RHEOLOGICAL BEHAVIOUR OF SOME BIO-COMPOSITES BASED ON MARINE CHITOSAN AND KETOCONAZOLE

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The study assessed the rheological behaviour and properties of bio-composites based on chitosan extracted from Pachygrapsus mormoratus, the stone crab found in the Black Sea and ketoconazole, an antifungal with proven properties. Several semi-solid formulations were prepared by incorporating marine chitosan in various proportions and ketoconazole into ointment bases. For the obtained compositions, the rheological behaviour was analyzed in order to evaluate the stability of the pharmaceutical forms.

Keywords: Chitosan, *Pachygrapsus mormoratus*, rheological properties, chitosan ointment

1. Introduction

The development of pharmaceutical formulations with topical application that contain bioactive compounds requires knowledge of their properties, but also the beneficial effects and side effects, in order to potentiate the therapeutical effects and reduce the unwanted ones. In recent years, the continuous identification of new and useful natural biomaterials has led to the exploration of the marine ecosystem in search of new biologically active compounds [1, 2, 3]. The exploitation of marine resources in pharmaceutical interest was based on clean resources, untouched by pollutants [4, 5, 6, 7, 8]. In the process of isolating bioactive compounds from marine resources offered by the Black Sea, several study directions can be highlighted [9, 10, 11].

Due to their remarkable properties, natural polymers are used in the field of regenerative medicine and for a wide range of pharmaceutical applications, for the treatment of wounds and burns [12, 13, 14, 15].

Chitosan is a natural, non-toxic and biodegradable polymer, that is extremely abundant in nature. Chitosan is a polycationic copolymer, consisting of glucosamine and *N*-acetylglucosamine units and obtained by deacetylation of chitin found in the shells of crustaceans, like crab, lobster, shrimp [16, 17]. This biopolymer presents biocompatibility, biodegradability [18], non-toxicity, mucoadhesion, antimicrobial activity [19], natural origin and low cost and

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chitosan-based materials are widely considered to be used in several areas of medicine and pharmacy [20]. Despite the fact that chitosan is a unique and versatile compound, just a few pharmaceutical products based on chitosan are available. Chitosan is a hygroscopic material and its physico-chemical parameters differ depending on the source from which it was extracted [21].

The molecular weight (MW) and the degree of deacetylation (DD) are the main physico-chemical parameters that influence the characteristics of chitosan [22]. Chitosan can be extracted from a variety of sources and it is commercially available in different ranges of Mw and DD, factors that can effect the properties of the chitosan, so it is very important to consider the influence of these parameters on the biomedical applications [23, 24]. The molecular weight is a very important parameter because a minimum and certain value of molecular weight is needed in order to obtain a particular property of chitosan [25]. The degree of deacetylation represents a structural parameter with a influence on the physicochemical properties of chitosan [26] and many biological properties, including biodegradation [27] and wound healing properties [28].

In this paper we aim to assess the rheological behaviour of chitosan-based ointments, in mixture with ketoconazole. The novelty of the paper derives from the investigation of a natural polymer - chitosan, extracted for the first time from the shells of the stone crab species, *Pachygrapsus marmoratus*, from the Black Sea and the realization of a new semi-solid pharmaceutical formulation with topical application in the form of ointment. This new product is obtained by combining marine chitosan with a chemical component - ketoconazole, which is an antifungal with proven properties, but when administered orally it is toxic to the liver. In order to reduce the toxicity of ketoconazole, we propose the association with marine chitosan, which acts as a carrier. In this context, the new preparation is intended to be used for topical application, thus expanding the range of antifungal, topical products. In the formulation of the new preparations, the most important aspect is the stability study, which we realized through the rheological study. In this sense, we focus on the rheological study of the new preparation made in several variants. For this purpose we present two variants for the chitosan extraction from the shells of *Pachygrapsus mormoratus* stone crabs, found in the Black Sea. The degree of deacetylation and molecular weight for each obtained chitosan was determined.

2. Experimental

2.1. Procedure for extracting chitosan from Pachygrapsus mormoratus stone crabs found in the Black Sea

Chitosan was obtained from the shells of *Pachygrapsus mormoratus* stone crabs found in the Black Sea. The crabs were harvested from the Black Sea coast, Năvodari-Vadu coastal area, Constanța county, between June and July 2017.

