Praca doktorska

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Wybrane skorupiaki z wód polskich jako alternatywne źródło chityny i chitozanu

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Chosen crustacean species from Polish waters as an alternative source of chitin and chitosan

by

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1. **Nuc Z.**, Dobrzycka-Krahel A. 2021. From chitin to chitosan–a potential natural antimicrobial agent. Progress on Chemistry and Application of Chitin and its Derivatives, 26, 23-40. DOI:10.15259/PCACD.26.003

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2. **Nuc Z.**, Brusotti G., Catenacci L., Grenha A., Pontes J.F., Pinto da Silva J., Ana da Costa A.M., Moro P., Milanese C., Grisoli P., Sorrenti M., Dobrzycka-Krahel A., Bonferoni M.C., Caramella C.M. 2023. *Astacus leptodactylus* (Eschscholtz, 1823) and *Faxonius limosus* (Rafinesque, 1817) as new, alternative sources of chitin and chitosan. Water, 15(17), 3024. DOI: 10.3390/w15173024.

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3. **Nuc Z.,** Brusotti G., Catenacci L., Grenha A., Pontes J.F., Pinto da Silva J., Ana da Costa A.M., Moro P., Milanese C., Grisoli P., Sorrenti M., Dobrzycka-Krahel A., Bonferoni M.C., Caramella C.M. Green extraction of chitin from two crustacean species: *Dikerogammarus villosus* (Sowinsky, 1894) and *Neomysis integer* (Leach, 1814) and characterization of chitin and chitosan obtained from them with standard method

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Wykaz skrótów

The following list describes the meaning of the most important abbreviations and acronyms used throughout the dessertation :

List of abbreviations/ Lista skrótów

FTIR	spektroskopia podczerwieni z transformacją Fouriera (*eng. Fourier – Transform Infrated Spectroscopy)*
HBA	akceptor wiązań wodorowych (*eng. Hydrogen Bond Acceptor*)
HBD	donor wiązań wodorowych (*eng. Hydrogen Bond Donor*)
IAS	obce gatunki inwazyjne (*eng. Invasive Alien Species*)
IC	stężenie hamujące (*eng. Inhibitory Concentration*)
MBC	minimalne stężenie bakteriobójcze (*eng. Minimum Bactericidal Concentration)*
MIC	minimalne stężenie hamujące (*eng, Minimum Inhibitory Concentration*)
Mw	średnia wagowo masa cząsteczkowa (*eng. molecular weight*)
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolinum bromide)
NADES	naturalne rozpuszczalniki głębokoeutektyczne (*eng. Natural Deep Eutectic Solvents)*
SEM	skaningowy mikroskop elektronowy (*eng. Scanning Electron Microscopy*)
TG	analiza termograwimetryczna (*eng. thermogravimetric analysis*)

Streszczenie

Chityna jest powszechnie występującym w środowisku biopolimerem, syntezowanym przez owady, grzyby, mięczaki, gąbki i skorupiaki. W procesie deacetylacji można uzyskać jej pochodną, zwaną chitozanem. Chitozan, dzięki wolnym grupom amidowym może łączyć się z różnymi cząsteczkami i powierzchniami, oraz wykazuje różnego rodzaju aktywność biologiczną. Uznaje się, że jest związkiem biokompatybilnym, charakteryzującym się niską toksycznością. O jego aktywności biologicznej decydują między innymi parametry takie jak masa cząsteczkowa i stopień deacetylacji, które różnią się w zależności od źródła polimeru. Dodatkowo, na właściwości chitozanu może mieć wpływ metoda ekstrakcji chityny oraz jej deacetylacji. Metoda ekstrakcji chityny wykorzystywana na skalę masową jest uznawana za szkodliwą dla środowiska ze względu na zużycie energii, produkcję dużej ilości ścieków, a używane w niej stężone odczynniki mogą powodować degradację izolowanego polimeru. Jedną z alternatywnych metod ekstrakcji chityny jest stosowanie mieszanin naturalnych rozpuszczalników głęboko eutektycznych (NADES).

Celem niniejszych badań było uzyskanie oraz analiza właściwości fizykochemicznych chityny oraz chitozanu z czterech gatunków skorupiaków, występujących w wodach polskich: raka błotnego *Pontastacus leptodactylus* (Eschscholtz, 1828), raka pręgowanego *Faxonius limosus* (Rafinesque, 1817), kiełża *Dikerogammarus villosus*, (Sowinsky, 1894) i lasonoga *Neomysis integer* (Leach, 1814), oraz ocena ich potencjału jako alternatywne źródła tych polimerów. Dodatkowo podjęto się ekstrakcji chityny z tych samych gatunków, przy pomocy naturalnych rozpuszczalników głęboko eutektycznych (NADES) oraz zbadano aktywność przeciwbakteryjną i cytotoksyczną uzyskanego chitozanu.

Niniejsze badania wykazały, że *P. leptodactylus*, *F. limosus* i *D. villosus* stanowią atrakcyjne źródło chityny i chitozanu. Wszystkie badane próbki chitozanu wykazały aktywność przeciwbakteryjną wyższą niż chitozan będący materiałem referencyjnym. Mikrostruktura próbek chitozanu uzyskanego z *P. leptodactylus* i *F. limosus* charakteryzowała się cechami pożądanymi w medycynie pod względem inżynierii tkanek, aplikacji w opatrunkach i lekach, takimi jak porowatość i obecność mikro włókien. Wszystkie badane próbki chitozanu wykazały działanie cytotoksyczne przy stężeniach powyżej 250 µl/mL i czasie ekspozycji 24 h. Na podstawie współczynnika dyspersji odrzucono *N. integer* jako atrakcyjne źródło badanych polimerów. Zastosowana

w niniejszych badaniach mieszanina NADES okazała się być efektywna tylko dla ekstrakcji chityny z pancerzy *P. leptodactylus*. W pozostałych przypadkach nie udało się całkowicie odseparować chityny od białek.

