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Dedicated to Professor Gheorghe Maria
on the occasion of his 70th anniversary

EXPERIMENTAL EVALUATION OF CHITOSAN CHARACTERISTICS ON THREE MODEL ASSAYS OF BIOLOGICAL INTERFERENCES

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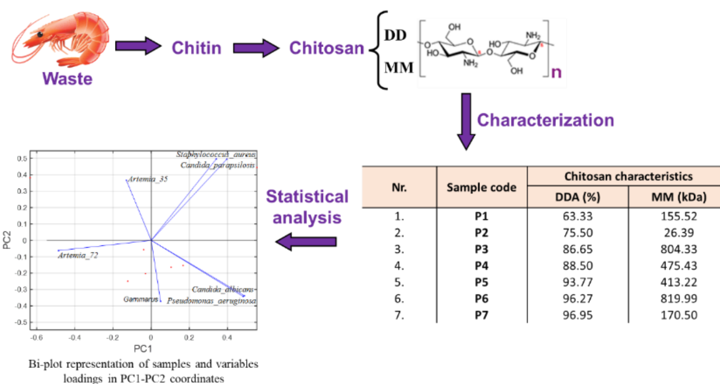
The degree of deacetylation (DDA) and molar mass (MM) are key factors influencing the biological activity of a given chitosan sample. There is growing interest in biocompatible and biodegradable polymers with demonstrated value, driven by their diverse biological and industrial properties, particularly considering the increase in antimicrobial-resistant strains. The seven chitosan samples were evaluated based on their biological effects, and a matrix of their characteristics was used in Partial Component Analysis (PCA) conducted on standardized data.



Nr.	Sample code	Chitosan characteristics	
		DDA (%)	MM (kDa)
1.	**P1**	63.33	155.52
2.	**P2**	75.50	26.39
3.	**P3**	86.65	804.33
4.	**P4**	88.50	475.43
5.	**P5**	93.77	413.22
6.	**P6**	96.27	819.99
7.	**P7**	96.95	170.50

INTRODUCTION

Both chitin and its deacetylated form, chitosan, are among the most important and widely used biopolymers with numerous applications in a wide variety of fields such as environmental engineering, aquaculture, medical, pharmaceutical, etc.[1–4] Chitosan has the ability to build a three-dimensional network which can bind to and presearve biologically active substances, such as small molecules, peptides, proteins, or other similar substances, from destruction.[5] On the other

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hand,
it has diverse biological activities, including antifungal, antimicrobial, antitumor, and antioxidant properties.[6–8]

Structurally, chitosan is a linear polysaccharide composed of N-acetyl-2-amino-2-deoxy-d-glucopyranose (acetylated unit) and 2-amino-2-deoxy-2-deoxy-glucopyranose (deacetylated unit), the repeating units being linked by β-(1→4)-glycosidic bonds. From physical – chemical point of view, chitosan is characterised by the molar mass (MM) and the degree of deacetylation (DDA). The presence of amino groups on the glycosidic chain, by protonation at pH less than 6.5, imparts to chitosan a good solubility and could be responsible for its antibacterial, antimicrobial and antifungal activity.[9,10]

Chitosan may be obtained from different natural sources such as shellfish waste, molluscs, insect sloughs (*exuviae*), etc. Recently, mollusc egg capsules were also identified as a valuable chitosan source.[11] From economic point of view, the extraction of chitosan from shrimp waste offers a sustainable and cost-effective way to valorise food waste, in particular seafood waste, mitigating the environmental impact while generating value-added goods.[12] Today, this eco-friendly approach to chitosan is in line with the laws of circular economy and One Health initiatives, which emphasise the transformation of waste into versatile functional biomaterials.[13] Due to the ability to modify its main physicochemical characteristics, such as MM and DDA, with a direct impact on its biological activity, shrimp chitosan is particularly attractive from scientific point of view.[14,15]