Chitin was extracted from the cleaned, washed, dried and crushed shells. The bio-technological process of obtaining chitosan from the shells of *Pachygrapsus mormoratus* crab, involves 4 stages: deproteinization - the stage in which proteins and carbohydrates are dissolved, demineralization - removal of calcium carbonate and other minerals, discoloration - extraction of carotenoids and pigments – and deacetylation – transformation of chitin into chitosan.

We developed a working biotechnology composed of two different variants of chitin extraction, which we named V1 (variant 1) and V2 (variant 2). The experiments showed different results depending on the concentrations of the reagents, the temperature conditions and the reaction times used. Thus, we obtained two types of chitosan, with different molecular weights and different physico-chemical characteristics, which we marked with *CT1* and *CT2*, respectively. The characterization of the two types of chitosan was achieved by determining the qualitative attributes of the physico-chemical properties, such as: the degree of deacetylation and molecular weight. To determine the degree of deacetylation (DD) of the chitosan samples, the titration method with standardized aqueous solution of NaOH was applied. The method for determining the molecular weight (Mw) was based on intrinsic viscosity measurements of chitosan solutions extracted from *Pachygrapsus mormoratus*.

2.2. Preparation of chitosan bio-composites

The bio-composites based on chitosan extracted from *Pachygrapsus mormoratus* stone crabs were formulated as semi-solid, ointment-type pharmaceutical forms. The ointment base was prepared from cetyl alcohol, vaseline, glycerin, liquid paraffin, Tween 80 and water. In this oitment base, the two types of chitosan (*CT1* and *CT2*) were incorporated, in two different proportions (1% and 2%) and ketoconazole (*K*) was added in a constant proportion of 1% in each formulation. The samples were homogenized using an electric blender, at a speed of 150 rpm for 10 minutes. We obtained five homogeneous preparations of semi-solid consistency. All the prepared samples kept their consistency and appearance over time. The compositions of the five samples is shown in *Table 1*.

Table 1.

Composition of samples

Sample	Code of sample	Ointment base (%)	Chitosan (%)	Ketoconazole (%)
Sample 1	*B*	100	-	-
Sample 2	*B/CT1a/K*	98.0	1.0	1
Sample 3	*B/CT1b/K*	97.0	2.0	1
Sample 4	*B/CT2a/K*	98.0	1.0	1
Sample 5	*B/CT2b/K*	97.0	2.0	1

2.3. Rheological study of chitosan-based ointments

The rheological behavior of the bio-composites was analyzed for each ointment sample. The cylindrical system method with a known geometry was used and the viscosity of the preparations was determined, knowing the rotational speed. The viscosity curves were obtained taking into account the apparent viscosity (viscosity value corresponding to a given shear rate), calculated according to equation (1):

$$\eta = f(D) \tag{1}$$

We obtained the rheograms for the prepared bio-composites knowing the values of the shear stress τ *(Pa)* and the shear rate D *(s^{-1})* and applying equation (2):

$$D = f(\tau) \tag{2}$$

The shear rate D (s^{-1}) was calculated using equation (3), taking into account the values of the rotational speed ω (rpm). R is a specific constant for each axis of rotation, used to calculate the shear rate D (s^{-1}) in correlation with the rotational speed ω (rpm) used.

$$D = \omega * R \tag{3}$$

The shear stress τ was calculated using equation (4):

$$\tau = \eta * D \tag{4}$$

A Reovasco Star R rotary viscometer was used for the viscosity measurements. The time interval between the measurements and the duration of each determination was 10 seconds, and the measurements were performed by increasing and decreasing the rotational speed in the range of 4-200 rpm.

3. Results and discussion

3.1. Extraction process and characterization of the obtained chitosanes

Chitosan is a cationic polymer with a polysaccharide structure, composed of (1-4)-D-glucosamine and *N*-acetyl-D-glucosamine and obtained by alkaline deacetylation of chitin extracted from the crab shells.

The reaction conditions, temperatures used and working times for the proposed extraction variants are shown in *Table 2* and *Table 3*.

Table 2.

Reaction conditions for extraction variant 1 (V1)

	Variant 1 (V1)		
Step	Reagent	Temperature	Time
Deproteinization	NaOH 4%	90 ^{0}C	2 h
Demineralization	HCl 1%	25 ^{0}C	24 h
Discoloration	$KMnO_4$/ $C_2H_2O_4$ 1%	25 ^{0}C	1 h
Deacetylation	NaOH 50%	100 ^{0}C	2 h

Table 3.