Słowa kluczowe: biopolimer, chityna, chitosan, rozpuszczalniki głęboko eutektyczne skorupiaki, *Pontastacus leptodactylus, Faxoniux limosus, Dikerogammarus villosus, Neomysis integer*

Abstract

Chitin is a biopolymer commonly found in the environment, synthesized by insects, fungi, mollusks, sponges, and crustaceans. It can be modified in the process of deacetylation into chitosan. Chitosan, thanks to its free amide groups, can bind to various molecules and surfaces and exhibit various biological activities. It is considered a biocompatible compound characterized by low toxicity. Its biological activity is determined by parameters such as molecular weight and degree of deacetylation, which vary depending on the polymer source. Furthermore, the chitin extraction and deacetylation method can impact chitosan properties. The chitin extraction method used on industrial scale is considered environmentally harmful due to energy consumption, and production of large amounts of wastewater. Furthermore, strong reagents, used in the process can cause degradation of the isolated polymer. One of the alternative methods for chitin extraction is based on mixtures made with natural deep eutectic solvents (NADES).

The aim of this study was to obtain and analyze the physicochemical properties of chitin and chitosan polymers from four crustacean species found in Polish waters: the narrow-clawed crayfish *Pontastacus leptodactylus* (Eschscholtz, 1828), the spinycheek crayfish *Faxonius limosus* (Rafinesque, 1817), gammarid *Dikerogammarus villosus* (Sowinsky, 1894), and mysid *Neomysis integer* (Leach, 1814), and to assess their potential as alternative sources of these polymers. Additionally, chitin was extracted from these same species using natural deep eutectic solvents (NADES). The antibacterial and cytotoxic activity of the obtained chitosan was assessed.

This study demonstrated that *P. leptodactylus*, *F. limosus*, and *D. villosus* constitute attractive sources of chitin and chitosan. All tested chitosan samples demonstrated antibacterial activity higher than the chitosan used as a reference. The microstructure of chitosan samples obtained from *P. leptodactylus* and *F. limosus* was characterized by

desirable characteristics in medicine in terms of tissue engineering, wound dressings, and drug applications, such as porosity and the presence of microfibers. All tested chitosan samples demonstrated cytotoxic activity at concentrations above 250 µl/mL and an exposure time of 24 hours. Based on the molecular dispersity index, *N. integer* was rejected as an attractive source of the tested polymers. The NADES mixture used in this study proved effective only for the extraction of chitin from *P. leptodactylus* shells. Chitin from *F. limosus*, *D. villosus* and *N. integer* wasn't fully separated form proteins.

Keywords: biopolymer, chitin, chitosan, deep eutectic solvents, crustaceans, *Pontastacus leptodactylus*, *Faxonius limosus*, *Dikerogammarus villosus*, *Neomysis integer*

1. Wprowadzenie

1.1. Chityna

Pojęcie „chityna" obejmuje polimery z grupy polisacharydów, składające się z grup N-acetyl-2-amino-2-deoksy-d-glukopiranozy i 2-amino-2-deoxy-d-glukozy połączonych wiązaniami β - 1, 4 - glikozydowymi. Łańcuchy chityny składają się z 1000 – 3000 jednostek, są ułożone warstwowo i połączone wiązaniami wodorowymi N-H…O=C między grupami amidowymi i karbonylowymi (Mucha, 2010). W zależności od ułożenia warstw łańcuchów chityny, oraz typu łączących je wiązań, możemy ją podzielić na trzy formy allomorficzne: α, β i γ (Jang i inni, 2004, Kaya i inni, 2017). α -chityna produkowana jest przez skorupiaki i owady, chociaż została również wykryta w łuskach ryb (Heryanto i inni, 2025), β-chityna występuje w kościach mątw i kałamarnic (Arrouze i inni, 2020), natomiast γ-chityna we włóknach kokonu ćmy *Orgyia dubia* (Kaya i inni, 2017). Chityna po procesie syntezy i połączeniu się w mikrofibryle, wiąże się z innymi cukrami, białkami, glikoproteinami i proteoglikanami (Muzzarelli, 2010) tworząc kompleksy, których proporcja w stosunku do siebie, a także stabilność różni się w zależności od źródła tego polimeru (Kaya i in., 2014).

1.2.Źródła chityny

Zdolność do biosyntezy chityny wykazują owady, pajęczaki, skorupiaki, grzyby należące do sprzężniaków, mięczaki i gąbki (Joseph i inni, 2021). Badania naukowe coraz częściej skupiają się na ekstrakcji chityny z grzybów, owadów i pajęczaków, ze względu na łatwy proces ekstrakcji oraz możliwość wyprodukowania dużej biomasy w krótkim czasie i niskim kosztem (Nwe i in., 2011; Machałowski i in., 2019). Pod względem dużej biomasy wygrywają również skorupiaki, ze względu na ich popularność w przemyśle gastronomicznym. Rocznie produkuje się potężne ilości odpadów po konsumpcji owoców morza, która wymaga utylizacji, a jednocześnie stanowi źródło wielu cennych związków - w tym chityny (Coppola i in., 2021). Pojawiają się również badania dotyczące pozyskiwania chityny z obcych gatunków inwazyjnych (IAS)(Khiari i in., 2020; Jabeur i in., 2022). Zgodnie z dyrektywą europejską 1143/2014 dotyczącą postępowania z IAS na terenie Unii Europejskiej, wszystkie gatunki klasyfikowane jako IAS muszą podlegać kontroli, a w przypadku stwarzania zagrożenia dla środowiska – usunięcia (Robertson i in., 2020). Istnieją różne metody pozbywania się osobników IAS ze środowiska i można podzielić je na chemiczne, biologiczne i fizyczne. Najbardziej przyjazne dla środowiska są metody fizyczne, takie jak na przykład pułapki (Wood i in., 2021). Wyłapane osobniki wymagają utylizacji i mogą stanowić dodatkowe źródło chityny. Chityna zawarta w pancerzach skorupiaków występuje w postaci kompleksów składających się z chityny, soli mineralnych i białek.

