing aspect and insufficient digestion ability. Nonetheless, in various parts of Europe, algae were used as a food product.

Alongside the Romanian coast, from a qualitative point of view, in the year 1935, the number of macrophytes was 77. Between 1970 and1980, only 68 species were recorded. According to other estimates, at the end of the 1990s, only 38 species of *Clorophytes* were recorded. These observations outline the significant qualitative decline of the Romanian shore macrophytobenthos. During the last seven decades, the progressive diminishing of the macroalgae flora has intensified, with severe consequences on the entire coastal ecosystem. In 199, only the southern part of the Romanian shore exhibited a more specific diversity. Vegetation impoverishment, especially after 1970, is due to natural and anthropologic causes which have deteriorated the marine system (severe frosting, argyle sedimentation on bottom rocks, a decrease in light penetration in the water column due to suspensions, increase in eutrophication) [1][2].

Along with the increase in eutrophication, significant qualitative changes were noted in the structure and function of the macrophytobenthos, starting with the oldest records up to the end of the year 2000. Due to important amounts of suspended particles and plankton, the transparency of the sea water registered significant changes, with a considerable decrease. The position of the compensation point changed in such a way that bottom plants that were growing at depths over 7-8 metres became shadowed. This has contributed to the decline of macrophytes, despite large quantities of nutrients. Consequently, due to a large variability of ecologic factors, these changes of the ecosystem and community structure has lead to certain phytocenses being replaced by others. The consequene has been a change in seasonal and multiannual dynamics of the algae communities. The interaction between various anthropologic factors on vegetation has lead to various results, from a structural simplification to complete disappearance. Following hydrotechnics construction work, the algae bed was covered with mud and/or sand. Under these circumstances, *Cystoseira Ag.* species were replaced by *Cladophora* and *Ceramium,* these macrophytes being usually seen in shore areas, at depths lower than 3 metres, were eutrophication tolerant species have emerged such as *Enteromorpha intestinalis* and *Cladophora vagabunda* [1], [3].

The most frequent species belong to the *Enteromorpha* and *Ceramium* genus, but also *Cladophora, Porphyra Ag.* and *Callithamnion Lyngb.* Usually, *Enteromorpha* species are

mixed in this green algae belt with *Cladophora* species, and, in particular, *Cl. sericea (Huds), Kutz* and, sometimes, *Cl. albida (Huds) Kutz.* and *Cl. laetevirens (Dillw.) Kutz* [4]. Although in a reduced number (qualitatively), the remaining species have developed considerable productivities on the available rocky substrates; the common algae populations, represented primarily by *Enteromorpha* and *Ceramium* species cover 80% of this substrate.

During the past years, it became obvious that the ecosystem presents **a marine algae excedent.** The phytochemical studies on the algae (inferior marine plants belonging to the Thallophyta systemic category) presents the possibility of their usage as raw material, due to the high context in poli-sugars, such as [5], [6]:

- alginates (the alginic acid and its metallic salts, organic bases or their derivates), characteristic for the brown algae,
- the agar-agar (co-polymer of D-galactoze with 3,6-anhydro-L-galactoze, partially esterified with sulphuric acid characteristic for the red algae,
- various L-type monozes-galactoze, 3,6-anhydro-D-galactoze, L-frucoze, D-maluronic and L-guluronic acids, cellulose, chracacteristic for the green algae.
- In addition, aminoacids (especially in green alage, such as *Ulva lactuca*) – among which the 8 that are essential for the human organism, and that impossible to be synthesized: izoleucine, leucine, lizine, metionine, fenil-alanine, treonine, triptofan, valine – are present in the algae.

Furthermore, the extraction of agar-agar from red algae Phyllophora nervosa, Ceramium rubrum, C. elegans, Callithamnion corymbosum, Polysiphonia violaceea) can provide vegetal geloze [3]. For the Romanian littoral area, the marine materials are easily accessible [4]. In literature on the matter, attempts of obtaining pharmaceutical products from the Romanian shore marine algae as alcoholic extracts incorporated in collagen hydrogels have been recorded [2]. The active principles identified in the Black Sea algae have drawn researchers' attention for their use in obtaining new pharmaceutical products [5][7][8].

The present study outlines the obtaining of new pharmaceutical formulations using marine algae, collagen, and hyaluronic acid. Each of these components has their own individual properties. The main goal is obtaining time-stable products with beneficial effects on various skin disorders.

2. Materials and Methods

The materials used are represented by green marine algae from the *Enteromorpha intestinalis* and *Cladophora vagabunda* species. Enteromorpha (Enteromorpha intestinalis),one of the mmost common green seaweeds to be found in shallow rockpools which copes with high temperatures and changes in salinity [3], [4]. From macroscopic and microscopic observations, the following descriptions can be made [3]:

Table 1. The macroscopic examination of the analysed marine algae [3], [5]

Cladophora Vagabunda	Enteromorpha intestinalis
Multiannual alga, 10–15 cm tall; Strong discoidal rhizoid; Filamental tale with ramifications, Bushy aspect; Filaments formed of long continuous cells; Terminal ramifications in groups of 4–6, bent as a sickle.	green multimulticellular alga, with a single-cell layer tale height – from a few cm to 1m, width – from 1 mm to 10 cm; disk-like rhizoid; short, cylindric cauloid; intestine-like filoide.

Table 2. The microscopic examination of the analyzed algae [3]

Cladophora Vagabunda	Enteromorpha intestinalis
Multiannual alga, 10–15 cm tall; Strong discoidal rhizoid; Filamental tale with ramifications, Bushy aspect; Filaments formed of long continuous cells; Terminal ramifications in groups of 4–6, bent as a sickle.	green multimulticellular alga, with a single-cell layer tale height – from a few cm to 1m, width – from 1 mm to 10 cm; disk-like rhizoid; short, cylindric cauloid; intestine-like filoide.

The algae used were in powder form.

Type I fibrillar collagen

Collagen hydrolysate is obtained from bovine skin. Collagen, under all its characteristic forms, presents as a polymer with triple helix structure, which individualizes through intense hydrophilia, variable ionic characters and diverse functionality (Fig. 3).

Fig. 1 *Cladophora Vagabunda*

Fig. 2 Enteromorpha intestinalis

It can be involved in a wide number of interaction systems with other micro- or macromolecular components. Currently, in vertebrates, at least 27 different types of

collagen are known, which exhibit a remarkable diversity in what regards molecular and supramolecular organisation, tissue distribution and function.

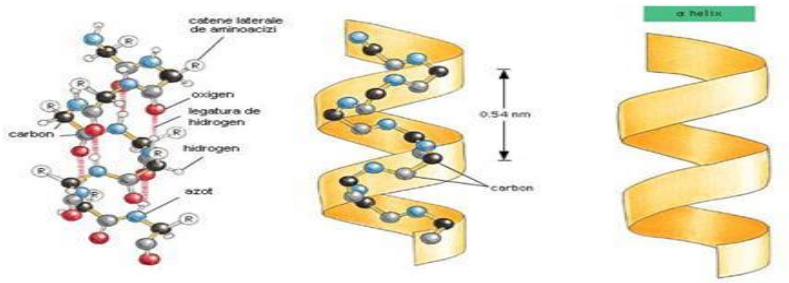

Fig 3 Collagen triple helix function

From this material, mixtures with varying collagen and Black Sea green algae composition were made (Fig. 4).

Fig 4 Products obtained from a collagen and different percentages of green algae

In order to select the product with the best stability, rheological studies were performed. In stability tests, the rotational viscometer Reovscostar R was used. Viscosity was measured at various rotation speed for the collagen products containing different percentages of green algae. Reading times were identical every 10 seconds, for 10 minutes.

Results and Discussions

The formulations were obtained from collagen gels and green algae. Rheological studies were performed through viscosity measurements at varying rotation speeds. Thus, we established the D (s^{-1}) shear rate gradient, for which the η (cPoise) viscosity was obtained. From experimental data analyses, rheological parameters were obtained for each formulation:

- For the P1 formulation with 4.76% green algae in collagen hydrolysate, the values from Table 1 were obtained.

Table 1. Rheological parameters for P1

Viscosity η (cP)	Shear rate D (sec $^{-1}$)	Shear stress τ (Pa)
Interval between 19700 cP and 3800 cP,	Interval between 4,8 (sec $^{-1}$) and 68 (sec $^{-1}$)	Interval between 80,37 Pa and 258,4 Pa.

- For the P2 formulation with 7% green algae in collagen hydrolysate, the values from Table 2 were obtained.

Tabelul 2 parametrii reologici pentru P2

Viscosity η (cP)	Shear rate D (sec $^{-1}$)	Shear stress τ (Pa)
Interval between 21323 cP and 8000 cP,	Interval between 6,8 (sec $^{-1}$) and 34 (sec $^{-1}$)	Interval between 145 Pa and 268,6 Pa.

Measurements were made both at rotation speed increase and decrease. Rotation speed was between 12 rpm and 200 rpm for formulation P1 and between 12 rpm and 100 rpm for formulation P2. For rheological analyses, graphics were used so as to follow rheograms (Fig. 5 and Fig. 6), variation of sheer stress with speed gradient (Fig. 7 and Fig. 9) and flow curves (Fig. 8 and Fig. 10).

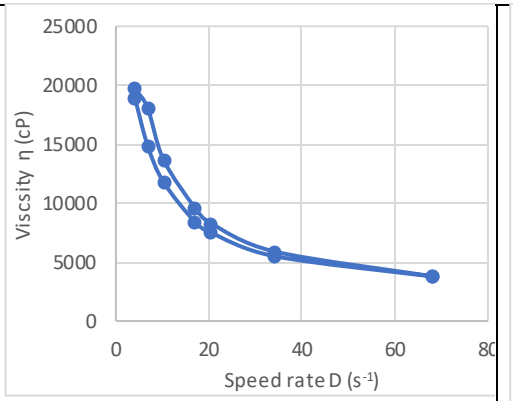

Fig. 5 Rheogram for formulation P1 (collagen and 4.76% green algae)

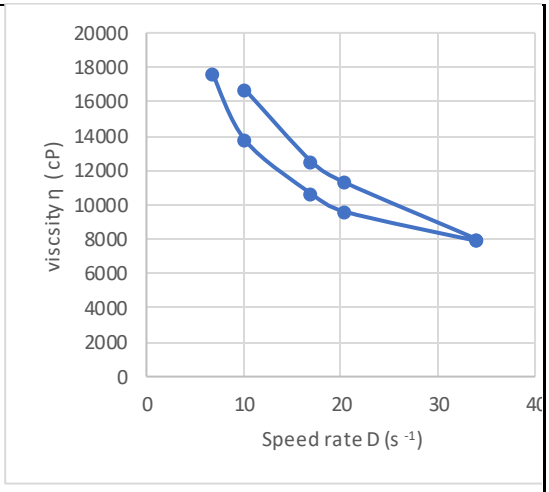

Fig. 6 Rheogram for formulation P2 (collagen + 7% green algae)

As it can be seen in figures 5 and 6, viscosity variation with speed gradient, the formulations have a pseudoplastic behaviour. Viscosity decreases as rotation speed increases. It can be noted that, at percentage of algae increases, the viscosity of the product increases. In figures 7 and 9, a more rapid increase of shear stress is noted at

the same shear rates, with an increase in algae percentage. Hysterzis loops are wider for formulation P2, which has a higher percentage of green algae.

From analysing flow curves (figures 8 and 10), a linearization of curves for both formulations can be observed at increases of shear rates of over 20 sec^{-1}. This means they exhibit a tendency for ideal plastic behaviour at high shear rates, over 20 sec^{-1}, which practically means that the gel no longer changes its structure at shearing rates over the mentioned value.

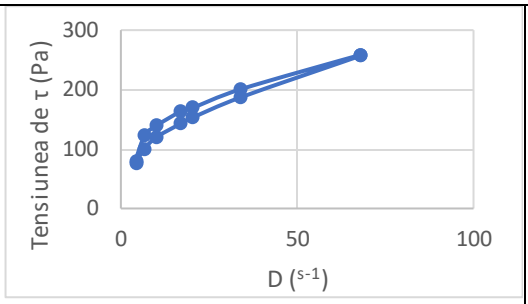

Fig. 7 Shear stress variation with speed gradient for P1 – collagen + 4.76% green algae

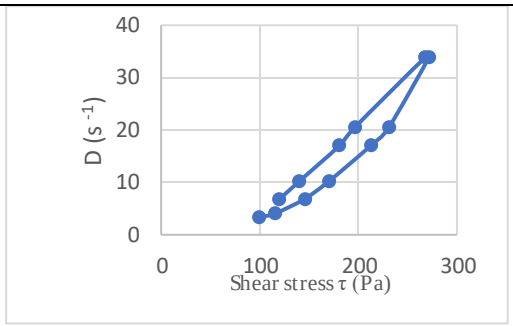

Fig. 8 Flow curve P1 collagen+ green algae 4.76%

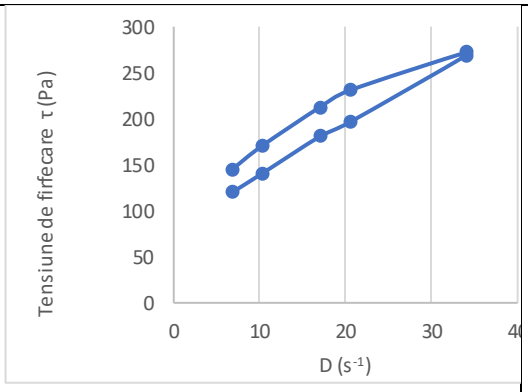

Fig.9 Variation in shear stress τ (Pa) with speed gradient for P 2 collagen + 7% green algae

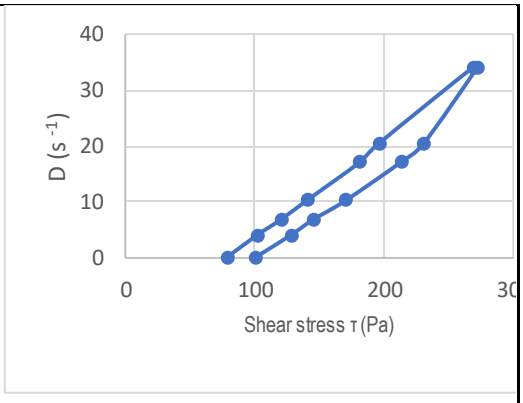

Fig.10 Flow curve for P2 collagen + 7% green algae

For a comparative study of the new pharmaceutical formulations and an appraisal of the interaction between algae and collagen components, which are represented by collagen gels containing marine algae powders from the *Enteromorpha intestinalis*

and *Cladophora vagabunda* species, from the rheological data, the following were taken into account:

- Shear rate domains on which viscosity can be measured;
- Rheogram shape;
- Viscosity values at reduced sheer rates;
- Destructuration resistance (value of shear rate at which sudden destructuration is produced).

To the points mentioned above, the aspect of the gel - opalescent – was added – which can represent a proof of the compatibility of the components.

Conclusions

Formulations with different green algae content in collagen hydrolysate were obtained.

From the present study, the following conclusions can be drawn:

- Formulations with marine algae in the same type of collagen hydrolysate have a pseudoplastic rheological behaviour, with a decrease in apparent viscosity as shear rates increase.
- Formulations are stable and have an opalescent aspect.
- After the linearization of flow curves over a certain shear rate, formulations are stable and no longer modify structure.
- Based on the values of rheological parameters, it can be noted that formulation P2, with a higher percentage of algae in the same collagen hydrolysate, has a superior stability.