Antimicrobial resistance represents a significant global challenge to public health and development. Antimicrobial resistance is a phenomenon in which bacteria, viruses, fungi, and parasites cease to respond to antimicrobials. The risk of disease transmission, severe illness, disability, and mortality is elevated as a consequence of drug resistance, which renders antimicrobial drugs ineffective and infections difficult or impossible to treat[16]. Therefore, in the past two decades, there have been significant efforts in discovering and developing new agents with antimicrobial potential[17]. Chitin and chitosan have been studied as antibacterial agents against several target organisms, including bacteria and fungi, through both *in vivo* and *in vitro* interactions in multiple forms (solutions, films, and composites)[18] primarily assessing the minimum inhibitory concentration (MIC) while also suggesting potential mechanisms for interaction. Certain research indicates that the biological activity of chitosan is greatly influenced by its MM and DDA. Although both characteristics independently influence the antibacterial activity of chitosan, it is hypothesised that the impact of MM on antimicrobial activity is more significant than that of the DDA.[19–21]

Currently, there is an increase in interest and demand for biocompatible and biodegradable polymers with proven value that are based on a multitude of biological and industrial properties, especially in the context of the alarming rise in antimicrobial-resistant strains.[22] In this investigation, chitosan samples were derived from chitin extracted from shrimp and subsequently deacetylated under controlled circumstances to achieve high degrees of deacetylation and varying molar masses. One of the objectives of our study was to evaluate the biological activities of chitosan obtained from shrimp waste on three experimental models: *in vitro* antimicrobial tests and *in vivo* cytotoxicity evaluation on two aquatic organisms. The second objective was to correlate the main physicochemical characteristics of chitosan (MM and DDA) with its antimicrobial and toxicological activity, thus highlighting the importance of optimising the extraction processes of chitosan from marine wastes.

EXPERIMENTAL

1. Materials and methods

1.1 Synthesis and characterisation of chitosan samples

The chitosan powders (P1-P7) were obtained by optimising the deacetylation step of chitin extracted from shrimp waste following deproteination and demineralisation steps. The procedure was presented in detail in previous work.[14] The mean MM and DDA of polymer molecules are the most important features that influence most structure-function relationships. The DDA characterisation was carried out using potentiometric pH measurements.[11,23] MM was determined by measuring the intrinsic viscosity of diluted acid solutions of chitosan using an Ostwald-type capillary viscometer (model 518 10, SI Analytics GmbHTM, capillary tube inner diameter Øi = 0.43 mm).[11,14] These characteristics are presented in Table 1.

Table 1

Characteristics of the tested biopolymer

No.	Sample code	Chitosan characteristics	
		DDA (%)	MM (kDa)
1.	**P1**	63.33	155.52
2.	**P2**	75.50	26.39
3.	**P3**	86.65	804.33
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1.2 Biological activity assessment

Three biological test models were employed: I – pathogenic microorganisms; II – larval model; III – adult model (benthic crustacean organism) (Table 2). The analysis was performed for 24 hours to assess the impact of short-term exposure. The MIC values and the subsequent lethal effects following exposure to chitosan solutions (acute toxicity) were recorded as final evaluations. The tested concentrations were between (0.05–6.2 mg/mL) for Model I, and two concentrations, 35 µg/mL and 72.67 µg/mL, for tracking the *in vivo* effects (Models II and III).

Table 2

Biological model used in experimental evaluation

Experimental model	Tested species	Testing system	Organism details	Exposure time (h)	Temperatures (°C)	Endpoints
Model microorganisms	*Staphylococcus aureus* (ATCC 23235)	*In vitro*	Gram-positive	24	37	MIC*
	Pseudomonas aeruginosa (ATCC 27353)	*In vitro*	Gram-negative	24	37	MIC*
	Candida albicans (ATCC 10231)	*In vitro*	*Fungus*	24	25-28	MIC*
	Candida parapsilosis (ATCC 22019)	*In vitro*	*Fungus*	24	25-28	MIC*
II. Larval model	*Artemia* sp.	*In vivo*	*Larvae* (naupliar stage)	24 h	22-25	Acute toxicity
III. Adult model	*Gammarus sp.*	*In vivo*	Adult and pre-adult organisms	24	22-25	Acute toxicity

*MIC = minimum inhibitory concentration

1.2.1. Model I – pathogenic microorganisms

The antimicrobial activity was evaluated on *Staphylococcus aureus* (ATCC 23235), *Pseudomonas aeruginosa* (ATCC 27353), *Candida albicans* (ATCC 10231), and *Candida parapsilosis* (ATCC 22019). The evaluation was performed in sterile plates (96 wells), and seven serial dilutions were performed.

The tested solutions (100 µL) were distributed in the wells, of which 50 µL were transferred to the wells of the same column, and successive dilutions were performed. Then, the standardised bacterial suspensions (density was adjusted) and 10 µL of resazurin dye were added.