1.3.Metody ekstrakcji chityny

Konwencjonalna metoda ekstrakcji chityny składa się z trzech etapów. Pierwszym jest demineralizacja, podczas którego, przy użyciu silnego kwasu usuwa się sole mineralne z biomasy. Najczęściej używany jest w tym celu stężony kwas solny (HCl). W drugim etapie, deproteinizacji, przy użyciu stężonej zasady, najczęściej wodorotlenku sodu (NaOH), usuwa się z materiału białka (poprzez zerwanie wiązań peptydowych) i lipidy (przez saponifikację). W trzecim etapie, który jest opcjonalny, materiał jest ponownie traktowany stężoną zasadą, w celu pozbycia się pigmentu (Broqua i inni, 2019). Metoda ta jest uznawana za względnie szybką i bardzo wydajną, jednak posiada parę istotnych wad. Ze względu na konieczność produkcji ciepła do przeprowadzenia deproteinizacji, metoda ta powoduje duże zużycie energii. Dodatkowo po każdym etapie należy przepłukać materiał wodą, w celu uzyskania obojętnego pH, co w warunkach produkcji powoduje zużycie dużej ilości wody. Kolejną wadą jest problem użycia

odczynników, któremogą powodować degradację chityny i wpływać negatywnie na jej właściwości (Gadgey and Bahekar, 2017).

Zgodnie z założeniami „Zielonej i zrównoważonej chemii" (Halpaap, 2021). Green and Sustainable Chemistry: Framework Manual.), a także rezolucji przyjętej przez Parlament Europejski 2020 r., w sprawie strategii w zakresie chemikaliów na rzecz zrównoważenia, wszystkie strony UNEP (United Nations Environment Programme) i UE powinny w miarę możliwości rezygnować lub zredukować do minimum zużycie substancji chemicznych szkodliwych dla środowiska (Dziennik Urzędowy Unii Europejskiej, C/2024/2894, 26.04.2024). W tym celu opracowywane są metody alternatywne, bardziej przyjazne środowisku. Można je podzielić na biologiczne i chemiczne. W metodach biologicznych wykorzystuje się bakterie do produkcji enzymów lub fermentacji. Za pomocą proteaz można pozbyć się do 90 % białek, jednak ze względu na to, że wymagają one środowiska zasadowego, nie nadają się do demineralizacji. Fermentacja natomiast może skutkować całkowitą ekstrakcją chityny, jednak jest ona czasochłonna i wymaga przygotowania odpowiedniej pożywki wspomagającej wzrost kultury, tak, aby zmaksymalizować jej wydajność (Kozma i inni, 2022).

Wśród metod chemicznych opracowywane są systemy cieczy jonowych (ILs) i naturalnych rozpuszczalników głęboko eutektycznych (NaDES), elektroforezy (Nowacki i inni, 2020), czy wyładowań bariery dielektrycznej (DBD- dielectric barrier discharge)(Borić i inni, 2020). Jednak największą popularnością cieszą się ILs i NADES. Ils charakteryzują się stabilnością termiczną i niską temperaturą topnienia. Nie są łatwopalne, ani lotne, a dodatkowo mogą po regeneracji zostać użyte ponownie. Jednak pomimo tego, że są efektywne przy usuwaniu białek, do demineralizacji konieczne jest użycie kwasu. Ich toksyczność i biodegradowalność zależy od składu mieszaniny. Duża lepkość Ils może jednak mieć negatywny wpływ na ekstrakcję (Zhu i inni, 2025). Mieszaniny NADES posiadają podobne cechy do ILs, takie jak biodegradowalność, możliwość ponownego wykorzystania, a ich lepkość i toksyczność również zależy od składu mieszaniny. NADES stanowią metabolity pierwotne. Mieszaniny wykorzystywane do ekstrakcji składają się z co najmniej dwóch składników. Po połączeniu obniża się ich punkt topnienia w porównaniu do temperatury każdego składnika z osobna. Efektywność tej metody ekstrakcji może wynikać z interakcji wiązań wodorowych między NADES a biomasą, prowadząc do rozpuszczenia chityny w mieszaninie (Huang i in., 2018).