References

[1] Sîrbu R., Zaharia T., Negreanu-Pîrjol B.S, Nicolaev S., Bologa A.,Psegalinschi I, The Black Sea ecosystem – important potential source for pharmaceutical industry, Journal of Environmental Protection and Ecology, 11 (4): 1336-1348, ISSN 1311-5065, 2010,
[2] R. Sîrbu, T. Zaharia, V. Maximov, A.m. Bechir, M. Mariş, B. Negreanu-Pîrjol, D.Mariş, T. Negreanu-Pîrjol, M. Leca, E. M. Cadar, R. M. Stoicescu, L. Mocanu, S. Jurja, Clean bio-technologies for obtaining new pharmaceutical formulations based on collagen gels and marine algae extracts for medical applications, Journal of Environmental Protection and Ecology, 11 (2), 654-665, 2010;
[3] R. Sirbu, T. Zaharia , A. M. Bechir, G. Lilios, S. Nicolaev , F. N. Roncea, Important Characteristics of the Marine Environment of the Romanian

Littoral Coast –Favourable for Pharmaceutical Utilisat ions, Journal of Environmental Protection and Ecology, 13, No 3A, 1842–1855, 2012;
[4] R. Sirbu, T. Zaharia, A. Bechir, G. Lilios, S. Nicolaev, Characterisation of Marine Habitats Ecosystem and the Macro-algae Biodiversity along the Romanian Black Sea Shore Journal of Environmental Protection and Ecology, 13, No 1, 190–197, 2012;
[5] Rodica Sîrbu, Ticuta Negreanu–Pîrjol, Emin Cadar, Bogdan-Stefan Negreanu-Pîrjol, Active Principles which are Important to Human Health Obtained from Ceramium Rubrum - Seaweed in the Black Sea, Academic Journal of Interdisciplinary Studies, MCSER Publishing, Rome-Italy, Vol 4 No 1 S2, p. 253-258, April 2015;
[6] Ticuţa Negreanu-Pîrjol, B. Negreanu-Pîrjol, R. Sîrbu, G. Paraschiv, A. Meghea, "Comparative studies regarding the antioxidative activity of some therapeutic marine algae species along Romanian Black Sea Coast", Journal of Environmental Protection and Ecology (ISSN 1311-5065), vol.13, No. 3A, p. 1744-1750, 2012;
[7] Emin Cadar, Cristina-Luiza Erimia, Aneta Tomescu, Paris Stelian, Rodica Sîrbu, Marine Algae from Black Sea - Important Resources in the Pharmaceutical and Medical Research, European Journal of Interdisciplinary Studies (EJIS), Vol.4 , Nr. 1, p 24-30, 2016;
[8] Constanţa SAVA, Rodica SIRBU, "Analytical study of flavonoids determination in algae from the Black Sea, Ovidius University Annals of Chemistry, Volume 21, Number 1, pp29-34, 2010.

Centering Romanian Healthcare System in Patient – Requirement of a European Health System

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Abstract

Currently, in Europe there is a close collaboration between the World Health Organization, the European Union and the Council of Europe with the declared aim to support the implementation of coordinated strategies for the implementation of patient rights, the concerns in this field intensifying, mainly, with the development of Amsterdam Declaration on the Promotion of Patients' Rights in Europe, adopted in 1994. To create a modern healthcare system, there is need for it to be cantered on patients' needs, to have dynamic and integrated structures, adaptable to the different and ever changing health needs of the society in general and of individuals in particular and which, not least, has to recognize the role of the patient as an active partner in health policies. In this context, this article examines the national legal framework governing the rights, duties, responsibilities and penalties applied in the field of patient rights. This article aims to analyse how patients' rights in the European Union area are implemented and enforced in the national legislation and the role that patients play in the Romanian health system.

Keywords: human rights, patient rights European legislation, the modernization of the national legislation, the standardization of the legislation, the health system, health policies

1. Introduction

Social, economic, cultural, ethical and political development in Europe, as well as the concepts patients' rights are based on (dignity, self-determination, respect for the person), have led to the emergence of a movement for the establishment and compliance of patients' rights both at European and national level.

Finland was one of the first European countries which adopted a patients' rights law in 1992 [1], subsequently followed by France, which adopted in 1994 [2] a charter of

patients' rights *"Charte du malade Hospitalisé",* as an annexed part of a ministerial order. The document stipulates, from the first sentences, that "*the hospitalized patient it is not only a patient. He is first of all a person with rights and obligations*".

2. Theory

In order to apply the principles of Patients' rights in Europe (WHO, 1994, Amsterdam), in Romania was adopted Law no. 46 of 21 January 2003 [3], relating to patients' rights, which enunciates the patients' classical rights (to information, confidentiality, consent etc.), as well as the penalties and responsibilities derived from the non-implementation and noncompliance with the law. The process of elaboration of the current law was a long and democratic process, which involved the participation of several competent institutions.

However, the regulations for the application of the law were developed with a time difference of almost a year [4], and recent studies show that the implementation process is a difficult one: the law it is not fully known to the medical personnel and the patients, and most of the latter don't know the rights and obligations incumbent upon them [5].

3. Results and Discussions

Analysing the provisions of the national document which regulates patient rights, we see that, alongside the classical rights arising from the basic principles of the European document, Law no 46/2003 addresses some new domains which have not been addressed in any of the previous Romanian regulations.

By analysing the provisions of Law no. 46/2003 *we find that the* **right to safety is not covered**.

Protecting and promoting health, priority objectives of the Romanian health system, cannot be achieved outside the legal aspects concerning patient safety and without the active involvement of the civil society in healthcare.

The safety of the Romanian patient relative to health services can only be solved by well informing the patients about the safety standards and measures, the remaining risks and complaint procedures and with the involvement of patient organizations in developing health policies.

Another legislative gap in this area is found with regard to the patients' right to personalized treatment. Considering that personalized medicine starts with the patient [6], as long as the Romanian healthcare system is cantered on the patient and its rights, we cannot speak about personalized treatment in the Romanian medical practice.

Another unregulated right for the Romanian patient is its right to the respect of waiting time. The health services must guarantee each patient the access to services they need in a predetermined period of time. The Romanian health service consumer

does not have this right of European patients respected, which, not being regulated, cannot be claimed by the national health system patients. If this right were to be included among the Romanian patients' rights, would also arise the **corresponding obligation of health services to repay the additional costs incurred upon the patients**.

4. CONCLUSIONS

In the *European Health Care Index 2014* [7] – the annual measurement system of the status of 36 national medical systems of Europe, published on 27 January in Brussels, Romania ranks 35 [8], with 453 points of the total of 1.000, being outranked by non-EU member states such as Serbia and Montenegro.

The results of the report on respecting European patients' rights in Romania are bleaker than those in 2011 [9]. Among the rights that have received a poor score, being rarely respected, are included the right to information (the lack of information posted on the websites of hospitals), the lack of informed consent standard forms in scientific research, information on the identity of patients was disclosed, the waiting list for selective surgery accessible to the public.

Other negative aspects that have been raised by the HCP 2014 report regard the lack of regular consultations with citizens' organizations, of partnership initiatives between health institutions and patients' organizations on the quality of medical services and the lack of interest of health units in evaluating patient satisfaction.

From the chart of the evolution of medical systems between 2006 and 2014 results that Romania has experienced a decline, from 530 points in 2006 to 453 in 2014.

By analysing the internal legal framework, correlated with the extensive case law of the Court of Justice of the European Union, we have observed that Law no. 46/2003 , the national document which governs the field of patients' rights, proclaims a series of classical rights arising from the basic principles of the European Charter of Patients' Rights, but these rights are addressed only in principle, there are no special provisions in the event of noncompliance.

References

[1] Active Citizenship Network, European Patient Rights Day Report, 5th European Patients' Rights Day: Putting Citizens at the Centre of EU Health Policy, 1-12th of April 2011, European Parliament, Brussels, Belgium, 2011.
[2] Charte du patient hospitalisé, annexée à la circulaire ministérielle n° 95-22 du 6 mai 1995, France, in Union Nationle des Associations Familiales (UNAF), Les chartes dans le domaine de la santé, Unaf, Décembre 2007.
[3] Cristina-Luiza Erimia, The emancipation of the European citizen by affirming the patients' rights, International Multidisciplinary Scientific Conference on

Social Sciences and Arts SGEM 2014, 3-9 September 2014, Bulgaria, Vol. I, pp. 823-830.
[4] European Alliance for Personalised Medicine (EAPM), Innovation and patient access to personalised medicine, Report from Irish Presidency Conference, 20-21 March 2013.
[5] Health Consumer Powerhouse, Euro Health Consumer Index 2014 Report, Health Consumer Powerhouse Ltd., 2015, ISBN 978-91-980687-5-7.
[6] Lag 785/1992 om patientens ställning och rättigheter, disponibil la http://www.axl.cefan.ulaval.ca/europe/Finlande-lois-diverses.
[7] Law no. 46/2003, patients' rights law, published in the Official Gazette of Romania, Part I, no. 70 of 3 February 2003.
[8] Methodological norms for the application of the Patients' Rights, published in the Official Gazette of Romania, Part I, no. 356 of 22 April 2004.
[9] The HCP survey includes 36 European countries and Scotland.

Studies on Improving Solubility in Different Media for Said Formulations with Topical Application

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Abstract

The studied compounds are gels with meloxicam, piroxicam and tenoxicam in different solvents. The problem of solubilization of these compounds is generated by their poor solubility in hydrophilic media. Also, when applying local non-steroidal anti-inflammatory drugs (NSAIDs) in the form of ointments, the local concentration of the active substance is much higher than in the untreated tissue. The amount of NSAIDs required to produce a therapeutic effect by topical administration is much lower than in oral administration. Side effects may, however, occur as a local irritation from the time a topical formulation is applied. This paper presents the solubilization tests performed for pharmaceutical compounds of the NSAID class in different hydrophilic media. The test conclusions are useful because their solubility can be increased by using solvents (polar or non-polar) and surfactants. The conclusions of the solubility tests help in choosing solvents in the formulation and technologicalization of semi-solid preparations with topical application.

Keywords: topical application, oxicams, piroxicam, meloxicam, tenoxicam.

Introduction

Oxicams represent the newest NSAID agents introduced in therapy after the 1980s. Their appearance is related to the processes recorded in the field of biopharmacy, respectively pharmacokinetics [1], [2].

The study of these derivatives was initiated by Lombardino and Wiseman, as early as 1970, starting with the predecessors of oxicams, followed by 1st generation oxicams and 2nd generation oxicams, in which the benzene nucleus of the heterocyclic system was replaced with other heterocyclic, in general sulfur (ex. tenoxicam), or the introduction of a hepta-atomic heterocycle in the case of meloxicam, [2], [3]. Although hundreds of derivatives with oxicam structure have been synthesized and experimented with, in therapy, following strict screenings, a few rigorously studied representatives, with a high therapeutic security remain. In addition to the anti-inflammatory activity, they possess a good analgesic, platelet antiaggregant, anti-arrhythmic activity. Oxicams are acid reactive substances, quite strong acids. Possible explanations for the high acidity refers to the involvement of hydrogen bonds that lead to the stabilization of the enolate anion. The molecules are ionized and therefore are distributed in the plasma, in the extracellular water, and at the same time are lipophilic due to the heteroaromatic nucleuses, as well as to the sulfonic group, so that the biological membranes are permeable to them [4]. These physicochemical properties are determined by the chemical, electronic and spatial structure (molecules have a flat shape), which foreshadows a possible adaptation for fixing on the active center of some enzymes (cyclooxygenase, phospholipase).

Material and Methods

Materials

Piroxicam (P), meloxicam (M), tenoxicam (T) (LaborMed Pharma, Romania), ethanol 96% (v/v) (Chimopar, Bucharest), propylene glycol (BASF Chem Trade GmbH, Germany), polyethylene glycol (PEG 400, BASF Chem Trade GmbH, Germany), tween 80, tween 85 (polysorbate 80, 85, Eigenmann & Veronelli, Italy), 1% sodium lauryl sulphate (Fluka Chemie AG, Sweden), paraffin oil solutol (Ra.M. Oil, Italy), oleic acid (Merck, Germany), cetyl stearyl alcohol (Cognis GmbH, Germany), lanolin (Lanolines De La Tossee, France), cetomacrogol self-emulsifying wax (B.P. 2009), distilled water (F.R. X), solutol H 15 (BASF Chem Trade GmbH, Germany), isopropyl miristat (Merck, Germany), [5].

Methods

Determining the solubility of piroxicam, meloxicam and tenoxicam in different solvents and adjuvants, using the saturation method by shaking the vial. For the purpose of determining the solubility of piroxicam, meloxicam and tenoxicam in different solvents, saturated solutions have been obtained by shaking for 96 h at 25°C, 50°C respectively [4], [5], due to the fact that the mixture is solid at normal

temperature. The composition and conditions used for obtaining their saturated solutions are shown in Table 1.

Apparatus

spectrophotometer, model CINTRA 10 E;

1 cm quartz cuvette (Hellma, Germany);

Analytical balance Sartorius BP 210 S, model FW 4798 (Germany).

Table 1. Composition and conditions for obtaining saturated solutions of Piroxicam, Meloxicam and Tenoxicam

No.	Carrier code	Carrier used	Temperature (ºC)
1	Aq	Distilled water	25
2	Et	Ethanol 96% (v/v)	25
3	Pg	Propylene glycol	25
4	PEG 400	Polyethylene glycol 400	25
5	T80	Tween 80	25
6	T85	Tween 85	25
7	LSS	1% sodium lauryl sulphate watery solution	25
8	UP	Paraffin oil	25
9	AO	Oleic acid	25
10	AC-UP	Cetyl stearyl alcohol/paraffin oil 1:17	50
11	AC-L-UP	Cetyl stearyl alcohol /lanolin/paraffin oil 1:1:17	50
12	CA-UP	Self-emulsifying wax /paraffin oil 3:7	50

Results and Discussions

Determining the solubility of piroxicam, meloxicam and tenoxicam in different solvents and adjuvants, [6].

In order to determine the calibration line for piroxicam, meloxicam and tenoxicam, standard solutions were obtained in ethanol 96% (v/v), "PStd_00", "MStd_00", "TStd_00" with the concentration 100 µg/mL. From the standard solutions were obtained the calibration solutions (in EtOH 96%) in increasing concentrations between 5-45 µg/mL. After thermostat control at 25ºC the spectre UV-VIS on the field 200-900 nm was registered, [7], [8]. The maximum absorption for piroxicam (in EtOH 96%) was obtained at the wavelength of 325 nm, for which the determinations for the unknown solutions were performed [8], [9].

In the case of Meloxicam, the main absorption maxima (in EtOH 96%) were obtained at the wavelengths of 205 nm, 270 nm and 355 nm, but the determination of the concentrations of the unknown solutions was only performed for the representative wavelength of 355 nm [9]. The main absorption maxima for Tenoxicam (in EtOH

96%) was obtained at the wavelengths of 267 nm and 358 nm, but the determination of the concentrations of the unknown solutions was only performed for the representative wavelength of 358 nm. For other solvents we generally observed a bathochromic shift of the absorption maximum, but due to the lack of appropriate quantities of the other solvents, the calibration curves could not be obtained for piroxicam, meloxicam and tenoxicam in such solvents [9]. Therefore, only the calibration curves of piroxicam, meloxicam and tenoxicam in ethanol 96% were used. In Figures 1, 2 and 3 are presented the calibration lines obtained for *piroxicam, meloxicam and tenoxicam*, at the wavelengths of 325 nm, 355 nm and 358 nm, respectively, corresponding to the absorption maximum in ethanol 96%, [10].

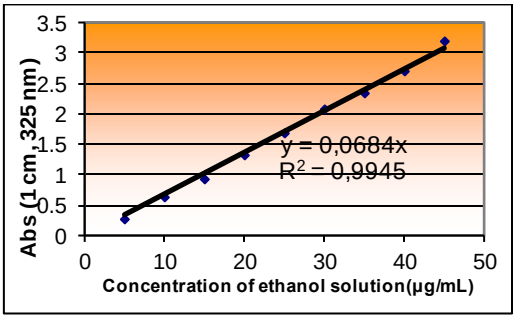

Figure 1. The calibration line of piroxicam for the absorption maximum at 325 nm

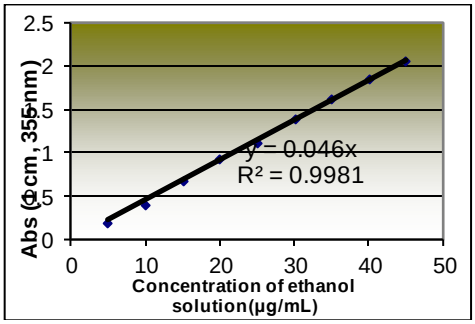

Figure 2. The calibration line of meloxicam for the absorption maximum at 355 nm

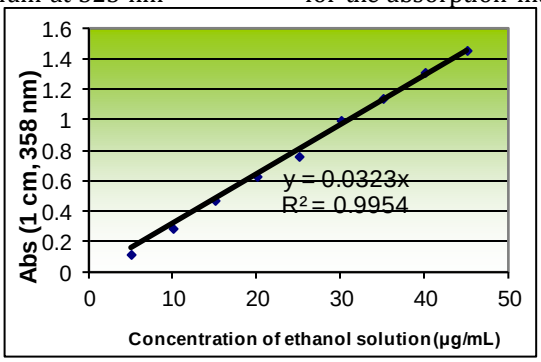

Figure 3. The calibration line of tenoxicam for the absorption maximum at 358 nm

The UV-VIS spectres for the calibration solutions of piroxicam on the relevant field of 230-400 nm, of meloxicam on the absorption field of 200-450 nm and of tenoxicam on the absorption field of 250-450 nm are presented in Figures 4, 5 and 6.