Reaction conditions for extraction variant 2 (V2)

	Variant 2 (V2)		
Step	Reagent	Temperature	Time
Deproteinization	NaOH 2%	28 ^{0}C	24 h
Demineralization	HCl 3.5%	25 ^{0}C	5 h
Discoloration	acetone - ethyl ether 1:1 v/v	25 ^{0}C	20 min.
Deacetylation	NaOH 40%	105 ^{0}C	24 h

In each of the extraction variants we followed the same steps, but we changed the concentrations of the reagents used and the reaction time, finally obtaining two different chitosans in terms of physico-chemical properties. Great importance must be given to each reaction, but also to the reagents and temperatures used in the extraction variants, because these factors influence the important parameters in the characterization and quality of the obtained chitosan.

The molecular weight (Mw) was determined by intrinsic viscosity measurements for each of the two chitosans we obtained. The molecular weight of *CT1* and *CT2* was calculated using the Mark-Houwink equation:

$$[\eta] = kM^a \tag{5}$$

where: M is the average viscosity of the molecular weight, [η] is the intrinsic viscosity, *k* and *a* are the constant values 1.424 x 10^{-5} (dL / g) and 0.96 respectively.

Fig.1 and Fig.2 show the reduced viscosity dependence on the concentration of chitosan solutions *CT1* and *CT2* and the values of molecular masses calculated by applying equation (5) for each chitosan sample.

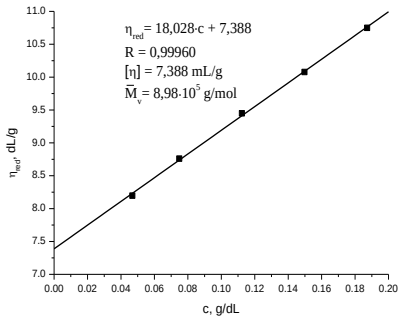

Fig. 1. Reduced viscosity dependence on *CT1* chitosan solution concentration

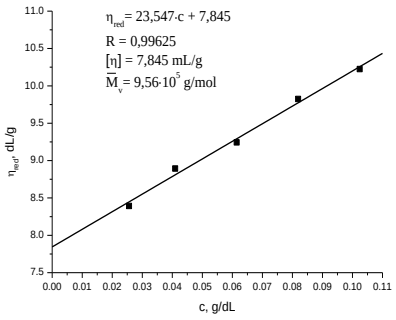

Fig. 2. Reduced viscosity dependence on *CT2* chitosan solution concentration

Extraction variant 1 (V1) resulted in a fine-powder, white-slightly yellowish chitosan (*CT1*) with a molecular weight of Mw = 8.98 x 10^5 g/mol and a degree of deacetylation of DD = 71.5%, and by applying extraction variant 2 (V2) we obtained chitosan *CT2*, with a coarser appearance, a brown color, a molecular weight of Mw = 9.56 x 10^5 g/mol and a degree of deacetylation of DD = 60.1%. The obtained results are summarized in *Table 4*.

Table 4.

Physical-chemical parameters of the obtained chitosanes

Extraction variant	Obtained Chitosan	Degree of deacetylation (DD)	Molecular weight (Mw)
V1	*CT1*	71.5%	8.98 x 10^5 g/mol
V2	*CT2*	60.1%	9.56 x 10^5 g/mol

3.2. Chitosan based bio-composites

The preparations obtained according to the formulas in Table 1 were stored and maintained at 4 ^{0}C until testing. All the analyzed samples were homogeneous and maintained their consistency for 7 days. Sample 1, the ointment base (*code B*) showed a fine appearance and a white color, the samples of bio-composites with chitosan in proportion of 1% (*code B/CT1a/K* and *code B/CT2a/K*) had a homogeneous appearance and a gray color, and for the samples with 2% chitosan (*code B/CT1b/K* and *code B/CT2b/K*) the appearance was slightly coarse and the color darker.

3.3. Rheological behavior of the chitosan-based bio-composites

Using the values obtained by experimental measurements for the rheological parameters shown in *Table 5* and by applying the calculation equations (1) - (4), we characterized the rheological behavior of the chitosan-based bio-composite samples.

Table 5.

Value ranges of rheological parameters for chitosan-based bio-composites

Sample code	*Viscosity η (cP)*	*Shear speed D (sec^{-1})*	*Shear stress (τ) Pa*
B	*1000 – 20450*	*1,36 - 68*	*27,81 – 68,00*
B/CT1a/K	*2470 – 54300*	*1,36 - 68*	*69,90 – 167,96*
B/CT1b/K	*2700 - 55300*	*1,36 - 68*	*70,31 – 183,6*
B/CT2a/K	*3100 – 51360*	*1,36 - 68*	*59,17 – 210,8*
B/CT2b/K	*4600 - 72400*	*1,36 - 68*	*98,46 – 312,8*

The rheological study of the chitosan-based bio-composites was performed by obtaining the rheogram, the viscosity curve and by linearization of the viscosity curve for each sample, and are presented in Fig. 3-7.