The positive control wells contained the culture medium and bacterial suspension for bacterial

growth, while the negative control wells contained the culture medium. The plates were sealed and subsequently incubated for 24 hours. During sample preparation, sensitivity control of *Candida sp.* strains (Fungitest) was also performed.

After incubation, the 96-well plates were visually examined to identify colour differences between the control and the tested samples.[24,25] Finally, the samples were compared by quantifying the minimum concentration at which inhibition is visible.

1.2.2. Model II – Artemia sp. assessment

The test was conducted in microplates utilising plexiglass wells with a volume of 1 mL. The study utilised *Artemia* sp. larvae sourced from the laboratory by introducing dehydrated cysts into artificially prepared saline water (35 g/L) using *Artemia* salt (Dohse Aquaristik GmbH & Co., Gelsdorf, Germany). Specific temperature conditions of 22–25°C were maintained for the development of the larvae, along with continuous aeration, in accordance with the ARTOXKIT protocol (ARTOXKIT M is a 24-hour mortality test utilising the anostracan crustacean *Artemia salina*. This assay adheres to ASTM Standard Guide E1440-91). These larvae are investigated for their cytotoxic effects' sensitivity and ease of quantification. The evaluation can be conducted for solutions that penetrate cell membranes as well as for particles that can be filtered and incorporated. The larvae initially do not feed for several hours; subsequently, they activate their digestive tract and commence feeding by filtering water.[26,27]

The analysed chitosan samples (Table 1) were weighed and solubilised in 1% acetic acid. For the purpose of this evaluation, solutions with small volumes, 5 and 10 µL/mL, were prepared at two different concentrations, 35 µg/mL and 72.67 µg/mL. The control variants included a water blank, a 1% acetic acid blank and commercial chitosan obtained from Sigma Aldrich. Each concentration was tested in triplicate. A minimum of 10 larvae *per* test hive was observed. Periodic evaluations were conducted, and effects were quantified at 24 hours (Table 2).

1.2.3. Model III – adult Gammarus sp. assessment

Testing was conducted on specimens of the amphipod genus *Gammarus*. This species is classified as a freshwater benthic organism, exhibiting a broad distribution in shallow aquatic environments. It is utilised in laboratory testing and *in situ* as an indicator of pollution. This study employed the principles of benthic aquatic organism testing.[28,29]

Specimen collection was conducted in accordance with the protocol, utilising a suitable nylon net to prevent injuries. Harvesting occurred in the Sibioara stream, Constanta County, in June 2022. The organisms were selected based on size and colour, which are indicators of normal physiological conditions (swimming ability, reaction speed to stimuli), lipid accumulation, and developmental stage. They were acclimated in large 50 L pots for a duration of 7–10 days and were periodically fed with plant debris sourced from the environment.

The organisms were evaluated under controlled experimental conditions in a static system to assess the acute effects of the substances analysed. Specimens selected ranged in size from 0.7 to 1.5 cm. Test solutions were prepared by dissolving chitosan in 1% acetic acid. Experiments were conducted in duplicates, with the number of specimens varying from 8 to 13 per experimental vessel. A batch maintained only in water was analysed as a negative control. Testing was conducted in plastic containers equipped with aeration systems. Each test vessel contained 100 mL of water and 0.5 mL of the test solutions. The organisms were provided with dehydrated plant tissue to prevent cannibalism. The assessment of effects involved measuring the physiological discomfort and mortality of organisms induced by chitosan presence at 24-hour intervals. This indicates the acute toxicity. The organisms were immersed in Davidson's fixative for a minimum of 24 hours for subsequent microscopic analysis. The gills were isolated through microdissection and examined using fluorescence microscopy in a fluorescein solution (1 mg/mL in DMSO), with excitation at λ=490 nm and emission at λ=520 nm.

RESULTS AND DISCUSSIONS

1. *In vitro* analysis

Analysing the values of the minimum inhibitory concentrations (MIC), it can be noted that chitosan exhibits antimicrobial inhibition in the exposed species. The lowest MIC values are recorded in the case of *S. aureus* and *C. parapsilosis* (Table 3). Inhibition of the *S. aureus* bacteria starts from the concentration of 10 µg/mL (P5, P7), and the maximum concentration observed is over 3 mg/mL.