1.4. Chitozan

Słaba rozpuszczalność chityny ogranicza możliwości jej aplikacji, jednak można to zmienić poprzez proces deacetylacji. Przyjmuje się, że chityna o stopniu deacetylacji większym niż 50 % zaliczana jest do kategorii chitozanu. Chitozan nie jest rozpuszczalny w wodzie ani w roztworach zasadowych, jednak w uwodnionych kwasach już tak. Posiada wolne grupy amidowe o dodatnim ładunku, które umożliwiają mu łączenie się z innymi cząsteczkami lub powierzchniami. Przyjmuje się, że jego reaktywne grupy znajdują się przy cząsteczkach węgla C2 (grupa aminowa), C3 i C6 (grupy hydroksylowe) (Aranaz i inni 2021). Dzięki obecności w jego strukturze wiązań glikozydowych i β (1-4) jest łatwo rozkładany w organizmie przez proteazy i hydrolazy. Rozkładają one chitozan na łatwe do wydalenia, nietoksyczne oligo- i monosacharydy, (Sikora i in., 2021). Chitozan, dzięki swoim reaktywnym grupom wykazuje aktywność przeciwbakteryjną, przeciwgrzybiczą, antywirusową i hemostatyczną (Gheorghiță i in., 2023). Dotychczas zaproponowano trzy mechanizmy działania przeciwbakteryjnego chitozanu. Jeden z nich polega na interakcji chitozanu, dzięki dodatniemu ładunkowi z negatywnie naładowanymi składnikami ściany komórkowej bakterii, doprowadzając do jej uszkodzenia (Liu i in., 2004). Drugi polega na tworzeniu przez chitozan filmu na powierzchni bakterii zapobiegając w ten sposób przedostawaniu się składników odżywczych i tlenu. Ostatni natomiast dotyczy chitozanu o małej masie cząsteczkowej (M_w), który jest w stanie przejść przez membranę bakterii i zahamować syntezę mRNA(Liu i in., 2007). Wszystkie te cechy, razem ze zdolnością do łączenia się z innymi cząsteczkami, biodegradowalnością i niską toksycznością sprawia, że chitozan jest atrakcyjnym polimerem pod względem zastosowania w lekach i szczepionkach w roli nośnika substancji aktywnych, w inżynierii tkanek (Sikora i in., 2021), a także w dziedzinach innych niż medycyna: w rolnictwie, włókiennictwie, oczyszczaniu wody i ścieków, przemyśle spożywczym i w kosmetyce (Mucha i in., 2010).

Ze względu na wcześniej wspomniane różnice pomiędzy polimerami chityny w zależności od ich pochodzenia, każdy z nich wymaga badań wstępnych. Zazwyczaj obejmują one analizę właściwości fizykochemiczne badanego polimeru, stopień deacetylacji (DD), masę cząsteczkową (M_w), krystaliczność i stabilność termiczną. Dodatkowo, w przypadku planowanej aplikacji badanego chitozanu w medycynie lub przemyśle spożywczym wykonuje się analizy na aktywność przeciwbakteryjną i cytotoksyczną.

1.5. Skorupiaki występujące na terenie Polski jako źródło chityny

Według danych Generalnej Dyrekcji Ochrony Środowiska (GDOŚ), na terenie Polski występuje 587 gatunków skorupiaków (GDOŚ, 27.09.2025). Wśród nich znajdują się gatunki powszechnie występujące w wodach przybrzeżnych Bałtyku, takie jak lasonóg *Neomysis integer* (Leach, 1814), który w latach 80' był przedmiotem badań dotyczących jego wykorzystania w akwakulturach, jako karma dla zwierząt (Kuhlmann, 1984) oraz gatunki obecne na rynku spożywczym, jak rak błotny *P. leptodactylus* (Eschscholtz, 1828) (Harliog˘lu i Harliog˘lu, 2004). W 2018 r. przeprowadzono ekstrakcję chityny z pancerzy osobników tego gatunku jednak jej opis obejmował tylko pasma absorpcji w podczerwieni i struktury powierzchni uzyskanej chityny (Küçükgülmez, 2018). Dokładniejsze zbadanie tego polimeru i metod jego ekstrakcji mogłyby pomóc przy utylizacji odpadów powstałych w wyniku jego konsumpcji, a także wskazać jego potencjalne zastosowanie.

Wśród skorupiaków występujących wodach polskich znajdują się również gatunki klasyfikowane jako IAS, takie jak przenoszący dżumę raczą rak pręgowany *Faxonius limosus* (Rafinesque, 1817) i kiełż *D. villosus* (Sowinsky, 1894), znajdujący się na liście 100 najgroźniejszych IAS. W 2025 r. przeprowadzono badania na osobnikach *F. limosus*, wyłapanych w Serbii, z Dunaju, które wykazały, że ich mięso jest bezpieczne do spożycia, zwracając uwagę na to, że idąc zgodnie z polityką zero – waste, pancerze raków również mogłyby zostać zutylizowane i warto je zbadać pod kątem chityny i innych cennych związków (Vidosavljević i in.,2025). Osobniki *F. limosus*, tak jak osobniki *D .villosus* można wyłapywać ze środowiska za pomocą pułapek (Wood i in., 2021; Bruno i in., 2023).

W niniejszych badaniach po raz pierwszy wyizolowano i scharakteryzowano pod względem właściwości fizyko-chemicznych chitynę i chitozan z gatunków: *F. limosus* (publikacja 2), kiełża *D. villosus*, oraz lasonoga *N. integer* (publikacja 3) w celu określenia, czy mogą stanowić jej alternatywne źródło. Dodatkowo przeprowadzono te same analizy dla chityny i chitozanu z gatunku *P. leptodactylus* (**publikacja 2**), w celu uzupełnienia luki w wiedzy dotyczącej ich właściwości. Podjęto się również optymalizacji metody ekstrakcji z użyciem mieszaniny NADES, pod względem stosowania jej w przyszłości na biomasie z badanych gatunków, idąc zgodnie z założeniami rezolucji UE C/2024/2894. Dodatkowo określono aktywność przeciwbakteryjną oraz cytotoksyczność chitozanu uzyskanego z badanych gatunków, w celu lepszego zrozumienia badanych polimerów.