In Table 2 are presented the values of the solubility of piroxicam, meloxicam and tenoxicam, as well as the growth factor of the solubility of these substances in different solvents and adjuvants, ranging between 1 and 1884. The solubility of the three oxicams in the studied *polar* solvents increased with the decrease of the polarity

of the solvent; for the respective solvents, the growth factor ranged between 1 and 486 in the case of piroxicam, between 1 and 503 in the case of meloxicam and, respectively, between 1 and169 in the case of tenoxicam. From the hydrophilic (polar) solvents used, polyethylene glycol 400 showed the highest capacity to dissolve the tree oxicams; the solubility of Meloxicam and tenoxicam in this solvent (5.03 respectively 12.82 mg/mL) was equal to, even higher than, the concentration in which they are include in topical preparations (5 respectively 10 mg/mL). Unlike meloxicam and tenoxicam, the solubility of piroxicam in this solvent (7.78 mg/mL) was lower than the concentration in which the active substance is used in topical preparations (10 mg/mL). In the studied *nonpolar* carriers, the solubility growth factor ranged between 2 and 253 for piroxicam, between 4 and 145 for meloxicam and between 3 and 8 for tenoxicam.

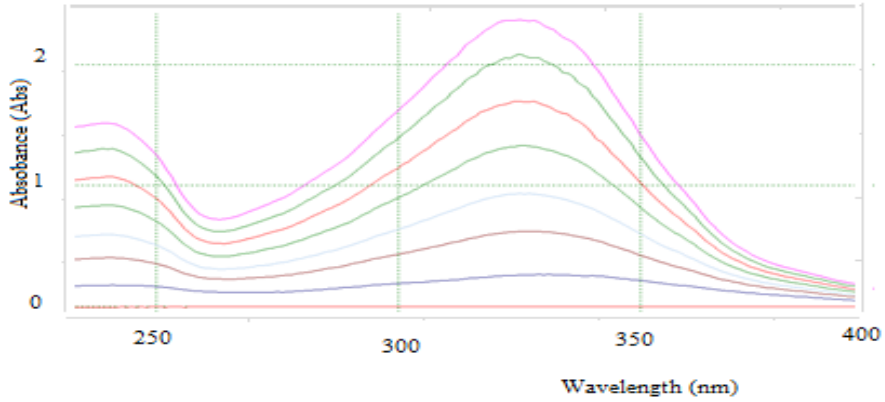

Figure 4. Overlapped absorption spectres for the calibration solutions of piroxicam (230-400 nm)

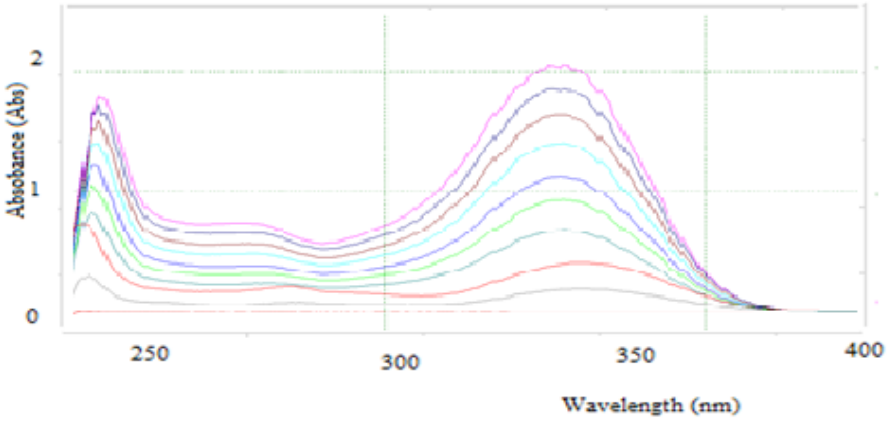

Figure 5. Overlapped absorption spectres for the calibration solutions of meloxicam (200-450 nm)

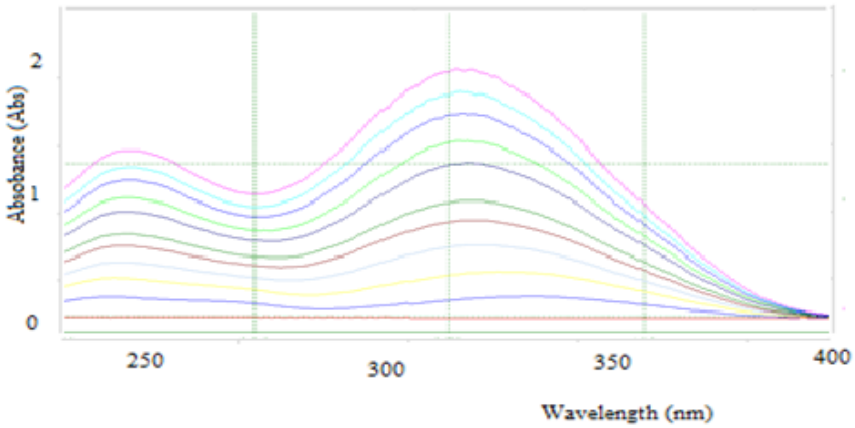

Figure 6. Overlapped absorption spectres for the calibration solutions of tenoxicam (field 250-450 nm)

The solubility of the three oxicams in most of the selected nonpolar carriers (liquid paraffin, cetyl stearyl alcohol/paraffin oil 1:17, cetyl stearyl alcohol /lanolin/paraffin oil 1:1:17, self-emulsifying wax /paraffin oil 3:7) was low, having values close to, generally lower than, those obtained in water and ethanol (for example, the solubility of tenoxicam in these nonpolar carriers, except for the Self-emulsifying wax /paraffin oil 3:7, presented lower values than its solubility in water).

In contrast, form the other two nonpolar solvents used, oleic acid presented an increased dissolution capacity for piroxicam and meloxicam, and isopropyl myristate acted similarly only in the case of piroxicam. Comparing the maximum values of the growth factor of the solubility of piroxicam, meloxicam and tenoxicam, calculated in the series of the polar solvents (PEG 400 corresponding values) with the high value of the same factor, calculated calculate din the series of nonpolar vehicles (the values corresponding to oleic acid and to isopropyl myristate), was observes that the former are significantly higher than the latter. The growth factor of solubility in the studied surfactants ranged between 8 and 1348 for piroxicam, between 9 and 1884 for meloxicam and between 8 and 529 for tenoxicam. The dissolution capacity of surfactants for the three studied oxicams varied in the following order: T85 > SH15 > T80 > LSS

Table 2. Values of the solubility of piroxicam, meloxicam and tenoxicam, as well as the growth factor in different solvents and adjuvants.

Carrier	Piroxicam		Meloxicam		Tenoxicam	
	Solubility (mg/mL)	Growth factor*	Solubility (mg/mL)	Growth factor*	Solubility (mg/mL)	Growth factor*

1	Water	0.016	1	0.010	1	0,076	1
2	Ethanol	0.620	40	0.325	33	1.196	16
3	Propylene glycol	1.668	104	0.462	46	0.611	8
4	Polyethylene glycol 400	7.777	486	5.029	503	12.816	169
5	Tween 80	12.701	794	5.430	543	6.154	81
6	Tween 85	21.577	1348	18.843	1884	40.178	529
7	1% sodium lauryl sulphate solution	0.135	8	0.087	9	0.618	8
8	Solutol H 15	19.622	1226	8.592	859	15.792	208
9	Liquid paraffin	0.037	2	0.003	-	0.014	-
10	Oleic acid	4.058	253	1.446	145	0.631	8
11	Isopropyl myristate	2.394	150	0.333	33	0.208	3
12	Cetyl stearyl alcohol/paraffin oil 1:17	0.303	19	0.040	4	0.029	-
13	Cetyl stearyl alcohol /lanolin/paraffin oil 1:1:17	0.302	19	0.046	5	0.012	-
14	Self-emulsifying wax /paraffin oil3:7	0.607	38	0.247	25	0.245	3

*The growth factor: represents the ratio between the solubility of the substance in 1 ml of solvent/ the solubility of the substance in 1 ml of water, at 25ºC

Nevertheless, differences were observed between the solubility of piroxicam, meloxicam and tenoxicam in each of these surfactants. It should be noted that the solubility of the three oxicams in the surfactants T85 and SH15 were a lot higher than the values of this parameter in the polar solvent PEG 400.

Conclusion

The results of this study have shown that although the three analysed oxicams (piroxicam, meloxicam and tenoxicam) are medicinal substances poorly soluble in water, their solubility can be increased significantly through the use of solvents (polar and nonpolar) and surfactants [9], [10]. Also, this study on solubility has helped to rationalize the selection of the carrier for formulation of skin hydrogels containing piroxicam, meloxicam or tenoxicam.

Thus, form the analysis of the obtained results, the following conclusions may be drawn:

From among the selected polar solvents, Piroxicam, meloxicam and tenoxicam presented the highest solubility in PEG 400 and the lowest in ethanol;

In the case of lipophilic carriers, the solubility of piroxicam, meloxicam and tenoxicam presented the highest values for oleic acid; moreover, Piroxicam had a relatively high solubility in isopropyl myristate;

Tween 85 and Solutol H15 were carriers in which piroxicam, meloxicam and tenoxicam presented the highest solubility.

Consequently, in formulating piroxicam, meloxicam or tenoxicam based hydrogels, PEG 400 is the most adequate co-solvent, due to the high solubilisation capacity, and ethanol will mainly act as absorption promoter, because it influences less the solubility of the three oxicams. In formulations containing a lipophilic phase, (creams, emulsions or micro emulsions-gels), oleic acid may be used mainly as promoter al of the skin penetration of the three oxicams, having a lower solubilisation capacity. Based on similar considerations, isopropyl myristate may be included in the same type of formulations, containing piroxicam. Tween 85 and Solutol H15 may be included as elective auxiliary substances both in the formulation of hydrogels and in that of emulsion systems, simultaneously fulfilling three roles: solubilizer, emulsifying and penetration promoter agents.

References

[1] Aurelia Nicoleta Cristea, Treatise on Pharmacology Medical Publishing House, Bucharest, 2005, pp. 617-635;
[2] Banchero M., Manna L., Ronchetti S., Campanelli P., Ferri A., Supercritical solvent impregnation of piroxicam on PVP at various polymer molecular weights, J Supercrit Fluids 49, 2009, 271-278;
[3] Dumitru Dobrescu, Pharmacotherapy, Medical Publishing House, Bucharest, 1989, pp. 133-138;
[4] Dumitru Lupuleasa, Ionela Belu, Ani Simona Sevastre, Mirela Mitu, Practical guide for the preparation of medicines, Vol I Second Edition, Craiova University Medical Publishing House, (2009), 171;
[5] Leucuţa S.E., Biopharmacy and pharmacokinetics, Dacia Publishing House, Clui-Napoca, 2001;
[6] Patel D.M., Shah R.R., Jagani P.D., Studies to enhance dissolution of piroxicam, Indian J Pharm Sci, 65 (3), 2003, 264-267;
[7] Rowe R.C., Sheskey P.J., Quinn M.E., Handbook of Pharmaceutical Excipients, 6th ed., Pharmaceutical Press, London, 2009, pp. 40-50;
[8] Savić S., Weber C., Savić MM, Goymann-Müller C., Natural surfactant-based topical vehicles for two odel drugs: Influence of different lipophilic

excipient son in vitro / in vivo skin performance, Int J Pharm 381, 2009, 220-230;

[9] Sîrbu, R., Negreanu-Pîrjol T., Tomescu A., Cherim M., Erimia CE, Tests for NSAID formulation for healthcare, 15th International Multidisciplinary Scientific GeoConference SGEM 2015, 16-25 June, 2015, Bulgaria, ISBN 978-619-7105-42-9, pp. 595-602,

[10] Wu K., Li J., Wang W., Winstead AD, Formation and Characterization of Solid Dispersions of Piroxicam and Polyvinylpyrrolidone Using Spray Drying and Precipitation with Compressed Antisolvent, J Pharm Sci 98 (7), 2009, 2422-2431 ;

Comparison of Extraction Methods of Chitin and Chitosan from Different Sources

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Abstract

Chitin and chitosan have become of great interest not only as underutilized resources, but also as new functional materials of high potential in various fields. The methods of isolating chitosan from different sources: shrimp (*Panaeus monodon*), crab (*Scylla olivicea* and *Scylla serrata*), locust (*Schistocerca gregaria*), honeybee *(Apis mellifera*), beelte (*Calosoma rugosa*) and fish (*Labeo rohita)* were compared. The same steps of demineralization and deproteinization were followed for the chemical extraction of chitin, but the concentration of reagents, the temperatures and reaction times were varied, which resulted in chitosans with different degrees of deacetylation.

Keywords: chitin, chitosan, extraction methods, different sources, deacetylation process

Introduction

Natural polymers are gaining more interest due to their biocompatibility and biodegradability in contrast to many synthetic polymers that have more limited properties. Chitin and chitosan are among the novel families of biological macromolecules that are studied as suitable functional materials, due to the excellent properties of these natural polymers, such as biocompatibility, biodegradability, non-toxicity and adsorption properties [Kumar, M.N.V.R., 2000].

Chitin and its deacetylated derivative chitosan are natural polymers composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). Due to their natural origin, both chitin and chitosan

cannot be defined as a unique chemical structure but as a family of polymers which present a high variability in their chemical and physical properties. This variability is related not only to the origin of the samples but also to their method of preparation [Aranaz, I., *et al*., 2009].

Chitin is the second most abundant natural polymer in nature after cellulose and it is found in the structure of a wide number of invertebrates (crustaceans' exoskeleton, insects' cuticles) and the cell walls of fungi [Pillai, C.K.S., *et al*., 2009]. Chitin is a white, hard, inelastic, nitrogenous polysaccharide and is insoluble in acidic aqueous media because it has a low amount of 2-amino-2-deoxy-D glucose units, while chitosan is soluble in acidic conditions due to the high amount of 2-amino-2-deoxy-D glucose units [Brunner, E., *et al.*, 2009].

Chitosan is the *N*-deacetylated derivative of chitin, although this N-deacetylation is almost never complete. Chitosan is a non-toxic, biodegradable polymer, a fiber-like substance, very much similar to cellulose [Hossain, M.S., *et al*., 2014].

The only difference between chitosan and cellulose is the amine (-NH2) group in the C-2 position of chitosan instead of the hydroxyl (-OH) group found in cellulose. Unlike cellulose, chitosan has the ability to chemically bind with negatively charged fats, lipids, cholesterol, metal ions, proteins, and macromolecules due to its positive ionic charges [Rout, S.K., 2001]. The chemical structures of cellulose, chitin and chitosan are presented in Fig. 1.

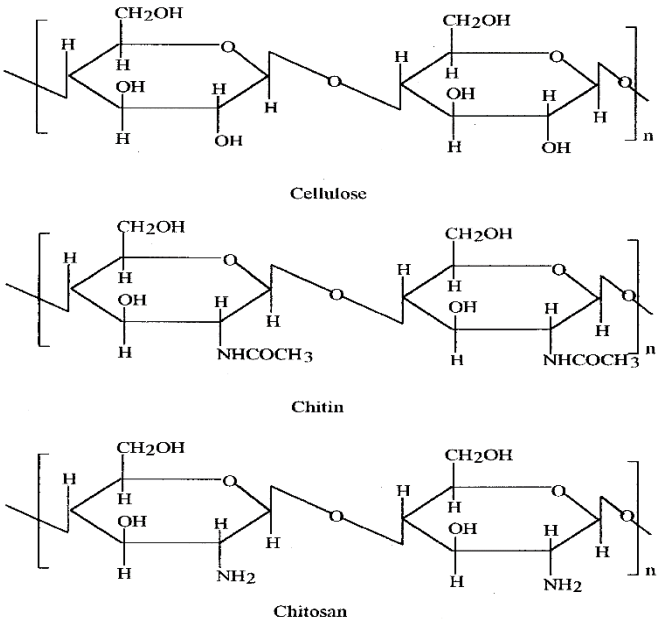

Fig.1. Chemical structures of cellulose, chitin and chitosan

Chitin can be extracted from various sources, however, commercial chitins are usually isolated from marine crustaceans, mainly because a large amount of waste is available as a by-product of food processing of marine products. Crustacean shells consist of 30-40% proteins, 30-50% calcium carbonate and calcium phosphate, 20-30% chitin and also contain pigments of a lipidic nature such as carotenoids (astaxanthin, astathin, canthaxanthin, lutein and β-carotene) and a high percentage of nitrogen (6.89%). These proportions may vary with species and with season and unfortunately crustacean shell wastes can be limited and subject to seasonal supply [Muxika, A., *et al*., 2017].