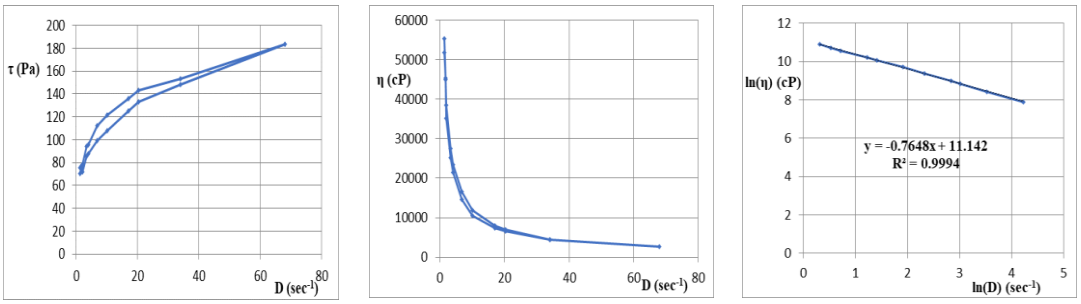

Fig. 3. Results of rheological study for bio-composite sample 1 *code B*
a) Rheogram; b) The viscosity curve; c) Linearization of the upward viscosity curve

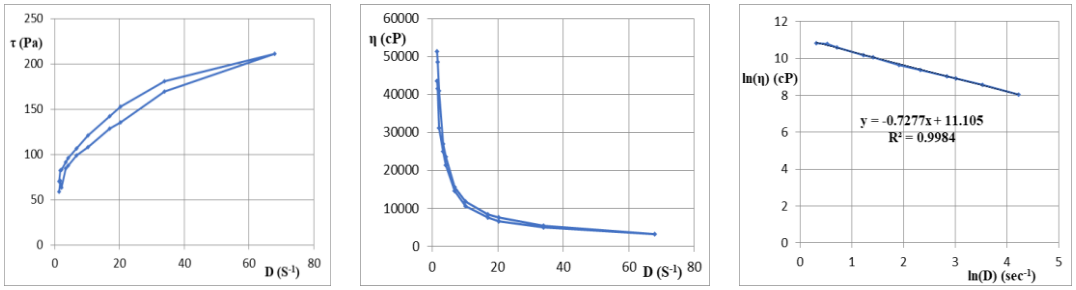

Fig. 4. Results of rheological study for bio-composite sample 2 *code B/CT1a/K*
a) Rheogram; b) The viscosity curve; c) Linearization of the upward viscosity curve

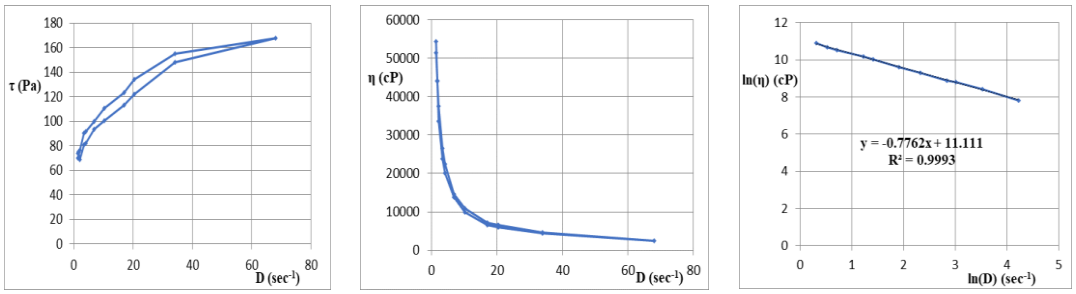

Fig. 5. Results of rheological study for bio-composite sample 3 *code B/CT1b/K*
a) Rheogram; b) The viscosity curve; c) Linearization of the upward viscosity curve