2. Hipotezy i cele badawcze

W oparciu o dotychczasowy stan wiedzy oraz dane literaturowe sformułowano następujące **hipotezy badawcze**:

i. Wybrane gatunki skorupiaków mogą stanowić alternatywne źródło chityny oraz chitozanu z nakierowaniem na zastosowanie medyczne

ii. Za pomocą metody „zielonej" z użyciem NADES można wyizolować chitynę z wybranych gatunków skorupiaków

Niniejsza praca powstała w celu wypełnienia luki badawczej dotyczącej potencjału wybranych skorupiaków wód polskich: *P. leptodactylus*, *F. limosus, D. villosus* i *N. integer* pod kątem wykorzystania ich jako źródła chityny i chitozanu. W związku z tym wyznaczono następujące cele badawcze:

- ekstrakcja chityny metodą konwencjonalną i porównanie jej właściwości fizyko-chemicznych z materiałem kontrolnym,
- optymalizacja metody z użyciem mieszaniny NADES do ekstrakcji chityny z wybranych gatunków skorupiaków,
- analiza materiału uzyskanego w wyniku ekstrakcji z użyciem mieszaniny NADES i porównanie jej właściwości fizyko-chemicznych z materiałem kontrolnym
- deacetylacja chityny uzyskanej poprzez konwencjonalną metodę ekstrakcji oraz analiza otrzymanego materiału wraz z porównaniem z materiałem kontrolnym.
- analiza materiału uzyskanego w wyniku deacetylacji pod kątem aktywności przeciwbakteryjnej i cytotoksycznej.

3. Metodyka i koncepcja badań

3.1. Przegląd literatury

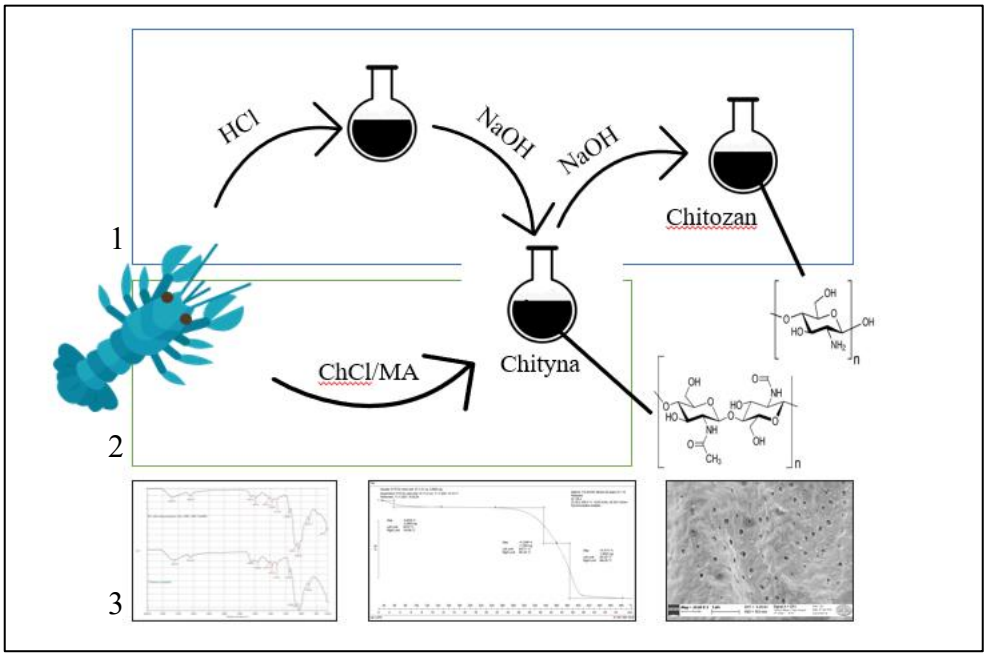
Przed rozpoczęciem badań, przeprowadzono przegląd prac dotyczących chityny, chitozanu, ich źródeł, metod ekstrakcji i ich wykorzystania, czego wynikiem była praca przeglądowa (**publikacja 1**) pod tytułem „From chitin to chitosan–a potential natural antimicrobial agent.", wydana w 2021 r.

3.2. Zbiór organizmów i przygotowanie materiału do badań

Osobniki raków **(publikacja 2)** gatunku *P. leptodactylus* otrzymano z akwakultury Gospodarstwa Rybackiego Bytów sp. z o.o., jesienią 2020 roku, natomiast osobniki raka *F. limosus* z Gospodarstwa Rybackiego Mielno sp. z o.o. (Jezioro Jamno) latem 2015 roku. Pancerze zostały odseparowane od tkanek miękkich według metody opisanej przez Rodriguez-Veiga i in. (2022). Osobniki *D. villosus* oraz *N. integer* **(publikacja 3)** zebrano w strefie brzegowej Zatoki Gdańskiej, przy Bulwarze Gdyńskim, za pomocą sitka, następnie zaklasyfikowano do odpowiedniego taksonu, wysuszono i zmielono w moździerzu. Tak przygotowany materiał został poddany ekstrakcji.

3.3. Ekstrakcja chityny

Ekstrakcję przeprowadzano na dwa sposoby **(publikacja 2 i 3)**. Część materiału (z każdego gatunku) wykorzystywano do ekstrakcji konwencjonalnej, natomiast pozostałość do ekstrakcji z użyciem mieszaniny NADES. Materiał uzyskany w wyniku ekstrakcji metodą konwencjonalną poddawano deacetylacji. Po każdej ekstrakcji i deacetylacji uzyskany materiał był ważony i poddawany analizom FTIR i TGA. Chitozan był dodatkowo analizowany skaningowym mikroskopem elektronowym (Rys. 1).