Table 3

MIC values (mg/mL)

Sample	MM (kDa)	DDA (%)	*Staphylococcus aureus* ATCC 23235	*Pseudomonas aeruginosa* ATCC 27353	*Candida albicans* ATCC 10231	*Candida parapsilosis* ATCC 22019
P1	155.52	63.33	3.60	3.60	3.60	3.60
P2	26.39	75.5	0.86	1.70	1.70	1.70
P3	804.33	86.65	0.22	3.50	3.55	1.70
P4	475.43	88.5	1.70	3.40	3.40	1.70
P5	413.22	93.77	0.10	3.40	3.40	1.70
P6	819.99	96.27	0.22	3.40	3.40	1.70
P7	170.5	96.95	0.10	3.30	3.30	1.60

Analysing the results on *P. aeruginosa*, a gram-negative bacterium, it can be stated that chitosan manifests similarly effects in low molar mass samples (MM < 100 kDa). The disparities seen in both bacterial species can be attributed to the composition and architecture of the cell wall, with the impacts arising from the obstruction of cell membrane functions due to the aggregation of chitosan molecules on the bacterial surface. The findings align with existing research indicating that large polymer molecules tend to create a film that envelops cell membranes.[30]

The examination of chitosan solutions on the two fungal strains (*Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019) in the presence of resazurin dye did not reveal an effect connected with the concentration levels of chitosan. The results regarding the link with the alteration in its molar mass were not significant. Variations in reactions were noted across the tested *Candida* species, with *C. parapsilosis* exhibiting sensitivity. Other investigations indicate that chitosan exhibits significant fungicidal activity against *C. albicans*; nonetheless, the mechanisms driving its antifungal effects remain ambiguous.[31]

C. parapsilosis infections are notably common, particularly in nosocomial settings, and the widespread application of antifungals, both prophylactically and therapeutically, is acknowledged as a significant contributor to antifungal resistance globally.[32]

2. *In vivo* testing and survival rate recording

The tested organisms exhibited variations in response following 24 hours of exposure to the analysed solutions. In Model II, the larvae exhibited notable survival variations, with rates of 59% in C4 and 61% in C6, respectively. In the additional tests, survival rates exceeded 80% (Fig. 1). The effects in the Model III were markedly distinct. The majority of registrations fell below the 50% threshold (Fig. 1). The mechanism of action in this instance aligns with microscopic examinations of gills and is predicated on the assumption that chitosan caused swift mortality through the suppression of respiratory epithelia. Osmoregulation is an essential physiological process that regulates overall metabolism and significantly influences the survival and reproduction of aquatic species. Freshwater crustaceans maintain hyperosmotic regulation of body fluids compared to their external environment, hence limiting water influx and ion depletion.[33]

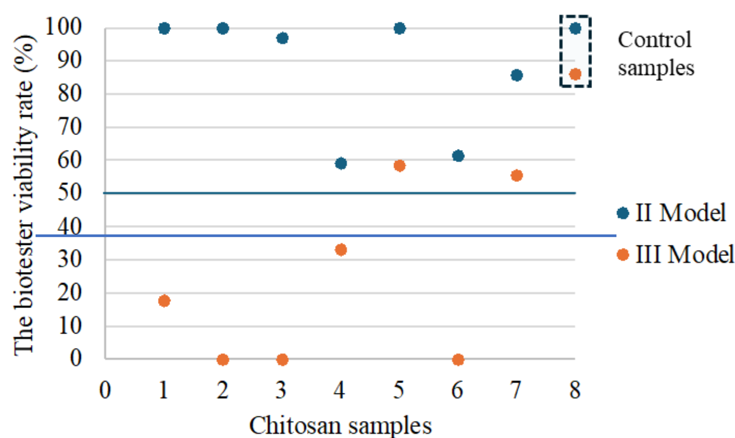

Fig. 1 – The biotester (Models II and III) viability rate (%) after 24 h exposure to chitosan samples (P1–P7).