Fig. 2 briefly present the different sources used for chitin and chitosan extraction.

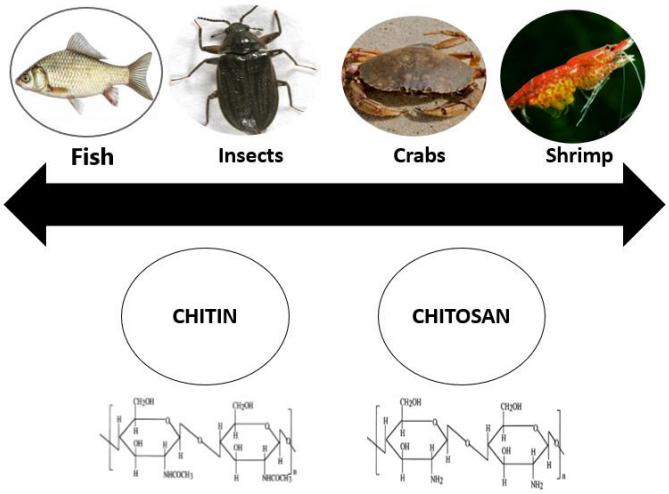

Fig.2. Different sources used for chitin and chitosan extraction

The chemical extraction of chitin involves a demineralization step in which the calcium carbonate is dissolved by acid treatment, followed by alkaline extraction to dissolve the proteins. A depigmentation step that removes the astaxantine is added in order to obtain a colourless product [Acosta, N., *et al*., 1993]. A brief scheme for obtaining chitin and chitosan is presented in Fig.3.

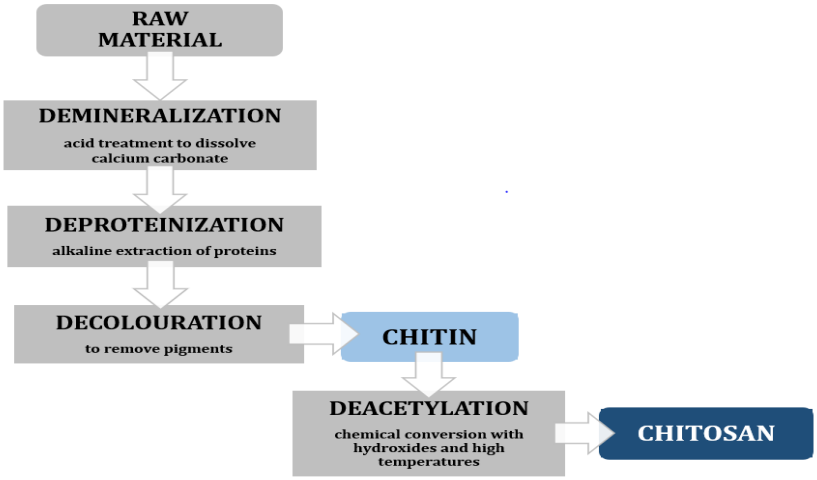

Fig.3. Scheme for obtaining chitin and chitosan

Chitin can be converted into chitosan by a deacetylation step. This step can involve enzymatic or chemical processes; however, the chemical conversion is preferred due to its lower cost and its suitability for mass production [Younes, I., *et al*., 2015]. Chemical deacetylation involves the treatment of chitin with hydroxides at high temperatures, usually above 80 ^{0}C. The treatments that use high NaOH concentrations (50–60%) and high temperatures (130–150 ^{0}C), have a very fast deacetylation step (2 h) [Lizardi-Mendoza, J., *et al*., 2016]. Fig.4 present the deacetylation process of chitin.

The ratio between the D-glucosamine and N-acetyl-D-glucosamine units of chitosan is considered as the degree of deacetylation [Bedian, L., *et al*., 2017], [Verlee, A., 2017]. In order for the polymer to become soluble in aqueous acidic media, the deacetylation degree of chitosan must reach 50%. When chitosan is dissolved in acidic environment, the amino groups in the chain protonate and the polymer becomes cationic, allowing it to interact with diverse types of molecules, thus turning chitosan into the only cationic marine polysaccharide. This positive charge may explain the antimicrobial activity of chitosan, because it interacts with the negatively charged cell membranes of microorganisms [Lizardi-Mendoza, J., *et al*., 2016].

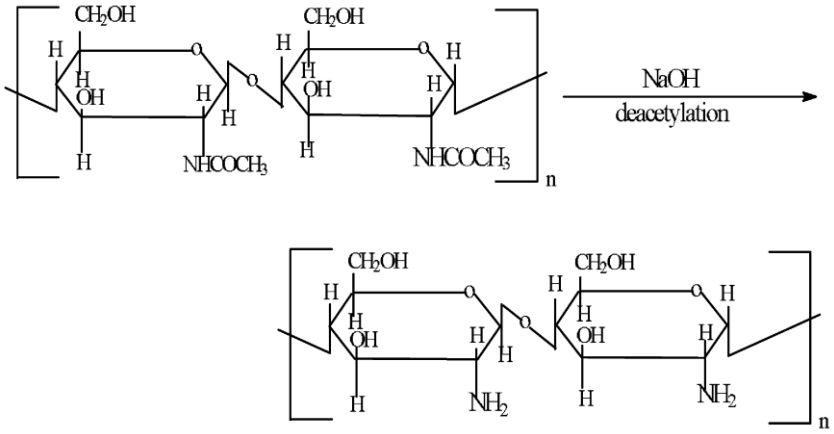

Fig.4. The deacetylation process of chitin

The characteristics of chitin and chitosan have a great effect on their properties and on their possible applications. Not every chitin or chitosan sample can be used for the same applications, and that is why a complete characterization of the samples is very important.

The main parameters affecting the properties of chitosan are the degree of deacetylation (DD) and the molecular weight (Mw). The degree of deacetylation is one of the most important chemical characteristics, which could influence the performance of chitosan in many of its applications [Baxter, A., *et al*., 1992], and the average Mw can have an influence on the viscosity development of aqueous solutions with a important role in the biochemical and biopharmacological applications [Tharanathan, R.N., *et al*., 2003].

Chitin and chitosan are currently receiving a great deal of interest as regards medical and pharmaceutical applications because of their interesting properties that make them suitable for use in the biomedical field, such as biocompatibility, biodegradability, non-toxicity and low immunogenicity [Harish, Prashanth K.V., *et al*., 2007], [Pillai, C.K.S., *et al*., 2009], [Nagahama, H., *et al*., 2008].

This paper present the chemical process for isolating chitin from different sources such as shrimp, locust, honey bee, beetle, crab and fish. The raw materials were subjected to demineralization and deproteinization to isolate the crude chitin. The obtained chitins were deacetylated to chitosan with different degrees of deacetylation. For the synthesis of chitosan from *P. monodon* shrimp two extraction methods were presented that differ by the order of the steps. Crab chitosan was obtained from two species of crab (*Scylla olivicea* and *Scylla serrata*) by two different methods that used different alkali solutions and conditions in the extraction steps, resulting in chitosans with different degrees of deacetylation.

Material and Method

The different sources used to extract chitin were shrimp (*Panaeus monodon*), crab (*Scylla olivicea* and *Scylla serrata*), locust (*Schistocerca gregaria*), honeybee *(Apis mellifera*), beelte (*Calosoma rugosa*) and fish (*Labeo rohita).*

The exoskeleton of the insects, of the shrimp and crab shells were scraped free of loose tissue, washed with tap and distilled water, oven dried until constant weight and grounded to pass through a 500 μm sieve.

Extraction of chitin and chitosan from shrimp *Penaeus monodon* specimens

According to Puvvada Y.S., *et al*. (2012) the crushed shrimps shells were boiled in sodium hydroxide 2% (w/v) for one hour in order to dissolve the proteins and sugars [Lertsutthiwong, P., *et al*., 2002] and then cooled for 30 minutes at room temperature [Lamarque, G., *et al*., 2005].

The demineralization step was carried out using 1% HCl, 1:4 (w/v) for 24 h, to remove the calcium carbonate. Then, the shells were treated with 2% NaOH for 1 h and the obtained chitin was washed with deionized water [Trung, T.S., *et al*., 2006].

For the deacetylation process, the obtained chitin was boiled in 50% NaOH for 2 h and then cooled for 30 minutes at room temperature. The sample was washed with 50% NaOH, filtered and oven dried at 110 ^{0}C for 6 h [Huang, M. *et al*., 2004] to obtain chitosan.

The extraction method used by Marei N.H., *et al*. (2016) starts with the demineralization process which involves an acid treatment with 1M HCl solution, 1:15 (w/v), at 25 ^{0}C. The resulted sample was washed with distilled water until neutral pH.

The deproteinization step was perfomed with 1M NaOH at 100 ^{0}C for 8 h, several times. The resulted sample was washed with distilled water until neutral pH and then with hot ethanol and later boiled in acetone to remove all the impurities. The resulted chitin was dried in an oven at 50 ^{0}C to constant weight [Rødde, R.H., *et al*., 2008].

Deacetylation was perfomed with 50% NaOH, 1:15 (w/v), at 100 ^{0}C for 8 h and then filtered and washed with hot distilled water until neutral pH. The obtained chitosan sample was oven dried at 50^{0}C for 24 h [Abdou, E.S., *et al*., 2008].

Extraction of chitin and chitosan from fish *Labeo rohita*

The method proposed by Kumari S., et al., 2017 involves a deproteinization process with 3% NaOH, at 80 ^{0}C for 30 minutes, followed by washing the sample with distilled water until neutral pH.

The sample was treated with 3% HCl, at 25 ^{0}C for 30 minutes for demineralization, then washed with distilled water and dried at room temperature [Kumari, S., *et al*., 2015].

The obtained chitin sample was deacetylated with 40% KOH, at 90 ^{0}C, for 6 h [Kumari, S., *et al*., 2016].

Extraction of chitin and chitosan from insects: locust (*Schistocerca gregaria*), honeybee (*Apis mellifera*) and beetles (*Calosoma rugosa*)

The demineralization process uses 1M HCl solution, with a solution to solid ratio 15 mL/g, at room temperature. The samples were washed with distilled water until neutral pH [Marei N.H., *et al*. 2016].

For deproteinization, the samples were treated with 1M NaOH at 100 ^{0}C for 8 h, followed by washing with distilled water and hot ethanol.

Decolouration was achieved by boiling the samples in acetone. The obtained chitins were oven dried at 50 ^{0}C [Majtán, J., *et al*., 2007].

The chitin samples were treated with 50% NaOH, 1:15 (w/v), at 100 ^{0}C for 8 h (deacetylation). The rezulted chitosans were washed to neutrality with hot distilled water and oven dried at 50^{0}C for 24 h [Kaya, M., *et al*., 2015].

Extraction of chitin and chitosan from crabs *Scylla olivicea and Scylla serrata shells*

Chitin and chitosan were extracted from the mud crab (*Scylla olivicea)* by the method proposed by Shahidi and Synowiecki (1991).

Before the actual extraction process, a carotenoid extraction step was perfomed by mixing the gounded dried shells with cod liver oil and heating in a water bath at 60 ^{0}C for 30 minutes.

The dried shells, free of carotenoids, were treated with 2% KOH, 1:20 (w/v), for 2 h at 90 ^{0}C for deproteinization. The sample was washed with water until pH=7 and dried in the oven at 60 ^{0}C for 24 h.

The shells were demineralized with 2.5% HCl, 1:20 (w/v), at 20 ^{0}C for 6 h. The sample was washed with water until pH=7 and dried in the oven at 60 ^{0}C for 24 h.

The decolouration step used acetone to treat the samples, for 10 minutes, followed by drying at room temperature for 2 h. The decolourized shells were washed with tap water, and dried at 60 ^{0}C for 24 h in the oven [Sarbon, N.M., *et al*., 2015].

The resulted chitin was deacetylated with 40% NaOH, 1:15 (w/v) at 105 ^{0}C for 2 h, then washed with deionized water until pH=7. The obtained chitosan was dried at 60 ^{0}C for 24 h [Yen, M.T., *et al*., 2009].

Extraction of chitin from the black crab *Scylla Serrata* shells is performed by the method of Kumari S., et al., (2017).

The grounded shells were subjected to a alkaline treatment with 3% NaOH, at 80 ^{0}C, for 30 minutes. The protein free sample were then washed with distilled water until neutrality and oven dried.

Demineralization was achieved with 3% HCl, at 25 ^{0}C, for 30 minutes. The sample was washed with water to remove the excess HCl and dried at 25 ^{0}C.

Chitosan was obtained by deacetylation of the chitin sample with 40% KOH, at 90^{0}C, for 6 h [Hajji, S., 2015].

Determination of degree of deacetylation (DD)

The direct titration method was used to determine the degree of deacetylation of chitosans extracted from different sources [Kucukgulmez, A., 2011].

Dried chitosan samples (0.2 g) were dissolved in 20 cm^3 0.1 M HCl and 25 cm^3 deionized water. After 30 minutes of continuous stirring, the second portion of deionized water (25 cm^3) was added and stirring continued for 30 minutes. When the chitosan samples were completely dissolved, the obtained solutions were titrated with a 0.1 mol·dm^{-3} NaOH solution using an automatic burette (0.01cm^3 accuracy). The degree of deacetylation (DD) of chitosans was calculated using the formula [Tolimate, A., *et al*., 2000]:

$$\boldsymbol{DD}[\%] = \mathbf{2.03}\frac{\boldsymbol{V_2 - V_1}}{\boldsymbol{m + 0.0042(V_2 - V_1)}}$$

where: *m* – weight of sample, *V1*, *V2* – volumes of 0.1 mol·dm^{-3} NaOH solution corresponding to the deflection points, 2.03 – coefficient resulting from the molecular weight of chitin monomer unit, 0.0042 – coefficient resulting from the difference between molecular weights of chitin and chitosan monomer units.

Results and discussion

The degree of deacetylation (DD) may range from 30 to 95% depending on the source and preparation procedure [Martino, A.D., *et al*., 2005]. The degree of deacetylation values are highly dependent on the source and method of purification [No, H.K., & Meyers, S.P., 1995], as well as the type of analytical methods employed, sample preparation and type of instrument used, and various other conditions that may influence the degree of deacetylation analysis.

The degree of deacetylation (DD) is an important parameter that affects the properties, such as solubility, chemical reactivity and biodegradability of the obtained chitosan [Lamarque, G., et al., 2005].

Chitosan extracted from shrimp *Penaeus monodon* by two methods with similar reaction conditions showed two different degrees of deacetylation. The extraction conditions for each method are presented in Table 1.

The method used by Puvvada Y.S., *et al*., 2012 led to a chitosan with a DD of 89%, while Marei N.H., *et al*., 2016 obtained a chitosan with a DD of 74%. This is probably because Marei N.H., *et al*., 2016 used a decolouration step, involving boiling the chitin sample in acetone, that may have affected the yield of chitin.

The deacetylation step was carried out in the same conditions of temperature and NaOH concentration, but a longer reaction time resulted in a lower DD value, due to the excessice removal of the acetyl groups from the polymer during deacetylation.

Table 1. Extraction conditions of chitosan from shrimp *Penaeus monodon*

[Puvvada Y.S., *et al*., 2012], [Marei N.H., *et al*., 2016]

	Extraction step	Reagent	Temperature	Time	DD
Shrimp *Penaeus monodon* (M1)	Deproteinization	NaOH 2%	100 ^{0}C	1h	89%
	Demineralization	HCl 1%	25 ^{0}C	24h	
	Decolouration	-	-	-	
	Deacetylation	NaOH 50%	100 ^{0}C	2h	
Shrimp *Penaeus monodon* (M2)	Demineralization	HCl 1M	25 ^{0}C		74%
	Deproteinization	NaOH 1M	100 ^{0}C	8h	
	Decolouration	Acetone	100 ^{0}C		
	Deacetylation	NaOH 50%	100 ^{0}C	8h	

Table 2 shows the extraction conditions of the method used by Kumari S., *et al*., 2017. It can be observed that no decolouration step was used, and the temperatures and reaction times are lower than in the case of shrimp extraction. Also, the alkali solution used for deacetylation is KOH in a concentration of 40%. In this conditions, the resulted DD value was 75%, higher than the value obtained from shrimp by the method of Marei N.H., *et al*., 2016.