Rys. 1. Schemat pokazujący kolejne etapy postępowania z analizowanym materiałem: ekstrakcję i deacetylację metodą konwencjonalną (1), ekstrakcję chityny z użyciem mieszaniny NADES (2), oraz przykładowe wyniki uzyskane z analiz kolejno: FTIR, TGA i SEM (3).

3.3.1. Ekstrakcja konwencjonalna

W celu przeprowadzenia ekstrakcji konwencjonalnej materiał wpierw demineralizowano za pomocą stężonego kwasu solnego (HCl), a następnie poddawano deproteinizacji, za pomocą wodorotlenku sodu (NaOH). Po każdym etapie materiał był płukany i wirowany aż do uzyskania neutralnego pH, suszony, ważony i poddawany analizom FTIR i TGA, w celu sprawdzenia wydajności stosowanej metody ekstrakcji. Następnie uzyskany materiał poddawano deacetylacji, z użyciem NaOH i ponownie wirowano i płukano do uzyskania obojętnego pH. Przepłukany materiał suszono, ważono i poddawano analizom FTIR i TGA. Ze względu na niezadowalający wynik pierwszej deacetylacji (który został oceniony na podstawie wyników uzyskanych z analiz i na podstawie słabej rozpuszczalności chitozanu (Sannan i in., 1976)), postanowiono zoptymalizować metodę. Wykonano kilka podejść, zmieniając czas trwania procesu (1 – 6 h), temperaturę (90 – 180 °C) i stężenie NaOH (50 – 60%). Na podstawie analiz FTIR, TGA i po sprawdzeniu rozpuszczalności uzyskanych materiałów, uznano, że najlepsze wyniki uzyskiwano prowadząc deacetylację w świeżym roztworze 60% NaOH, w temperaturze 180 °C, przez 2 h.

3.3.2. Ekstrakcja chityny z użyciem mieszaniny naturalnych rozpuszczalników eutektycznych (NADES):

Ekstrakcja chityny z użyciem NADES, w odróżnieniu od ekstrakcji metodą konwencjonalną, wykonywana jest w jednym etapie, jednak przed jej rozpoczęciem należy przygotować mieszaninę. Składa się ona z co najmniej dwóch związków, z których jeden jest akceptorem a drugi dawcą wiązań wodorowych (HBA:HBD)(Jurić i in., 2021). W niniejszej pracy jako HBA wykorzystywano chlorek choliny (ChCl; $C_5H_{14}ClNO$), który jest najczęściej używany w badaniach dotyczących ekstrakcji z mieszaninami NADES, oraz jest klasyfikowany przez FDA jako „związek na ogół bezpieczny" (GRAS). Jako HBD wybrano kwas malonowy (MA; $C_3H_4O_4$), zgodnie z badaniami z 2017r. (Zhu i in.), które wykazały, że mieszanina ChCl : MA daje najlepszą wydajność ekstrakcji chityny z pancerzy homara spośród testowanych mieszanin. Ekstrakcję NADES przeprowadzono w kilku wariantach **(publikacja 2 i 3)**:

a) 1.5 g próbki mieszano ze świeżo przygotowaną mieszaniną NADES przez 2 h w łaźni wodnej, temperaturze 80 °C. Następnie filtrowano przez papierowy sączek próbkę z użyciem pompy próżniowej. Uzyskany filtrat wirowano przez

5 min. w wirówce (Eppendorf 5408R) przy 4000 obr./min., następnie płukano wodą destylowaną (25 mL) i wirowano ponownie. Cykl powtarzano 7 razy, do uzyskania obojętnego pH. Wysuszony osad zawieszano w 20 mL 10% H_2O_2 i mieszano za pomocą mieszadła mechanicznego w temperaturze 80 °C. Po 2 h próbki ponownie siedem razy wirowano (jak powyżej) i płukano 25 mL wody destylowanej, po czym uzyskany materiał suszono w temperaturze 80 °C przez 48 h.

b) 1.5 g próbki mieszano ze świeżo przygotowaną mieszaniną NADES przez 2 h w łaźni wodnej, temperaturze 100 °C. Następnie za pomocą pompy próżniowej filtrowano przez papierowy sączek próbkę. Uzyskany filtrat wirowano przez 5 min. W wirówce przy 4000 obr./min., następnie płukano wodą destylowaną (25 mL) i wirowano ponownie. Cykl płukania powtarzano 7 razy, do uzyskania obojętnego pH, , po czym uzyskany materiał suszono w temperaturze 80 °C przez 48 h. Wysuszony osad płukano wrzącą wodą, a następnie ponownie suszono w piecu w temperaturze 80 °C przez 48 h.

c) 1.5 g próbki mieszano z 10 mL 10% kwasu cytrynowego ($C_6H_8O_7$), w temperaturze pokojowej, przez 1 h. Następnie próbkę mieszano ze świeżo przygotowaną mieszaniną NADES przez 2 h, w łaźni wodnej, w temperaturze 100 °C. Następnie za pomocą pompy próżniowej filtrowano przez papierowy sączek próbkę. Uzyskany filtrat wirowano przez 5 min. W wirówce przy 4000 obr./min., następnie płukano wodą destylowaną (25 mL) i wirowano ponownie. Cykl płukania powtarzano 7 razy, do uzyskania obojętnego pH, po czym uzyskany materiał suszono w temperaturze 80 °C przez 48 h.