Active compensatory absorption of ions from water is carried out through the gills into the circulatory system. Ion transport is accomplished through ion channels, which are highly represented in specific cells of the gill epithelium of the ionocyte type. Two major components involved in osmoregulation play a key role in ion uptake: the basolaterally located Na+/K+-ATPase pump and the apically located V-H+-ATPase pump.[34,35]

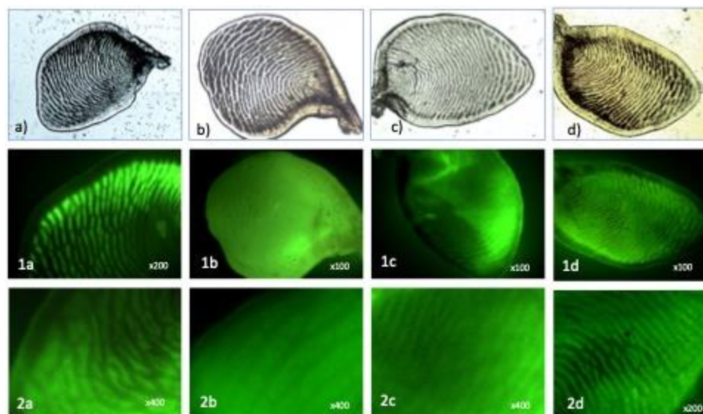

Fig. 2 – Microscopic details of gill structures in amphipods after exposure to chitosan solutions (35 μg/mL) for 24 h, for the relevant samples (a – control; b – P3; c – P4, d – P5) and different magnitudes 200× (1) and 400× (2).

The fast mortality of *Gammarus* specimens can be attributed to the obstruction of ion exchange control and structural modification of the gills. The observed findings indicate distortion of the canalicular structures and a more pronounced loss of gill morphology in specimens subjected to P3, P4, and P5 (Fig. 2). These data suggest that the effects of chitosan are contingent upon its molecular properties and the specific pathway and nature of its interaction with biological systems.

3. Statistical analysis

The initial phase of data analysis involved assessing potential relationships between the examined biological features and the primary characteristics of chitosan (degree of deacetylation and molecular mass). Table 4 consolidates the findings.

Table 4

Correlation between the examined biological features and the DDA and MM of chitosan

Properties	DDA – *Gammarus* viability	MM – *Gammarus* viability	DDA – *Artemia* viability at 72 μg/mL	DDA – *Artemia* viability at 35 μg/mL	MM – *Artemia* viability at 72 μg/mL	MM – *Artemia* viability at 35 μg/mL	DDA – *S. aureus* MIC	MM – *S. aureus* MIC
Person's correlation coefficient –	0.40	–0.31	0.15	–0.40	–0.40	–0.46	–0.84	–0.40

Table 4 shows a significant negative connection between chitosan DDA and its effect on the MIC value for S. aureus. This suggests that a higher DDA will result in a reduced MIC value, hence enhancing the antibacterial activity. The negative moderate correlation coefficients between the MM and Artemia viability suggest that a reduction in MM due to chitosan may enhance viability, whereas the DDA shows no any dependence on the Artemia viability at elevated chitosan concentrations. The relationship between the survival of benthic crustacea and chitosan characteristics is less significant, indicating that a higher DDA and lower MM would be advantageous.

A more detailed statistical treatment was performed using Partial Component Analysis (PCA).[36,37] Such multivariate statistical methods (clustering, PCA, Linear Discriminant Analysis LDA) are proved to be adequate tool in various experimental data processing.[38,39] This multivariate tool allows a variable reduction in coupling the real variables into the so-called principal components (PCs), which are orthogonal, by defining a linear relation between the variables. The coefficients of the real variables in each PC are called "loadings", and they stand for the

contribution of each variable in the PCs. The PCs encompass the data variability in decreasing order: the first PC will be responsible for the greatest variability, the second PC, the second greatest variability, and so on. The projection of samples in the PCs coordinates may highlight some samples grouping according to similar behaviour. Analysing the contributions of each real variable in the first PCs, some conclusions about the most relevant variables that differentiate the samples might be drawn.

The seven chitosan samples were characterised according to their biological effects, and the matrix of sample characteristics presented in Table 5 was implemented in the PCA analysis carried out for standardised data.

Table 5

Experimental data in biological assays

Chitosan	***Gammarus* viability**	***Artemia* viability at 72 µg/mL**	***Artemia* viability at 35 µg/mL**	***S. aureus* MIC**	***P. aeruginosa* MIC**	***C. albicans* MIC**	***C. parapsilosis* MIC**
P1	17.65	43.33	100.00	3.60	3.60	3.60	3.60
P2	0.00	100.00	100.00	0.86	1.70	1.70	1.70
P3	0.00	82.61	97.06	0.22	3.50	3.55	1.70
P4	33.33	75.00	59.26	1.70	3.40	3.40	1.70
P5	58.33	76.19	100.00	0.10	3.40	3.40	1.70
P6	0.00	38.09	61.29	0.22	3.40	3.40	1.70
P7	55.56	90.91	85.71	0.10	3.30	3.30	1.60

In PCA, the variables referring to crustacea viability are denoted by their names and the concentration of the solution used (Artemia_35, Artemia_72, Gammarus), while for the recorded MIC value, the variables were denoted by the corresponding bacteria or fungi names.