Table 2. Extraction conditions of chitosan from fish *Labeo rohita* [Kumari S., *et al*., 2017]

	Extraction step	Reagent	Temperature	Time	DD
Fish *Labeo rohita*	Deproteinization	NaOH 3%,	80 ^{0}C	30 min.	75%
	Demineralization	HCl 3%	25 ^{0}C	30min.	
	Decolouration	-	-	-	
	Deacetylation	KOH 40%,	90 ^{0}C	6h	

The degree of deacetylation of the chitosans derived from insect chitins, under similar conditions are showed in Table 3. All three samples presented high values of DD, over 90%.

The DD of the chitosan extracted from locust *Schistocerca gregaria* is the highest, with a value of 98%, followed by the chitosan isolated from honey bee *Apis mellifera*, 96% and from beetles *Calosoma rugosa*, 95%.

The DD values of chitosan obtained from these insects are the highest for all the samples compared, meaning that insects are a valuable source for chitosan extraction.

Table 4 presents two methos for isolating chitosan from the shells of two crabs, namely *Scylla olivicea* and *Scylla serrata*.

It can be observed that the method proposed by Sarbon N.M., *et al*., 2015 resulted in a chitosan with a lower DD value (53%), than that of the chitosan obtained by the method of Kumari S., *et al*., 2017. This low DD may be influenced by the different alkali solution used in the deproteinization step and also by the reaction time, which is longer in this case. The acetone used in the decolouration step of the chitin extracted from *Scylla olivicea* crab may have influenced the low value of DD.

Table 3. Extraction conditions of chitosan from locust *Schistocerca gregaria,* honeybee *Apis mellifera* and beetle *Calosoma rugosa* [Marei N.H., *et al*., 2016]

	Extraction step	Reagent	Temperature	Time	DD
Locust *Schistocerca gregaria*	Demineralization	HCl 1M	25 ^{0}C		98%
	Deproteinization	NaOH 1M	100 ^{0}C	8h	
	Decolouration	Acetone	100 ^{0}C		
	Deacetylation	NaOH 50%	100 ^{0}C	8h	
Honey bee *Apis mellifera*	Demineralization	HCl 1M	25 ^{0}C		96%
	Deproteinization	NaOH 1M	100 ^{0}C	8h	
	Decolouration	Acetone	100 ^{0}C		
	Deacetylation	NaOH 50%	100 ^{0}C	8h	
Beetle *Calosoma rugosa*	Demineralization	HCl 1M	25 ^{0}C		95%
	Deproteinization	NaOH 1M	100 ^{0}C	8h	
	Decolouration	Acetone	100 ^{0}C		
	Deacetylation	NaOH 50%	100 ^{0}C	8h	

Table 4. Extraction conditions of chitosan from crab *Scylla olivicea* [Sarbon N.M., *et al*., 2015] **and *Scylla serrata*** [Kumari S., *et al*., 2017]

	Extraction step	Reagent	Temperature	Time	DD
Crab *Scylla olivicea*	Deproteinization	KOH 2%	90 ^{0}C	2h	53%
	Demineralization	HCl 2,5%,	20 ^{0}C	6h	
	Decolouration	Acetone		10 min.	
	Deacetylation	NaOH 40%	105 ^{0}C	2h	

Crab *Scylla serrata*	Deproteinization	NaOH 3%	80 ^{0}C	30 min.	70%
	Demineralization	HCl 3%	25 ^{0}C	30min.	
	Decolouration	-	-	-	
	Deacetylation	KOH 40%,	90 ^{0}C	6h	

Following the comparison made between the extraction methods for obtainig chitosan from different sources, it can be observed that a valuable source of chitosan are insects, with DD values of over 95%. In the case of shrimp and crab chitosan, the differences in DD was due to the reaction conditions and the decolouration step used to remove the pigments. This step is necessary to obtain a colourless product, but influences the characteristics of the polymer.

Conclusion

Taking into account all the samples compared in this review it can be observed that good quality chitosan can be extracted for a variety of natural sources, such as marine crusteceans (shrimp, crabs), fish scales and insects (locusts, bees and beetles). The degree of deacetylation is very much dependent on the source and preparation procedures and can be easily influenced by the reaction conditions. The highest DD values were observed for the samples prepared from insects (DD = 95%-98%).

References

[1] Abdou, E.S., Nagy, K.S.A., Elsabee, M.Z. (2008). Extraction and characterization of chitin and chitosan from local sources. Bioresour. Technol., 99(5), 1359–1367.

[2] Acosta, N., Jimenez, C., Borau, V., Heras, A. (1993). Extraction and characterization of chitin from crustaceans. Biomass Bioenerg., 5(2), 145-53.

[3] Aranaz, I., Mengíbar, M., Harris, R., Paños, I., Miralles, B., Acosta, N., Galed, G., & Heras, Á. (2009). Functional Characterization of Chitin and Chitosan. Current Chemical Biology, 3, 203-230.

[4] Baxter, A., Dillon, M., Anthony Taylor, K.D., Roberts, G.A.F. (1992). Improved method for i.r. determination of the degree of N-acetylation of chitosan. I. J. Biol.Macromol.,14(3),166-9.

[5] Bedian, L., Villalva-Rodríguez, A.M., Hernández-Vargas, G., Parra-Saldivar, R., Iqbal, H.M.N. (2017). Bio-based materials with novel characteristics for tissue engineering applications – a review. Int. J. Biol. Macromol., 98, 837–846.

[6] Bowman, S.M. & Free, S. J. (2006). Thestructure and synthesis of the fungal cell wall. BioEssays, 28(8), 799–808.

[7] Brunner, E., Ehrlich, H.P. et al., (2009). Chitin-based scaffolds are an integral part of the skeleton of the marine demosponge Ianthella basta. Journal of Structural Biology, 168(3), 539–547.

[8] Campana-Filho S. P., de Britto, D., Curti, E., et al., (2007). Extracao estruturas e propriedades de α- e β-quitina. Qu´ımica Nova, 30(3), 644–650.

[9] Hajji, S., Ghorbel-Bellaaj, O., Younes, I., Jellouli, K., Nasri, M., (2015). Chitin extraction from crab shells by Bacillus bacteria. Biological activities of fermented crab supernatants. Int. J. Biol. Macromolec., 79, 167–173.

[10] Harish Prashanth, K.V., & Tharanathan, R.N. (2007). Chitin/chitosan: modifications and their unlimited application potential-an overview. Trends in Food Science and Technology, 18(3), 117–131.

[11] Hossain, M. S. & Iqbal, A. (2014). Production and characterization of chitosan from shrimp waste. J. Bangladesh Agril. Univ., 12(1),153–160.

[12] Huang, M. et al. (2004). Uptake and cytotoxicity of chitosan molecules and nanoparticles: Effects of molecular weight and degree of deacetylation. Pharmaceutical Research, 21(2), 344-353.

[13] Kaya, M., Baran, T., Asan-Ozusaglam, M., Cakmak, Y., Tozak, K., Mol, A., Mentes, A., Sezen, G. (2015). Extraction and characterization of chitin and chitosan with antimicrobial and antioxidant activities from cosmopolitan Orthoptera species (Insecta). Biotechnol. Bioprocess., 20(1) 168–179.

[14] Kucukgulmez, A., Celik, M., Yanar, Y. (2011). Physicochemical characterization of chitosan extracted from Metapenaeus stebbingi shells. J. Food Chem., 126(3), 1144–1148.

[15] Kumar, M.N.V.R. (2000). A review of chitin and chitosan applications. Reactive & Functional Polymers, 46, 1–27.

[16] Kumari S., Annamareddy, S.H.K., Abanti, S., Rath, P.K. (2017). Physicochemical properties and characterization of chitosan synthesized from fish scales, crab and shrimp shells. International Journal of Biological Macromolecules, 104, 1697–1705.

[17] Kumari, S., Rath, P., Sri Hari Kumar, A. (2016). Chitosan from shrimp shell (Crangon crangon) and fish scales (Labeo rohita): extraction andcharacterization. Afr. J. Biotechnol. 15(24), 1258–1268.

[18] Kumari, S., Rath, P., Sri Hari Kumar, A., Tiwari, T.N. (2015). Extraction and characterization of chitin and chitosan from fishery waste by chemical method. Environ. Technol. Innov., 3, 77–85.

[19] Lamarque, G. et al. (2005). Physicochemical behavior of homogeneous series of acetylated chitosans in aqueous solution: role of various structural parameters. Biomacro-molecules, 6(1), 131-142.

[20] Lamarque, G., Cretenet, M., Viton, C., Domard, A., (2005). New route of deacetylation of a-and b-chitins by means of freeze–pump out–thaw cycles. Biomacromolecules, 6, 1380–1388.

[21] Lertsutthiwong, P., How, N.C., Chandrkrachan, S. & Stevens, W.F. (2002). Effect of chemical treatment on the characteristics of shrimp chitosan. Journal of metals, materials and minerals, 12(1), 11-18.

[22] Lizardi-Mendoza, J., Monal, W.M.A., Valencia, F.M.G. (2016). Chemical characteristics and functional properties of chitosan, in: S. Bautista-Banos, G. Romanazzi, A.Jiménez-Aparicio (Eds.), Chitosan in the Preservation of Agricultural Commodities. Academic Press, Elsevier Inc., Cambridge, 3–31.

[23] Majtán, J., Bíliková, K., Markovic, O., Gróf, J., Kogan, G., Simúth, J. (2007). Isolation and characterization of chitin from bumblebee (Bombus terrestris). Int. J. Boil.Macromol. 40(3), 237–241.
[24] Marei, N.H., Abd El-Samie, E., Salah, T., Saad, G.R., Elwahy, A.H.M., (2016). Isolation and characterization of chitosan from different local insectsin Egypt. International Journal of Biological Macromolecules, 82, 871–877.
[25] Martino, A.D., Sittinger, M., Risbud, M.V. (2005). Chitosan: a versatile biopolymer for orthopaedic tissue-engineering. Biomaterials, 26, 5983–5990.
[26] Muxika, A., Etxabide, J., Uranga, P., Guerrero, K. de la Caba. (2017). Chitosan as a bioactive polymer: Processing, properties and applications. International Journal of Biological Macromolecules, 105, 1358–1368.
[27] Nagahama, H., Nwe, N., Jayakumar, R., Koiwa, S., Furuike, T., & Tamura, H. (2008). Novel biodegradable chitin membranes for tissue engineering applications. Carbohydrate Polymers, 73(2), 295–302.
[28] No, H.K., Meyers, S.P. (1995). Preparation and Characterization of Chitin and Chitosan-A Review. J. Aquat. Food Prod. Technol., 4(2), 27–52.
[29] Pillai C. K. S., Paul, W., & Sharma, C. P. (2009). Chitin and chitosan polymers: chemistry, solubility and fiber formation. Progress in Polymer Science, 34(7), 641–678.
[30] Pillai, C.K.S., Paul, W., & Sharma, C.P. (2009). Chitin and chitosan polymers: chemistry, solubility and fiber formation. Progress in Polymer Science, 34(7), 641–678.
[31] Puvvada, Y. S., Vankayalapati S., Sukhavasi S. (2012). Extraction of chitin from chitosan from exoskeleton of shrimp for application in the pharmaceutical industry. International Current Pharmaceutical Journal, 1(9), 258-263.
[32] Rødde, R.H., Einbu, A., Vårum, K.M. (2008). A seasonal study of the chemical composition and chitin quality of shrimp shells obtained from northern shrimp (Pandalus borealis). Carbohydr. Polym., 71(3), 388–393.
[33] Rout, S.K. (2001). Physicochemical, Functional, and Spectroscopic analysis of crawfish chitin and chitosan as affected by process modification. Dissertation
[34] Sarbon, N.M., Sandanamsamy, S., Kamaruzaman, S.F.S., Ahmad, F. (2015). Chitosan extracted from mud crab (Scylla olivicea) shells: physicochemical and antioxidant properties. J. Food Sci. Technol., 52(7), 4266–4275.
[35] Shahidi, F, Synowiecki, J. (1991). Isolation and characterization of nutrients and value-added products from snow crab (Chionoecetes Opilio) and shrimp (Pandalus Borealis) processing discards. J. Agric. Food Chem., 39(8), 1527–1532.
[36] Tharanathan, R.N., Kittur, F.S. (2003). Chitin: The Undisputed Biomolecule of Great Potential. Crit. Rev. Food Sci. Nut. 43(1), 61-87.
[37] Tolimate, A., Desbrieres, J., Rhazi, M., Alagui, A., Vincendon, M., Vottero, P. (2000). On the influence of deacetylation process on the physicochemical characteristics of chitosan from squid chitin. Polymer, 41, 2463–2469.

[38] Trung, T.S., Thein-Han, W.W., Qui, N.T., Ng, C.H., Stevens, W.F. 2006. Functional characteristics of shrimp chitosan and its membranes as affected by the degree of deacetylation. Bioresour. Technol., 97(4), 659-63.

[39] Verlee, A., Mincke, S., Stevens, C.V. (2017). Recent developments in antibacterial and antifungal chitosan and its derivatives. Carbohydr. Polym., 164, 268–283.

[40] Yen, M.T., Yang, J.H., Mau, J.L. (2009). Physicochemical characterization of chitin and chitosan from crab shells. Carbohydr. Polym., 75, 15–21.

[41] Younes, I., Rinaudo, M. (2015). Chitin and chitosan preparation from marine sources. Structure, properties and applications. Mar. Drugs, 13, 1133–1174.

[42] Zapata, P. A., Rojas, D. F., & Atehortua, L. (2012). Production of biomass, polysaccharides, and ganoderic acid using nonconventional carbon sources under submerged culture of the lingzhi or reishi medicinal mushroom, Ganoderma lucidum (W.Curt.:Fr.) P. Karst., (higher basidiomycetes). International Journal of Medicinal Mushrooms, 12(2), 197–203.

Residual Marine Algae Biomass - An Important Raw Material for Obtaining a Soil Biostimulator-Regenerator

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Abstract

This paper highlight a study regarding the valorification of residual marine algae biomass along Romanian Black Sea Coast, which recorded in the last summer period an accumulation of a large seaweed biomass quantities. The valorization of this waste was conceived as a result of theirs nutritional potential for improvind degraded soils of Dobrudja region, Romania and as complex capitalization of this biomass. It was establish the biotechnological process for the new biosolid nutrient composite obtaining. The parameter physical-chemical results of raw material used for biofertilizer obtaining, emphasized an increased organic charge compared with the inorganic compounds. Also, an increased value of total organic nitrogen and an optimum pH limits, was registered. Regarding the nutrients concentrations values, it could be noticed that the new biosolid fertilizer contains organic matter and valuable nutritive elements (N, P) could be contributed to the enhancement of the soil quality. The new biosolid biostimulator-regenerator obtained from natural residues bring a complex of nutrients for degraded soils by the presence of organic residual compounds, through the proteins, lipids, nutrients (nitrogen, phosphorus) and mineral salts content, necessary for plants nutrition and improvement of soils quality.

Keywords: residual marine algae biomass, soil, biostimulator and regenerator

Introduction

In the recent years there is a concern and an increased demand for organic agricultural fertilizer by valorification of various biological wastes. It is known that the purpose of developing, natural biofertilizers for agriculture using mixtures of grass and manure or high nitrogen binding bacteria *Azospirillum brasilense*, vegetable waste or *Bacillus megaterium*, *Bacillus macerans*, protein additives undergo fermentation or waste of skin, as organic mass soil nutrient (Lacatusu R., 2008).

In order to meet new demands for organic waste fertilizers solutions began to emerge based on different compost vegetable waste, sewage sludge, food waste, etc. (Lacatusu R., 2008; Nastac M., 2015).

There are also known the processes for obtaining agricultural fertilizer from organic waste by mixing and stirring the components, achieving the final product in liquid form or suspension. This process raises issue adopts the too acidic *p*H of the products or packaging, storage and transport of manufactured products, which are likely due to the presence of water, to ferment and to degrade (Negreanu-Pirjol T., 2017).