3.4. Analiza spektroskopii podczerwieni z transformacją Fouriera (FTIR)

W celu upewnienia się, że ekstrakcja oraz deacetylacja materiału przebiegła prawidłowo, był on poddawany analizie FTIR, za pomocą spektrofotometru Spectrum One FTIR (Perkin Elmer, Wellesley, MA USA), wyposażonego w MIRacleTM ATR (Pike Technologies, Madison, Wisconsin, USA) w zakresie 4000 – 650 cm^{-1} w rozdzielczości 4 cm^{-1} (64 skany). Pomiary spektrum pochłaniania podczerwieni były wykonywane w trzech powtórzeniach.

3.5. Analiza termograwimetryczna (TGA)

W celu zbadania stabilności termicznej uzyskanych próbek, oraz porównania ich z materiałem kontrolnym, przeprowadzano analizę termograwimetryczną (TGA). W celu przeprowadzenia analizy, na jedno powtórzenie używano 5 mg próbki, która była umieszczana w aluminiowym naczynku, a następnie była poddawana skanowaniu w częstotliwości 10 K min^{-1}, w temperaturze od 30 °C do 500 °C, w atmosferze azotowej (50 mL min^{-1}). Po kalibracji analizatora termograwimetrycznego, każdą analizę wykonywano w trzech powtórzeniach.

3.6. Analiza mikroskopii elektronowej skaningowej (SEM)

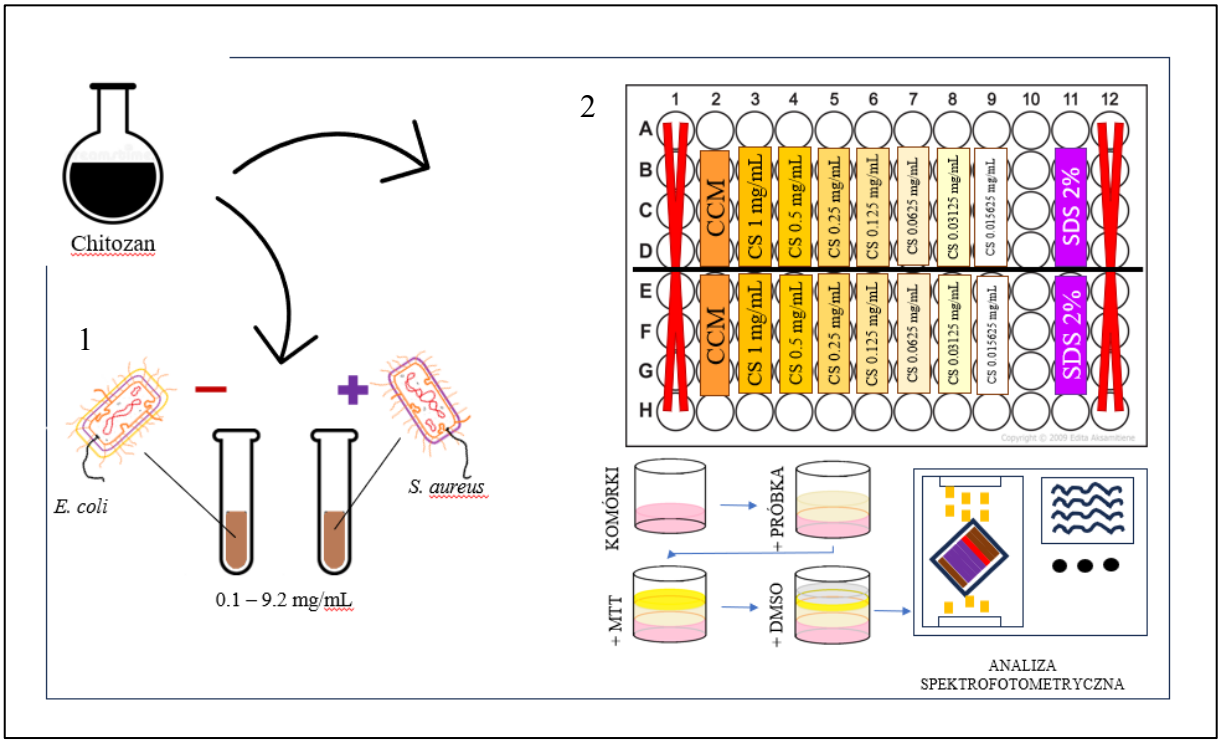
W celu określenia morfologii powierzchni otrzymanych próbek, poddawano je analizie przy pomocy skaningowego mikroskopu elektronowego (EVO MA10 instrument, Carl Zeiss, Oberkochen, Niemcy). Próbki były pokrywane złotem, a następnie wykonywano pomiary w bardzo wysokiej próżni o napięciu elektronowym 5 kV i odległości roboczej 8.5 mm.

3.7. Określenie masy cząsteczkowej chitozanu

Rozmieszczenie masy cząsteczkowej uzyskanych polimerów było określane za pomocą chromatografii HPSEC (High-Performance Size Exclusion Chromatography) przeprowadzanej za pomocą systemu składającego się z następujących modułów: odgazowywacza (Biotech Degasi GPC), pompy (Waters 515 HPLC) i detektora współczynnika refrakcji (Knauer K-2300). Dwie kolumny Shodex OHpak SB-806M HQ of 300 × 8.0 mm były używane wraz z kolumną 50 × 6.0 mm OHpak SB-G 6B (Showa Denko K.K., Tokio, Japonia). Fazę płynną stanowił roztwór 0.2 M NaNO3+0.5 M CH_3COOH (0.02% NaN_3), przepływający przez kolumny z prędkością1 mL/min. Próbki chitozanu rozpuszczano w eluencie w proporcji 5 mg/mL i zostawiano na noc, a następnie wirowano przez 10 min. Przy prędkości 4000 obr./min. Filtrowano przez filtry o średnicy 0.45 μm. Krzywa kalibracyjną przygotowywano na podstawie standardu polistyrenosulfonianu rozpuszczanego w eluencie w proporcji 5 mg/mL.