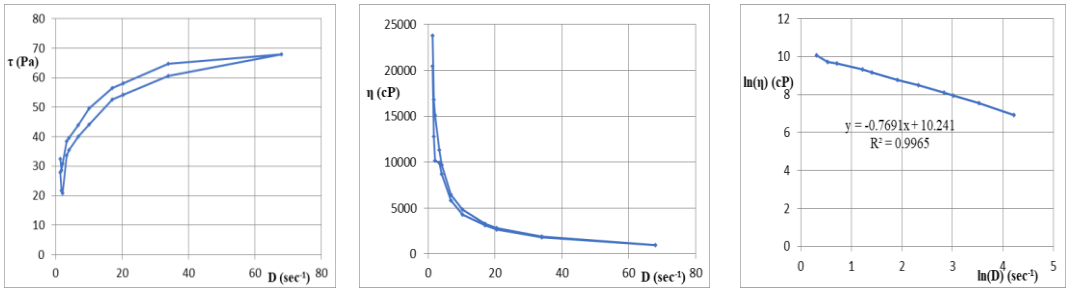

Fig. 6. Results of rheological study for bio-composite sample 4 *code B/CT2a/K*
a) Rheogram; b) The viscosity curve; c) Linearization of the upward viscosity curve

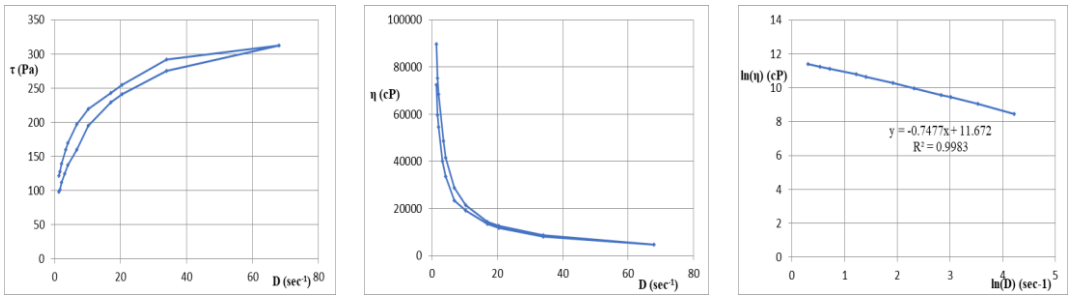

Fig. 7. Results of rheological study for bio-composite sample 5 *code B/CT2b/K*
a) Rheogram; b) The viscosity curve; c) Linearization of the upward viscosity curve

The rheograms show a pseudoplastic character of the prepared semi-solid formulations. Viscosity decreases with increasing rotational speed. After the linearization of the flow curves over a certain value of the shear rate, the prepared formulations maintain their structure. From the analysis of the obtained rheograms and graphs it was observed that the bio-composite samples prepared with chitosan *CT2* (sample 4 and sample 5), have a higher viscosity than the samples with chitosan *CT1*, due to the value of the molecular weight which is higher. At the same time, in the samples prepared with chitosan in proportion of 2% (*B/CT1b/K* and *B/CT2b/K*) an increase of the viscosity was observed at the same shear rate, compared to samples containing 1% chitosan. The viscosity curves obtained for the studied samples show a linearization of these curves at an increase in shear rate over 20 sec^{-1}, which means that they have an ideal plastic character at shear rates higher than 20 sec^{-1}. The analysis of the obtained results and rheograms reveals a non-Newtonian, pseudoplastic and thixotropic character of the studied bio-composite samples. This aspect leads to a stabile structure of the analyzed semisolid pharmaceutical forms. The results obtained by us are in agreement with the literature data on stability studies of chitosan composites [21, 24].

4. Conclusions

Bio-composites based on chitosan extracted from the shells of the *Pachygrapsus mormoratus* stone crabs found in the Black Sea were formulated as ointment type, semisolid pharmaceutical forms. The bio-composited intended for topical applications, incorporated two types of chitosan (*CT*) with different molecular weights, added to the samples in different proportion and ketoconazole (*K*) in order to assess the rheological behaviour each ointment.

The results obtained from the rheological study, highlight a non-Newtonian, pseudoplastic and thixotropic character of the prepared ointment samples. The viscosity curves obtained for each sample show a linearization of the curves when increasing the shear rate over 20 sec^{-1}, which shows a tendency

of ideal plastic behavior at high values of shear rate and a preservation of the structure of the analyzed pharmaceutical forms.

In conclusion, to reduce the toxicity of ketoconazole, I associated it with a natural compound of marine origin, chitosan, which has the function of a carrier. Chitosan increases antifungal efficacy, having its own antifungal effect. In conclusion, we propose products with topical application obtained from natural resources, which are stable, effective as antifungal drugs with low toxicity, thus expanding the area of antifungal preparations.

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