Use of algae in agriculture is focused on the fact that fertilizers based on seaweed have some properties considered to be very valuable: they mellow the soil, absorb and retain moisture, they are not attacked by pests and they contain large amounts of nutrients, nitrogen, sodium, potassium.

In the vegetative bodies, in addition to macroelements, micronutrients like Mn, Zn, Cu, Fe and other complex substances such as vitamins, auxins are present as well. Application of seaweed as fertilizer can contribute in this way to the reinclusion of these elements in the biological circuit (Negreanu-Pirjol B., 2011; Negreanu-Pirjol T., 2011; Zăgan S., 2011).

Algae administration can be done in several ways: by direct introduction into the soil layers alternating with other natural fertilizers, introduction after washing or as extracts or powders. But the algae products do not totally replace the organic or natural fertilizers, therefore, usually they are administered along with these; currently complex fertilizers achievement is wanted (Negreanu-Pirjol T., 2011; Gomoiu M.T., 1978).

Talophytic macroalgals algae spread from the Romanian Black Sea coast as brown and red algae have the most important role, green algae having a more reduced one (Bavaru A., 1977).

Ulva rigida is widespread in the Romanian Black Sea area, being more abundant between Costineşti and Mangalia. This algae's vegetative body has a light green to dark green color, with foliaceous blade looks of irregular shape, sometimes with numerous breaks in the middle, fixed on the substrate through a fixation system consisting of dark color rizoids. It can reach sizes of 5 to 30 cm or even more. Maximum development appear to be in winter-spring, at low water depth. It is

encountered as well in polluted areas and sometimes in abundance in nutrients rich areas (Bavaru A., 1977).

Enteromorpha intestinalis is a multicellular alga, with a hollow vegetative body at the beginning, usually simple, sometimes very little branched, and then it detaches from the substrate and becomes lamellar. It may reach up to 1 m high and 1 mm to 10 cm wide. The vegetative body is fixed to the substrate, and its base issues fixation rizoids that unite, resulting a disk. It is a eurihalin species – it tolerates environments with salinities ranging from very low (fresh water), medium (brackish water) to very high (seas and oceans) salinity. It is able to rapidly colonize different unpopulated environments, often being the first of algae species to be established on the coasts stones. It stands in highly contaminated waters, even polluted (Bavaru A., 1977).

Ceramium rubrum, the most popular red alga in the Black Sea is presented as a filamentous clump, of dark red color, attached to substrate through rizoids. The filaments have dichotomic ramification. This species is noted by a marked polymorphism. It is an annual species, sometimes largely colonizing the rocky substrates in the mid-and infralitoral and it may be epiphytic (on other larger algae). On our coast it is encountered along the entire coastal area, all year long, with greater development during spring and summer (Bavaru A., 1977).

Algae, like all communities of marine organisms, are subject to all conditions of the marine environment, being a number of ecological factors with great importance for the development of macrophytes algae as: substrate, light, hydrodinamics, temperature, and salinity (Fig. 1) (Bavaru A., 1977; Sîrbu R., 2014; Baweja P., 2016).

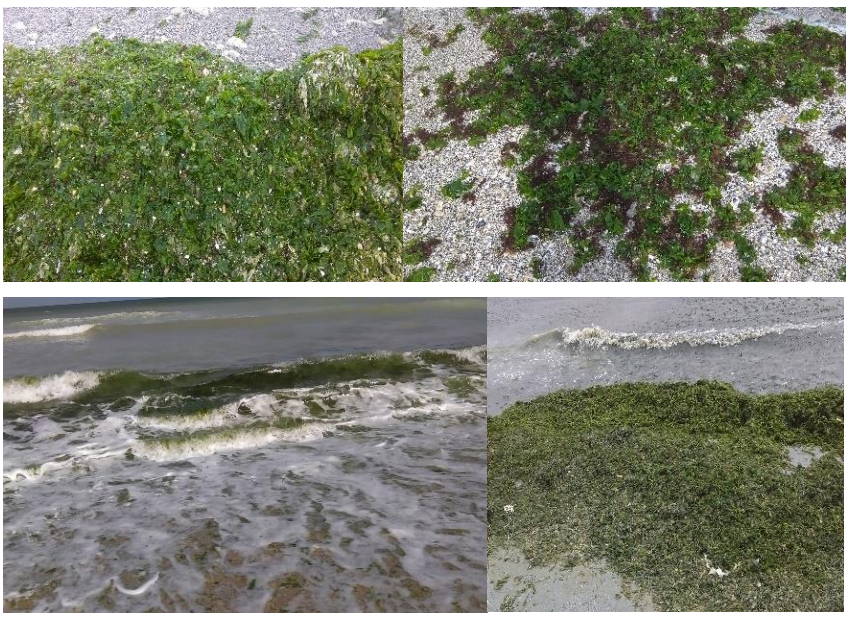

Fig. 1. Residual marine biomass along the south Romanian Black Sea Coast

The present paper emphasize the obtaining process of a new biosolid biostimulator-regenerator, as result of a mixture between residual marine biomass, macrophytobenthos: green, red and brown algae (such as *Clorophyta, Rodophyta, Phaeophyta*), in order to restore degraded soils poor in nutrients and organic substances, allowing recovery of marine algae biomass along Romanian Black Sea Coast, in the medio-littoral habitat (Biris-Dorhoi E.-S, 2018).

This new biosolid biostimulator-regenerator will be used as an innovative environmental non-conventional technology for agriculture, forestry, land reclamation and regeneration in relation to the benefits of marine waters protection. Through the biotechnology applied, the new bio-fertilizer will keep some of the qualities of residual marine biomass.

Material and Methods

Marine biomass sampling and analysis

The collecting of marine biomass samples was carried out between June - September 2018 - 2020, with minimum two samples per month; the sampling were from the Romanian beaches of Black Sea seaside in the Mamaia - Pescarie – 2 May Gulf – Vama Veche, Constanta County. All sample types were analyzed after collection in laboratory, in maximum 5 days (for all determined parameters we used averaged value, triple work samples were taken for each collecting point). The last decade of July - September, was characterized by the presence of abundant residual green macroalgae biomass, from which representative samples were taken. In the studied period, high atmosphere temperatures were recorded, over 35 °C and for sea water over 26°C. Residual marine biomass from Romanian Black Sea coast was dominated by green macroalgae (*Cladophora vagabunda*, *Ulva* and *Enteromorpha* species group), relative abundant were red species *Ceramium rubrum* and few pieces of *Porphyra leucosticta* and *Phylophora*; brown species *Cystoseira barbata* appeared only in the south littoral (Vasiliu F., 1984; Vasiliu F., 1996; Sava D., 2007; Sava D., 2006; AOAC 2016).

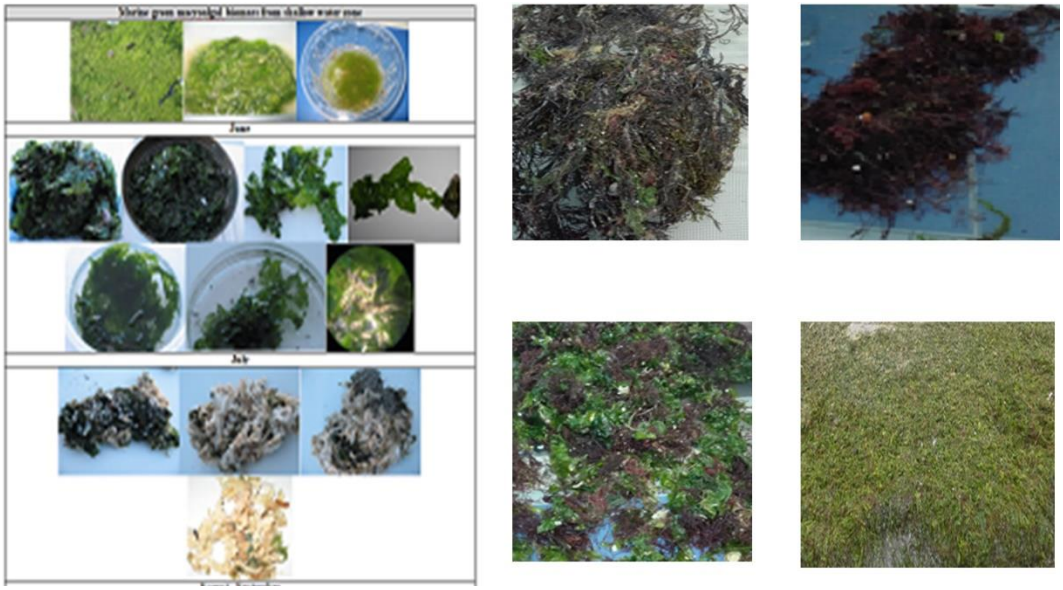

Fig. 2. Benthic macroalgal species from the shoreline of Romanian Black Sea Coast

In a first stage was carried out the separation of vegetal/macroalgae biomass and the triage and proper identification of organisms, made on the basis of taxonomic characters emphasized by stereomicroscope and Nikon microscope; considered quantitative parameters were occurrence frequency and dominance of an increased number of species (Fig. 2).

For physical-chemical and biochemical characterization of marine biomass, were used the follows methods:

Determination of *p*H was achieved using a Consort electronic *p*H-meter with temperature sensor.

Total phosphorus / phosphate, Kit Aquaquant1.14445.0001 (Merck)

Chlorides, Kit Aquaquant1.14401.0001 (Merck)

Nitrates, Portable UV-vis spectrophotometer Spectroquant, Merck

Nitrite, Kit Aquamerck1.11170.0001 (Merck)

Sulphides, Kit Aquaquant1.14416.0001 (Merck)

Sulfates, Portable spectrophotometer Spectroquant, Merck

Ammonium, Kit Aquaquant1.14428.0001 (Merck)

Total salts dissolved, WTW Portable TDS-meter

Determination of loss by drying was done under the provisions of the applicable European Pharmacopoeia (PhE 10.0 Ed.)

Determination of residue on ignition was performed under the provisions of the applicable European Pharmacopoeia (PhE 10.0 ED.)

Determination of total protein by Kjeldahl method was performed according to the official method of analysis for plant products European Pharmacopoeia (PhE 10.0 Ed.)

The quantitative determination of soluble proteins was done by Lowry method (Lowry O.H., 1951)

The quantitative determination of carotenoids was done by HPLC in ranversed stage (Dietz J.M. 1988; Hui Ni, 2005)

Determination of ascorbic acid in algae extracts was done by iodometric method described in European Pharmacopoeia (PhE 10.0 Ed.)

Sample preparation for heavy metals determination, AAS method

Heavy metals (Cu, Zn, Cr, Mn, Pb, Cd), by Atomic Absorption Spectrophotometry was analyzed.

For each raw material, a number of 3 samples with different dried masses were analyzed. A mixture of concentrated acids H_2SO4 96%, H_3PO4 85%, HF 40%, HNO_3 65%, was used for mineralization. After the complete digestion, the content of the digestion vessels was decanted in 50 mL flasks to be analyzed. For the metal content determination, measurements were performed by Atomic Absorption Spectrometry in both Graphite Furnace (GF-AAS) and Flame (FL-AAS) (Welz, 1999; Welz, 2005).

Apparatus used: High Resolution Continuum Source Atomic Absorption Spectrometer ContrAA-700, Analytik Jena AG, Germany, with autosampler for dilution sample, on acetylene flame technique, sequential analysis, at specific wavelengths, Cu (λ = 324.7 nm), Zn (λ =213.9 nm), Cr (λ= 357.9 nm), Mn (λ = 279.5 nm), Pb (λ =217 nm), Cd (λ = 228.8 nm) (Bucur Arpenti M., 2014, Cadar E., 2019).

For biochemical determinations, raw material consisted of a mixture of macrophyte algae collected along Black Sea coast, were stored in air for 48 hours for pre-drying. Aqueous extracts of algae were obtained as scheme in Fig. 3, Fig. 4. All determinations were made with double samples.

Cold aqueous extract:

Algae were suspended in distilled water, in relation 1:5 (m:m) and were kept cold (9 °C) for 84 h. Then the liquid was decanted and then clarified by filtration.

FRESH ALGAE

↓

SUSPENSION IN DISTILLED WATER (1 : 5) 48 hours at 4 °C

↓

DECANTING

↓

FILTRATION

Fig. 3. Scheme on how to obtain algal extracts (cold method)

Warm aqueous extract:

Algae were suspended in distilled water in relation 1:5 (m:m) and were maintained at 48°C for 4 hours. After cooling, the liquid was decanted, then clarified by filtration. The scheme to obtain the two extracts

FRESH ALGAE

↓

SUSPENSION IN DISTILLED WATER (1 : 5)

↓

WARMING (48 °C, 4 h)

↓

DECANTING

↓

FILTRATION

Fig. 4. Scheme on how to obtain algal extracts (warm method)

Results

Physical-chemical and biochemical characterization of residual marine biomass

The residual marine biomass (mixture between different algae species) was analized regarding the physical-chemical parameters (Table 1). Aqueous algal extracts was analized regarding the physical-chemical and biochemical parameters (Table 2).

Codification of sample type/ collected location: *1* - Total dried residual vegetal marine biomass / Romanian Littoral; *2* – Fresh macro algal green biomass/ Mamaia-Pescarie; *3* - Fresh macro algal green biomass / 2 May; *4* - Fresh macro algal green biomass / Vama Veche; *5* - Mixture residual marine macroalgae (green, brown, red with vegetative body) / 2 May; *6* - Mixture residual marine macroalgae (green, brown, red with vegetative body) / Vama Veche.

In the Table 1 are presented the average values for the mineral content of marine biomass for different collecting stations, as follows:

Table 1. Physical-chemical parameters of residual marine biomass, in dried substance

Parameter	Samples					
	1	2	3	4	5	6
*p*H	7.31	7.19	7.63	7.13	7.28	7. 34
Temperature (°C)	29	30	30	32	30	32
Total phosphorus (mg /g)	0.0024	0.0024	0.0024	0.114	0.0016	0.0018
PO_4^{3-} (mg/g)	0.0076	0.0076	0.0070	0.0070	0.068	0.073
Cl^- (mg/g)	0.0030	0.0014	0.0035	0.0012	0.0044	0.0048
NO_3^- (mg/g)	2.230	2.250	1.487	1.198	2.432	2.133
NO_2^- (mg/g)	0.01	0.001	0.011	0.011	0.130	0.120
S^{2-} (mg/g)	0.001	0.032	0.006	0.066	0.051	0.033
SO_4^{2-} (mg/g)	0.014	0.03	0.012	0.046	0.068	0.050
NH_4^+ (mg/g)	0.015	0.005	0.025	0,005	0.005	0.021
Total dissolved salts (mg /g)	3. 33	2.5	3.33	3.17	3.8	2.31
Cu (mg/kg)	3,023	3,640	3,432	3,028	4,326	4,280
Zn (mg/kg)	16,15	17,31	17.98	16,19	18,95	18,93
Cr (mg/kg)	2,041	1,370	1,620	1,467	1,506	1,880
Mn (mg/kg)	0,129	0,130	0,144	0,006	0,146	0,158
Pb (mg/kg)	1,112	1,270	1,198	1,010	1,113	1,126
Cd (mg/kg)	0,081	0,092	0,083	0,088	0,112	0,230

In the Table 2, are presented the average values for the physical-chemical and biochemical parameters of the aqueous algal biomass extracts (*p*H, dry substance, β-caroten, ascorbic acid, soluble protein, total Kjeldahl protein).

Table 2. Physical-chemical and biochemical determinations for aqueous algal biomass extracts

Sample	*p*H	Dry substance (%)	β-caroten (µg/dL)	Ascorbic acid (mg/mL)	Soluble protein (µg/mL)	Total Kjeldahl protein
Cold algae extract	5,60	0,560	112,3	1,890	0,948	1,966
Warm algae extract	6,62	0,577	68,3	0,862	1,048	2,130

Discussions

Regarding physical-chemical determinations of residual marine biomass:

Not significant differences between residual marine biomass collected from all stations, Mamaia-Pescarie - 2 May - Vama Veche, Constanta County were observed;

*p*H values recorded are within acceptable limits, without significant variations;

Total phosphorus content was registered in normal variation interval, greater quantity in samples 5 and 6 (mixture of algae), but all samples are included in the normal values;

Samples containing brown and red algae (5 and 6), shows higher values for other parameters: phosphates, nitrates, nitrites, sulphides, sulfates, total dissolved salts;

Sulfate ion shows values closer to the standard rules, but in larger quantities in sample 5;

The amount of ammonium ions shows slight variations in observation;

The heavy metal content has been low for this type of marine biological samples and are within the limits imposed by the standards values for surface water;

Were not observed significant differences between marine biomass residues collected from all three stations;

Higher zinc content, without exceeding the admissible limits for biological samples from the marine environment, was observed.