The graphical representation of data variability is represented by the box and whiskers diagram (Fig. 3) of standardised values (0y axis) according to the relation:

$$\text{Stand_val}_i = \frac{X_i - \text{mean}(X)}{\sigma(X)}$$

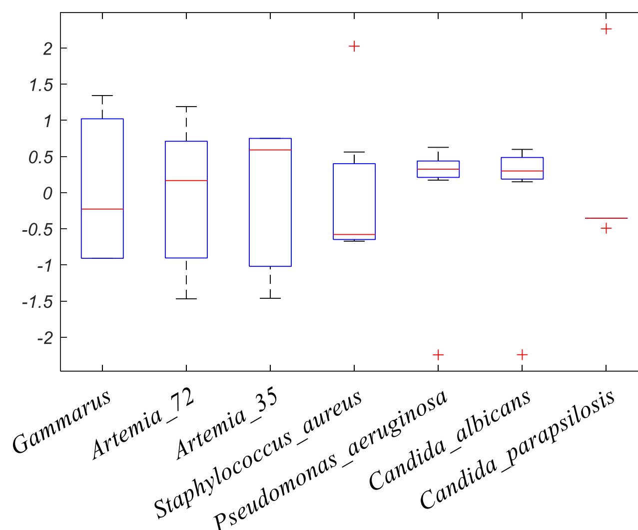

Fig. 3 – Box and whiskers diagram for the standardised value of variables used in PCA.

PCA performed for the seven samples corresponding to the chitosan types and the seven variables reflecting their biological responses (Table 5) resulted in data lumping in three main PCs that encompassed about 88% of data variability (PC1, 42.6 %, PC2, 24.8%, and PC3, 8.2%).

The variable loadings on these three PCs showed that the viability of the *Artemia larvae* at 72 mg/mL, the MIC values for *Pseudomonas aeruginosa* and *Candida albicans* had the highest loadings in PC1, which were responsible for the greatest variability between samples. PC2 encompassed MIC values for *Staphylococcus aureus* and Candida parapsilosis. Fig. 4 shows the importance of real variables considered in the first two PCs.

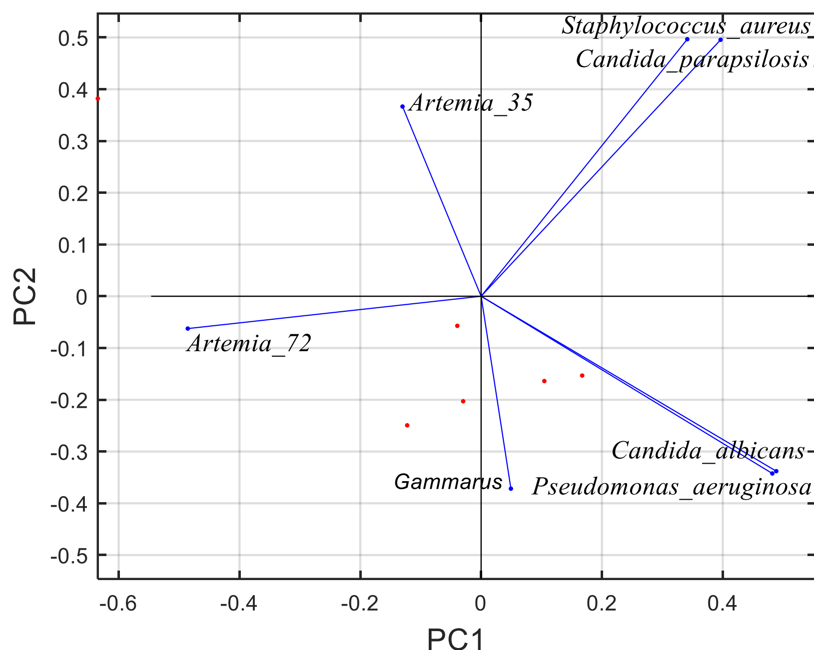

Fig. 4 – Bi-plot representation of samples and variables loadings in PC1-PC2 coordinates.