Regarding biochemical determinations of residual marine biomass:

Simple and reproducible methods were used to prepare cold and warm maceration extracts from the harvested algae biomass;

The values of biochemical parameters obtained for residual marine biomass in generally were comparable and not recorded a significant variation; all values parameters are in the acceptable limits for this type of samples.

Increased quantities of proteins in mixture algae biomass were registered (Table 2).

Technological process for obtaining the biosolid biostimulator-regenerator of soils

The process for obtaining the new biosolid nutrient composite, as mixture variants no. 1- 4, (Table 3), involved the following steps: The marine algae biomasses were conditioned by dehydration, at ambient temperature, for 72 hours. All biomass components were sprayed with a grinding machine, powders obtained were sieved through a sieve with mesh diameter of 90 μm. *p*H potentiometric method of each formula was determined, resulting in values ranging from 7.09 to 7.89. It was wetted with distilled water, with volumes of 75-95 mL, then left to macerate for 24 hours at

room temperature. After 24 hours, the solid product was passed through the meshes of a sieve with diameter of 1.25 μm, when non-uniform grains were obtained, which were subjected to the drying process, the oven at 50 °C. Dry granules were sieved again for uniformity. Circular shaped granules were obtained, whose color intensity was correlated to the mineral charcoal content. For specific smell elimination, mineral coal, granular sawdust, dry powder strongly odoriferous plant vegetation or dry powder citrus fruit peel, granulated in a granulator were used (Negreanu-Pîrjol B.-S., 2012; Negreanu-Pirjol T., 2019). It was obtained a biosolid nutritive composite, granular powder and homogeneous, different colours, from green-brownish until brown, with physical-chemical characteristics of the Table 4.

Table 3. Variants of new biosolid nutrient composite

Raw material	Varian t 1	Varian t 2	Variant 3	Variant 4
	Weight parts, g			
Green marine algae biomass	30	60	10	20
Brown marine algae biomass	30	20	60	10
Red marine algae biomass	30	10	20	60
Mineral charcoal and other absorbents	10	10	10	10

Table 4. General physical-chemical average parameters of biosolid nutrient composite, in dried substance

Parameter	Mixture of marine algae biomass
*p*H	7,09 - 7,43
Total soluble salts	1 - 20%
Phosphates	traces
Chlorides	0,2 %
Nitrates	0,5%
Sulphates	0,1%
Ammonium	traces

Conclusions

Regarding physical-chemical parameters analyzed of residue marine biomass, we conclusion that no significant differences between marine biomass collected from all three stations were observed. The values of physical-chemical parameters obtained for the three categories of residual marine macroalgae biomass generally were comparable and without recorded a significant variations; all values parameters are in the acceptable limits for this type of samples;

The new biosolid nutrient composite as organic fertilizer can replace some classical solid fertilizers by direct application to soil;

The biocomposite has a *p*H close to soil *p*H, being neutral to slightly alkaline, with low chlorine content, valuable for salty soils and have good stability (in time and light) of the physical-chemical and biochemical properties;

Used as raw material, the organic residue proposed are easily accessible, with minimal costs for their collection, which gives a low-price end product biofertilizer;

The biostimulator-regenerator effect of new biocomposite for strengthening and start up for plant protection, plant nutrition and growth promotion, unconditional soil features and could be used in agriculture, horticulture, viticultura for soil quality improvement.

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References

[1] AOAC, Official methods of analysis, (2016) Official Methods of Analysis of AOAC International 20th Edition method 978.04

[2] Bavaru A., Contribution of algal association study from rocky substratum of Black Sea coast, PhD Thesis, Ed. Bucuresti University, 195, 1977

[3] Baweja P., Kumar S., Sahoo D., Levine I., Chapter 3. Biology of Seaweeds, https://doi.org/10.1016/B978-0-12-802772-1.00003-8, in Seaweed in Health and Disease Prevention, Editors Joël Fleurence and Ira Levine, Academic Press, 2016, pp. 41-106, https://doi.org/10.1016/C2014-0-02206-X

[4] Biris-Dorhoi E.-S., Tofana M., Chis S.M., Lupu C.E., Negreanu-Pirjol T., Wastewater treatment using marine algae biomass as pollutants removal, Rev. Chim. (Bucharest), 2018, vol. 69, issue 5, 1089-1098

[5] Bucur Arpenti M., Negreanu-Pîrjol T., Ehlinger T., Paraschiv G.M., Tofan L., Heavy Metal Content Analysis of Siutghiol Lake Water and Sediment to Assess its Pollution Level, Rev. Chim. (Bucharest), 2014, vol. 65, issue 9, 1108 – 1113

[6] Cadar E., Sirbu R., Negreanu Pirjol B.S., Ionescu A.M., Negreanu Pirjol T., Heavy Metals Bioaccumulation Capacity on Marine Algae Biomass from Romanian Black Sea Coast, Rev. Chim. (Bucharest), 2019, vol. 70, issue 8, 3065 – 3072

[7] Dietz JM, Sri Kantha S, Erdman JW Jr., Reversed phase HPLC analysis of α- and β-carotene from selected raw and cooked vegetables, Plant Foods for Human Nutrition (Dordrecht, Netherlands), 1988, 38(4):333-341

[8] European Pharmacopoeia (Ph.Eur.) 10th Edition, Council of Europe, https://www.edqm.eu/en/european-pharmacopoeia-ph-eur-10th-edition

[9] Gomoiu, M.-T., Marine Eutrofication Syndrome in the Northwestern Part of the Black Sea, Science of the Total Environmental, Supp. Else. Scie. Publ. B. V., 1978, 683 – 692.

[10] Hui Ni, Guo-qing He, Hui Ruan, Qi-he C., and Feng C., Application of derivative ratio spectrophotometry for determination of β-carotene and astaxanthin from Phaffia rhodozyma extract, Journal of Zhejiang University Science, 2005, vol. 6 (6), 514–522

[11] Lacatuşu R., Lacatuşu A.R., Lungu M., Breaban I.G., Macro- and microelements abundance in some urban soils from Romania, Carpathian Journal of Earth and Environmental Sciences, 2008, 3, 1, 75-83

[12] Lăcătuşu R., Ştefănescu S.L., Lăcătuşu A. R., Breabăn I. G., Indicator for the evaluation the soil fertility level, Lucrări Ştiinţifice USAMV Iaşi, 2008 ,vol. 51, 967-972

[13] Lowry OH, Rosebrough NJ, Farr AL, Randall R.J., Protein measurement with the Folin phenol reagent, J. Biol. Chem., 1951, 193 (1), 265–75

[14] Nastac M., Negreanu–Pirjol T., The impact of massive algae development on the marine ecosystem, 15th International Multidisciplinary Scientific GeoConferences – SGEM 2015 - Nano, Bio and Green – Technologies for a Sustainable Future, 18 – 24 June 2015, Albena, Bulgaria, Conference Proceedings, Book 3, Vol. 2, 699-706

[15] Negreanu-Pîrjol T., Negreanu-Pîrjol B.-S., Gorun E., Nițu B., Năstac M., Marine algae biomass as a useful resource in green fertilization, 17th International Multidisciplinary Scientific GeoConferences–SGEM 2017 - Nano, Bio and Green–Technologies for a Sustainable Future, 29 June – 5 July 2017, Albena, Bulgaria, Conference Proceedings, Section Micro and Nano technologies, Advances in Biotechnology, Vol. 17, Iss. 61, 797 – 806

[16] Negreanu-Pîrjol B., Negreanu-Pîrjol T., Paraschiv G., Bratu M., Sîrbu R., Roncea F., Meghea A., Physical-chemical characterization of some green and red macrophyte algae from Romanian Black Sea littoral, Scientific Study & Research-Chemistry & Chemical Engineering, Biotechnology, Food industry, 2011, XII (2), 173 – 184

[17] Negreanu-Pîrjol B., Negreanu-Pîrjol T., Tofan L., Sirbu R., Sava C., Meghea A., Physicochemical and microbiological characterisation of two biological wastes of Romanian Black Sea Coast, J Environ Prot Ecol, vol. 12, No. 4A, pp. 2205-2217, 2011

[18] Negreanu-Pîrjol B., Negreanu-Pîrjol T., Zăgan S., Paraschiv G.M., Zăgan R., Sîrbu R., Study regarding dynamic of groundwater quality after some unusual

meteorological phenomena, Rev. Chim. (Bucharest), 2012, vol. 63, No. 1, 110 – 115

[19] Negreanu-Pîrjol B., Negreanu-Pirjol T., Paraschiv G. M., Sirbu R., Meghea A., Physical-chemical characterization of a new biofertilizer used for degraded soils, 12th International Multidisciplinary ScientificGeoConference „Modern Management of Mine Production, Geology and Environmental Protection" – SGEM 2012, 17 – 23 June 2012, Albena, Bulgaria, Conference Proceedings, Vol. IV, Section Soils, 169 - 176

[20] Negreanu-Pîrjol T., Negreanu-Pîrjol B.-S., Popoviciu D.R., Mirea M., Vasile M., Micro-, macroelements and heavy metals in the algae component of a new biostimulator-regenerator for grapevine soils, 19th International Multidisciplinary Scientific GeoConferences–SGEM 2019, 9–11 December 2019, Vienna, Austria, Conference Proceedings, Nano, Bio, Green and Space: Technologies for a Sustainable Future, Issue 6.3, Section Advances in Biotechnology, Vol. 19, paper 19, 141-148

[21] Negreanu-Pîrjol, B.-S., Negreanu-Pîrjol, T., Meghea, A., Nastac, M., Paraschiv, G.M., Bratu, M.M., Sîrbu, R., Roncea, F.N., Bucur, L.A., Badea, N., Meghea, I., Baltă, A.G., Gheorghiu, A.K., Zuliang, L., Are Pedersen, Ecological fertilizer biocomposite and process for obtaining, 2012, RO Patent 126038 A0/ 30.03.2012

[22] Negreanu-Pîrjol T., Negreanu-Pîrjol B., Paraschiv G.M., Meghea I., Niţu B., „Potential improvement of the residual marine biomass as nutrient for eroded soils and agriculture purpose", 11th International Multidisciplinary Scientific GeoConference „Modern Management of Mine Production, Geology and Environmental Protection" – SGEM 2011, 20 – 25 June 2011, Albena, Bulgaria, Conference Proceedings, Vol. III, Section Ecology and Environmental protection, 553 – 565

[23] Sava D. Samargiu M.,D., Paraschiv, G.M., Macroalgal Flora from the Romanian Black Sea Coast. Diversity, Ecology, present tend; The 8th National Conference of Environmental Protection and 5th National Conference of Ecosangenesis, May 2007, 100-104

[24] Sava, D., Algele Macrofite de la litoralul românesc al Mării Negre, Ovidius University Press, Constanta, 2006, 15 – 118

[25] Sîrbu R., Negreanu-Pîrjol T., Paris S., Negreanu-Pîrjol B.S., Sanda J., Zaharia M., Tomescu A., Important bioactive compounds from marine algae–potential source of pharmaceutical industry, 14th International Multidisciplinary Scientific GeoConferences – SGEM 2014, 17 – 26 June 2014, Albena, Bulgaria, Conference Proceedings, Section: Advances in Biotechnology, Vol. I, 381 – 388

[26] Vasiliu F., Macrophytic Algal Production from Romanian Black Sea Coast; PhD Thesis, Bucuresti University, 1984, 210 pp.

[27] Vasiliu, F., Ecological Consideration of Macrophytic Species List from Romanian Black Sea Coast, Naturalia, Piteşti, St. cerc. 1996, II - III, 432 – 444

[28] Welz B., Sperling M., Atomic Asorption Spectrometry, 3rd edition, Wiley-VCH, 1999

[29] Welz B., Becker-Ross H., Florek S., Heitmann U., High-Resolution Continuum Source AAS, ed. Wiley-VCH, 2005

[30] Zăgan S., Negreanu-Pîrjol B., Negreanu-Pîrjol T., Gorun E., Zăgan R., Effect of ultraviolet radiations on the microbiological quality of drinkable groundwater, Environmental Engineering and Management Journal, 2011, vol. 10, No. 11, 1687 – 1692

Preliminary Data Regarding Total Chlorophylls, Carotenoids and Flavonoids Content in Flavoparmelia Caperata (L.) Hale Lichens Species

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Abstract

Flavoparmelia caperata L. Hale (common greenshield lichen) is an Ascomycete, foliose lichen usually growing on tree bark in most mesophytic-forested habitats in Romania. Lichen samples were collected from three stations in Romania (Craiova, Timişoara, Hoia-Baciu Forest, Cluj) and extracted in 96% ethanol. Both dry lichen tissue powder and extracts were analyzed by UV-Vis spectrophotometry for determining photosynthetic pigment concentrations (chlorophylls a and b and total carotenoids). Dry tissue samples had chlorophyll a concentrations of 95.12-109.59 mg/kg DW, chlorophyll b concentrations of 88.53-110.98 mg/kg and total carotenoids 88.89-102.85 mg/kg. Alcoholic extracts of fresh lichen tissue showed an extreme variability, with 715.97-10331.50 mg/kg chlorophyll *a*, 527.77-

8124.20 mg/kg chlorophyll *b*, 1125.72-8714.90 mg/kg carotenoids pigments, with the highest values in samples from Craiova. A lower variability was observed in flavonoid contents, with 461.78-966.02 mg/kg DW in dry powder and 815.56-2734.135 mg/kg in ethanolic extracts. Extracts of lichens from Craiova had a decreased content of total flavonoids compared with the other two lichens ethanol extracts.

Keywords: *Flavoparmelia caperata* (L.) Hale, total chlorophylls, carotenoids, flavonoids

Introduction

Flavoparmelia caperata (L.) Hale – the common greenshield lichen – is a medium to large-sized, foliose Ascomycete lichen (Lecanorales Order, Parmeliaceae Family) (Fig. 1). With medium to large dimensions (up to 20 cm in diameter), the foliage is green, turning to a specific yellowish shade when dry. It has a variate color, a lobed shape, with granular soredia. On the back it is blackish, with brown edges. It has rounded, flattened lobes, 6-10 mm, with granular sores, brown apothecia, sessile and unbranched rhizoids, abundant especially on the edge. Like most Parmeliaceae, the phototrophic symbiont is a green algae of the genus *Trebouxia*. It is a mesophytic species, abundant especially in areas with a Mediterranean climate. The greenshield lichen grows on tree bark (rarely on rocks), on various woody species, throughout Mediterranean, sub-Mediterranean and temperate forests (alpine regions excluded) and only exceptionally on the ground. It also prefers an acidic or subacidic *p*H, tolerates direct exposure to sunlight and moderately eutrophic environments [1].

Lichens were used since ancient times, for various folk medicine applications. *Flavoparmelia caperata* is used in some regions of Chile for treating dispepsia, bronchitis, diabtetes, tuberculosis, hemorrhages, spermatorrhea etc.

In Romania it is frequently found in oak forests (on various species of the genus *Quercus*). It can also be found on the bark of maples, birches, lindens, walnuts, carobs, beeches, hornbeams, trees of *Prunus* genus, including the orchards. Sometimes it also colonizes the bark of conifers, the wood of dead trees or the saxicol environment [2].

Regarding the chemical composition of lichen tissues, there are differences related to the age and region of the thallus. Iron and copper are mainly concentrated in symbiotic algae. The lower cortex of the young parts concentrates elements such as Ni, Si, Ti, probably having a protective role. The marrow, especially that of my old areas of the thallus, accumulates calcium oxalate. Non-metals (P, S, Cl, are more abundant in the upper layers) [3].

Flavoparmelia caperata easily accumulates heavy metals in the atmosphere particulate. Due to its abundance and large size, it is a valuable bioindicator of metals pollution. Thus, in areas with industrial activities such as coal-fired power plants or

metallurgical plants, there were significant increases in tissue concentration of As (4-39 mg / kg), Ce (7-708 mg / kg), Cr (3-863 mg / kg), Co (0.4-6.8 mg / kg), Fe (790-17.830 mg / kg), La (1.6-236 mg / kg), Ti (87-1850 mg / kg) , V (2-27 mg / kg), Zn (19-141 mg / kg) [4].