The analysis of angles between the vectors corresponding to various variables in the bi-plot graph (Fig. 4) indicates a strong positive association between the efficacy of chitosans against *Candida albicans* and *Pseudomonas aeruginosa*, as well as between the MIC values for *Staphylococcus aureus* and *Candida parapsilosis*. A weak to moderate positive correlation exists between the viability of *Artemia* larvae at a dosage of 35 mg/mL and the effect on *S. aureus*. The bioactivity against *S. aureus* is optimal at low MIC values, indicating that chitosan, which exhibits low MIC values, reduces the rate of survival of *Artemia* larvae. A comparable observation which can be articulated about the weak to moderate positive correlation between the viability of benthic crustacea (Gammarus) and the effects on *Pseudomonas aeruginosa*: chitosan, which exhibits low MIC values, is also associated with reduced crustacea viability.

Moreover, the representation of chitosan samples in PC1-PC2 coordinates (Fig. 5) suggests that a sample grouping is possible.

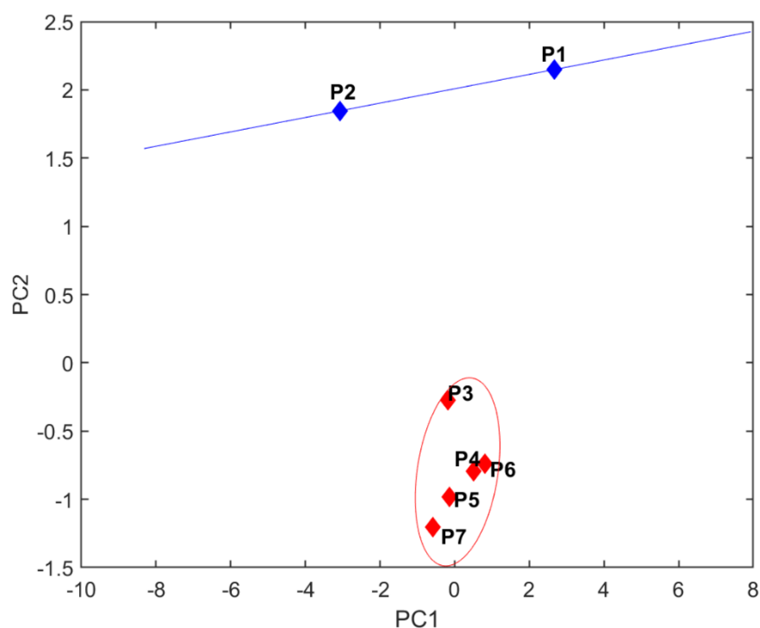

Fig. 5 – Sample representation in PC1–PC2 coordinates.

The first group (P1 and P2 samples) correspond to low DDA values, while the second group (P3–P7) correspond to chitosan samples with high DDA. As for the MM values, the P1 and P2 chitosan samples also have low to moderate MM. The chitosan form in P7 possesses a MM of 170 kDa; yet, due to its increased DDA, it exhibits biological activity that is comparable to samples with both high MM and high DDA. This may indicate that DDA plays a major role in determining the behaviour of chitosan in these specific biological assays.

CONCLUSIONS

Chitosan is a polymer that can exhibit varying interactions with biological systems due to variations in its physicochemical properties. The analysis of antibacterial and antifungal activity (Model I) revealed that inhibition was contingent upon the type of microorganism involved. The antibacterial activity of the studied chitosan can be correlated with the mode of interaction at the membrane level and the structural type of the cell wall. The observed results and species differences are promising and may facilitate new research avenues concerning the antifungal properties of chitosan. The toxicity or cytotoxicity assessed *in vivo* was low or moderate in the larval model (Model II), whereas it was significantly heightened in Model III (adult organisms). This indicates a correlation between the effects and the mode of penetration, similar to that observed in the microorganism models. In the larval model, chitosan was introduced *via* the digestive tube, as *larvae* filter particles. In Model III, the polymer interacts at the gill level during respiration, resulting in alterations in ionic homeostasis and a swift decline in viability. The findings indicate that, from a biological perspective, chitosan can be analysed in relation to its interaction mechanisms at the membrane level. The effects are determined by the molecular characteristics of chitosan and the mechanisms involved in its penetration into biological systems.

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