Other bioaccumulated elements by this lichens species are B (6-19 mg / kg), Ga and In (less than 0.1 mg / kg), Li (0.4-0.7 mg / kg), Pb (7-22 mg -kg), Sr (11-40 mg / kg), Tl (0.6-1.5 mg / kg) and Zn (17-24 mg / kg). Ba, K, Mg, Na, Tl and Zn elements accumulate especially in the young areas of the thallus, and As, B, Cd, Cr, Cu, Fe, Ga, In, Li, Ni, Pb and Se preferentially in the central areas [5]. After other reported determinations, concentrations of about 25 mg / kg Cu, 5,000 mg / kg Fe, 80 mg / kg Zn, 80 mg / kg Pb can be achieved [6].

On the other hand, it has been found that this species has a medium to low resistance to air pollution, growing abundantly only in unpolluted ecosystems [7].

Recently research emphasized that *Flavoparmelia caperata* contains significant and easily extractable amounts of carbohydrates, proteins, steroids, tannins and triterpenes, as primary metabolites, and atranorine, usnic acid and protocetraric acid, as secondary metabolites [8].

Flavoparmelia caperata contains about 90 mg / g phenolic compounds, 34 mg / g flavonoids and a remarkable antioxidant capacity among other lichen species (550 μg / mL IC50) [9]. Studies have shown that there is a seasonal variation in the content of antioxidants (polyphenols, flavonoids), with minimal values in the vernal season [10].

Usnic acid (about 0.35% by weight) present in this lichen species, has antimicrobial properties, especially against the genus *Mycobacterium* [11]. Also, this compound and other secondary metabolites, have some efficacy in inhibiting α-amylase, with a possible role in diabetes improving [12]. Antimicrobial abilities were also noticed against *Bacillus subtilis, B. cereus*, *Staphylococcus aureus, Enterococcus faecium, Erwinia amylovora* etc. bacteria strains, also against fungi of Aspergillus genus. Antiproliferative properties have been demonstrated, for example against colon adenocarcinoma cells [9, 13, 14].

Other potentially valuable compounds reported in *Flavoparmelia caperata* species are as follows, heavy polysaccharides - homoglucans (lichen, isolichenan, pustulan, evernan, nigeran) and heteroglycans (galactomannan, glucomannan), with antitumor, immunomodulatory, antiviral properties, etc. [15, 16].

Among other valuable active principles in *Flavoparmelia caperata*, chlorophylls (the main photosynthetic pigments) are known for their anti-inflammatory, anti-carcinogenic properties [17 - 19]. They are a source of magnesium and inhibit kidney stone formation. Carotenoids are the precursors of melanin and retinol, key compounds for skin and eye health, also, with antioxidant and antiproliferative properties [19, 20]. Flavonoids (low-mass polyphenolic compounds) are strongly antioxidant, antiviral, antibacterial, antifungal, antiproliferative and antitumoral

compounds [26, 27]. The paper reveals the comparative preliminary results regarding the total chlorophylls, carotenoids and flavonoids content determination in *Flavoparmelia caperata* (L.) Hale, collected from three different Romanian pedo-climatic area, in the aim to emphasize the therapeutic potential of this lichens species.

Material and Methods

Fig. 1. *Flavoparmelia caperata* (L.) Hale lichens species [29 - 31]

Fig. 2. Appearance of the *Flavoparmelia caperata* (L.) Hale lichens powder

Sampling:

Lichen samples were collected in the period summer-autumn of 2018-2019, from three sampling sites of Romania, as follows:

City center Park of Timișoara

Hoia-Baciu Forest of Cluj-Napoca

„Alexandru Buia" Botanical Garden of Craiova

Part of the lichen samples were air-dried for determining dry biomass percentage and ground to powder using an electrical grinder (Fig. 2). Part of the samples were extracted with 96% ethanol at 1% final concentration for all samples.

Analysis:

For chlorophyll *a*, chlorophyll *b* and total carotenoid determinations, an amount of 1 g ground tissue powder was extracted in 100 mL 80% acetone (triplicate samples for each species). The extract was filtered at normal pressure through Whatman blue band filter paper and the spectrophotometric absorbance was read (using a S106 WPA UV-Vis spectrophotometer) against an 80% acetone blank, at 470 nm, 647 nm and 663 nm of wavelengths [18, 19]. Absorbance values were used to calculate

carotenoids pigments concentration, according to the specific trichromatic equations [21 - 25].

For flavonoid determinations, an amount of 1 g ground tissue powder was extracted in 5 mL methanol p.a. and filtered (triplicate samples). An aliquot of 0.5 mL of extract was diluted in 4 mL water and 8 mL methanol mixture, and the spectrophotometric absorbance was read against a methanol blank, at 340 nm wavelength [27, 28].

Results and Discussion

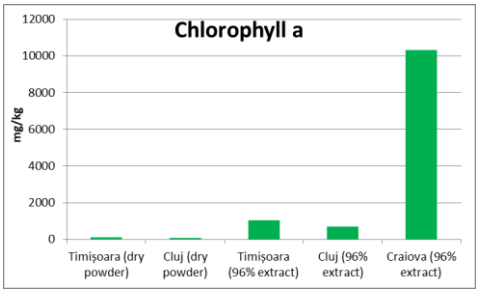

Fig. 3. Average concentration of chlorophyll a in dry powder and ethanolic extracts of *Flavoparmelia caperat*a samples from selected stations

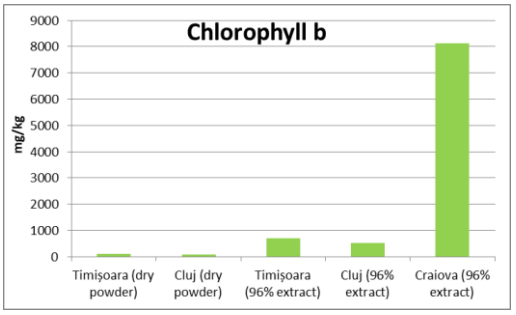

Fig. 4. Average concentration of chlorophyll b in dry powder and ethanolic extracts of *Flavoparmelia caperata* samples from selected

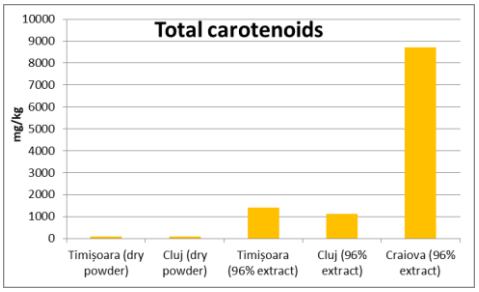

Fig. 5. Average concentration of total carotenoids in dry powder and ethanolic extracts of *Flavoparmelia caperata* samples from selected stations

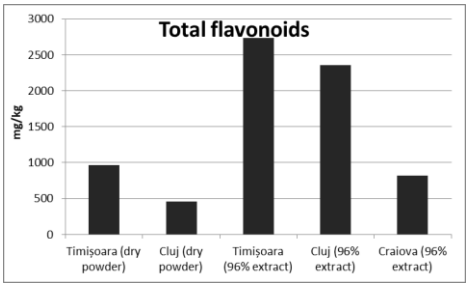

Fig. 6. Average concentration of total flavonoids in dry powder and ethanolic extracts of *Flavoparmelia caperata* samples from selected stations

Dry tissue samples had chlorophyll *a* concentrations of 95.12-109.59 mg/kg DW, chlorophyll *b* concentrations of 88.53-110.98 mg/kg and total carotenoids 88.89-102.85 mg/kg.

Alcoholic extracts of fresh lichen tissue showed an extreme variability, with 715.97-10331.50 mg/kg chlorophyll *a*, 527.77-8124.20 mg/kg chlorophyll *b*, 1125.72-8714.90 mg/kg carotenoids, with the highest values in samples from Craiova.

A lower variability was observed in flavonoid contents, with 461.78-966.02 mg/kg DW in dry powder and 815.56-2734.135 mg/kg in ethanolic extracts. Extracts from Craiova samples had lower content than other ethanolic extracts.

Conclusion

The preliminary results regarding the total chlorophylls, carotenoids and flavonoids contents of *Flavoparmelia caperata* (L.) Hale lichens species, emphasize a major geographical and pedo-climatic differences with impact on chemical composition.

The significant lower values obtained in the case of lichens drying tissue compared with hydroalcoholic extracts for all four bioactive compounds classes, were highlighted.

References

[1] Ach. (*Roccellaceae*) lichens collected from Eastern Blacksea Region, Turkey. J. Appl. Pharm. Sci., 3(2): 143-147.
[2] Aprile G.G., Di Salvatore M., Carratù G., Mingo A., Carafa A.M., 2010. Comparison of the suitability of two lichen species and one higher plant for monitoring airborne heavy metals. Environ. Monit. Assess., 162: 291-299.
[3] Aydin S., Kinalioğlu K., 2013. The investigation of antibacterial activities of ethanol and methanol extracts of *Flavoparmelia caperata* (L.) Hale (*Parmeliaceae*) and *Roccella phycopsis*
[4] Babiah P.S., Upreti D.K., John S.A., 2014. Fungicidal efficacy of a foliose lichen *Flavoparmelia caperata* (L.) Hale against phytopathogenic fungi. Int. J. Curr. Res. Biosci. Plant Biol., 1(5): 38-44.
[5] Eldahshan, O.A., Singab, A.N.B., Carotenoids, 2013, J. Pharmacogn. Phytochem., 2(1), 225-234.
[6] Ghorbanli M., Tehran T.A., Niyakan M., 2012. Seasonal changes in antioxidant activity, flavonoid, anthocyanin and phenolic compounds in *Flavoparmelia caperata* (L.) Hale and *Physcia dubia* (Hoffm.) Lettau from Babol forest sites in north of Iran. Iranian Journal of Plant Physiology, 2(3): 461-469.
[7] Godinho R.M., Wolterbeek H.T., Pinheiro M.T., Alves L.C., Verburg T.G., Freitas M.C., 2009. Micro-scale elemental distribution in the thallus of *Flavoparmelia caperata* transplanted to polluted site. J. Radioanal. Nucl. Chem., 281(2): 205-210.
[8] Inanç A.L. 2011. Chlorophyll: structural properties, health benefits and itsoccurrence in virgin olive oils, Akademik Gıda, 9(2), 26-32.

[9] Kivrak, I, Kivrak, 2014, Antioxidant properties, phenolic profile and nutritional value for *Sorbus umbellata* fruits from Turkey, Austin J Nutr Food Sc., 2(8), 1043-1048.

[10] Lichtenthaler H.K., Buschmann C. 2001. Chlorophylls and carotenoids: Measurement and characterization by UV-VIS spectroscopy. In Wrolstad R.E. (ed.), Current Protocols in Food Analytical Chemistry, John Wiley & Sons Inc., Hoboken, F4.3: 1-8.

[11] Mandujano O.N.C., 2010. Aislamiento del ácido úsnico de *Flavoparmelia caperata* y su determinación cuantitativa por espectroscopía UV, en diez líquenes. Rev. Soc. Quim. Peru, 76(4): 389-399.

[12] Mitrović T., Stamenković S., Cvetković V., Nikolić M., Baošić R., Mutić J., Anđelković T., Bojić A., 2012. Epiphytic lichen *Flavoparmelia caperata* as a sentinel for trace metal pollution. J. Serb. Chem. Soc., 77(9): 1301-1310.

[13] Mitrović T., Stamenković S., Cvetković V., Tošić S., Stanković M., Radojević I., Stefanović O., Čomić L., Đačić D., Ćurčić M., Marković S., 2011. Antioxidant, antimicrobial and antiproliferative activities of five lichen species. Int. J. Mol. Sci., 12: 5428-5448.

[14] Negreanu-Pirjol T., Sirbu R., Mirea M., Negreanu-Pirjol B.-S., Antioxidant activity correlated with chlorophyll pigments and magnesium content of some green seaweeds, European Journal of Medicine and Natural Sciences, Vol. 3, No. 1 (2019), p. 16 – 22.

[15] Popoviciu D.R., Negreanu-Pîrjol T., Bercu R., Carotenoids, flavonoids and total phenolic compounds concentration in fruits of milkflower cotoneaster (*Cotoneaster lacteus* W.W.Sm.), Annals of the University of Craiova, Editura Universitaria, Craiova, 2019, Series: Horticulture, Food products processing technology, vol. XXIV (LX), oct. 2019, pp. 476 – 481.

[16] Popoviciu D.R., Negreanu-Pîrjol T., Bercu R., Total carotenoid, flavonoid and phenolic compounds concentration in willowleaf cotoneaster (*Cotoneaster salicifolius* Franch.) Fruits", European Journal of Medicine and Natural Sciences - EJMN, September - December 2020, Vol. 4, Iss. 3, pp. 1 – 6.

[17] Popoviciu D.R., Negreanu-Pîrjol T., Carotenoid, flavonoid and total phenolic content of *Sorbus torminalis* fruits, Al E. M. M. (ed.), 2019, Rom.-Arab. Intern. J. Geo-Bio-div. VIII(1): 20-25, "Monachus" Group of Scientific Research and Ecological Education Publication.

[18] Popoviciu, D.R., Negreanu-Pirjol, T., Motelica, L., Negreanu-Pirjol B.S., Carotenoids, Flavonoids, Total Phenolic Compounds Content and Antioxidant Activity of Indigenous *Pyracantha coccinea* M. Roem. Fruits, Revista de Chimie (Bucharest), 71(4), 2020, 258-266.

[19] Popoviciu, D.R., Negreanu-Pirjol, T., Motelica, L., Negreanu-Pirjol B.S., Carotenoids, Flavonoids, Total Phenolic Compounds and Antioxidant Activity of Two Creeping *Cotoneaster* Species Fruits Extracts, Revista de Chimie (Bucharest), 71(3), 2020, 136-142

[20] Rashmi S., Rajkumar H.G., 2014. Preliminary phytochemical screening of different solvent extracts of lichens from Kodagu district, Karnataka. J. Pharmacogn. Phytochem., 3(4): 209-212.
[21] Showman R.E., Hendricks J.C., 1989. Trace element content of *Flavoparmelia caperata* (L.) hale due to industrial emissions. J. Air Poll. Control Assoc., 39: 317-320.
[22] Stanković M.S., 2011, Total phenolic content, flavonoid concentration and antioxidant activity of *Marrubium peregrinum* L. extracts, Kragujevac J Sci, 33, 63-72.
[23] Szabo I., Vonhaz G., Fodor A., Bungău S., Ţiţ D.M., 2012, The quantitative analysis through spectrophotometry of flavonoids and polyphenols from vegetable products *Hibisci trioni* herba, radix and fructus, Analele Univ Oradea, Fasc. Protecţia Mediului, 18, 73-80.
[24] Takeda T., Shimizu N., Watanabe S., Edagawa Y., Ito Y., Narui T., Shibata S., 2003. Further studies on the structure of polysaccharides from the lichen *Flavoparmelia caperata* (L.) Hale, Chem. Pharm. Bull., 51(12): 1436-1438.
[25] Valadbeigi T., Shaddel M., 2016. Amylase inhibitory activity of some macrolichens in Mazandaran province, Iran. Physiol. Pharmacol., 20: 215-219.
[26] Vicol I., 2011. Lichenii epifitici, indicatori ai calităţii mediului în ecosisteme forestiere din aria metropolitană a Municipiului Bucureşti. în: Nicolau, M., Ballo, A., Duda, S. (eds.). International Symposium „The environment and industry", Bucharest 16-18.11.2011. Vol. 2, Edit. EstFalia, Bucureşti: 303-309.
[27] Vicol I., 2013. Distribution of *Flavoparmelia caperata* (L.) Hale in Romania. Ann. Univ. „Al. I. Cuza". S II: Biologie Vegetală, 59(2): 65-73.
[28] Vicol I., A Study Regarding The Impact Of Forestry Management On Lichen Flora Within Forests From Bucharest Surroundings (Romania), Muzeul Olteniei Craiova. Oltenia. Studii şi comunicări. Ştiinţele Naturii. Tom. 27, No. 1/2011

Web

[1] http://www.stridvall.se/lichens/gallery/Flavoparmelia?page=1
[2] http://iucn.ekoo.se/iucn/species_view/103353/
[3] https://www.gbif.org/species/2606178