3.8. Określenie aktywności biologicznej chitozanu

W ramach badania aktywności biologicznej chitozanu analizowano jego aktywność przeciwbakteryjną oraz aktywność cytotoksyczną (Rys. 2).



Rys. 2. Badanie aktywności przeciwbakteryjnej (1) i cytotoksycznej (2) uzyskanego chitozanu.

3.8.1. Określenie aktywności przeciwbakteryjnej chitozanu

3.8.1.1. Kultury bakterii

Testy zostały przeprowadzone na hodowli bakterii *E. coli* szczepu (ATCC 10536), która służyła jako model bakterii Gram ujemnych i *S. aureus* (ATCC 6538), które służyły jako model bakterii Gram dodatnich. Hodowle bakterii były hodowane na pożywce TSB w 37 °C. W celu przygotowania bakterii do analiz i odseparowania ich od pożywki, były wirowane przez 20 min. przy prędkości 3000 obr./min., a następnie umieszczane w PBS (pH 7.3). Uzyskana zawiesina była rozcieńczana tak, aby ilość komórek wynosiła 1 x 10^7 – 1 x 10^8 CFU/mL.

3.8.1.2. Przygotowanie próbek do testów na aktywność przeciwbakteryjną

Ze względu na to, że chitozan nie jest rozpuszczalny w dimetylosulfonotlenku (DMSO), materiał otrzymany po deacetylacji był rozpuszczany w świeżo przygotowanym wodnym roztworze 3.6% HCl, a następnie w wodzie destylowanej w proporcji 1 g/ 6.2 mL/100mL, w celu tworzenia soli. Tworzenie soli powoduje protonowanie grup aminowych chitozanu, zwiększając jego rozpuszczalność. Następnie materiał był poddawany 24 h dializy, w temperaturze pokojowej, przy stałym mieszaniu (za pomocą

mieszadełka magnetycznego). Woda była wymieniana co 2 h. Następnie próbki były mrożone, i liofilizowane. Liofilizacja pomaga zwiększyć rozpuszczalność soli chitozanowej, ze względu na tworzenie bardziej porowatej struktury, w porównaniu do prostego suszenia.

3.8.1.3. Testy na aktywność przeciwbakteryjną chitozanu

Minimalne stężenie hamujące (MIC) i minimalne stężenie bakteriobójcze (MBC) zostały określone metodą dwukrotnego seryjnego rozcieńczenia w pożywce Iso-Sensitest, zgodnie z procedurą Clinical and Laboratory Standards Institute (CLSI) (NCCLS, 1999). Przed rozpoczęciem testów, zliczano kolonie bakterii inokulum początkowego. Wynosiło ono 1.0 x 10 CFU/mL. Próbki soli chitozanowej rozpuszczano w wodnym roztworze 10 % DMSO, w stężeniach 0.1 mg/mL – 9.2 mg/mL. Po 24 h inkubacji w 37 °C ponownie zliczano kolonie bakterii. Za MIC przyjmowano najniższe stężenie roztworu, dla którego obserwowano zahamowanie wzrostu kolonii bakterii, natomiast za MBC przyjmowano najniższe stężenie roztworu dla którego obserwowano redukcję wstępnego inokulum o > 99.9%. Wszystkie testy były wykonywane w trzech powtórzeniach (NCCLS, 2003). Pozytywną kontrolę stanowił wzorcowy roztwór ampicyliny.

3.8.2. Określenie cytotoksyczności chitozanu

3.8.2.1. Kultura komórek A549

Do testów wykorzystywano komórki A549 uzyskane z American Type Culture Collection (ATCC, Manassas, VA, USA) inkubowane w kolbach o powierzchni 75 cm^2 w temperaturze 37 °C w warunkach: 5% CO_2, 95% wilgotności powietrza atmosferycznego. Pożywka CCM była wymieniana dwa razy w tygodniu.

3.8.2.2. Testy MTT

Test był wykonywany zgodnie z procedurą International Organization for Standarization (IOS) w trzech powtórzeniach dla każdej próbki chitozanu i czasu ekspozycji. Próbki chitozanu były rozpuszczane w 1% kwasie octowym (CH_3COOH), w stężeniach od 15.62 µg/mL do 1000 µg/mL. 24 h przed przeprowadzeniem testów, do 96 – studzienkowych płytek przekładano komórki tak, aby w jednej studzience znajdowało się 1 x 104 komórek. Po 24 h pozbywano się CCM, którą zastępowano roztworami z chitozanem. Jako pozytywną i negatywną kontrolę stosowano odpowiednio roztwory CCM i SDS w tężeniu 2 %.Po 3 h, 24 h i 48 h ekspozycji próbki były zastępowane 30 µl MTT w stężeniu 5 ng/mL (w PBS pH 7.4), a następnie inkubowane przez kolejne 2 h. Następnie dodawano po 50 µL DMSO, w celu rozpuszczenia kryształów formazanu. Mierzono absorbancję

próbek za pomocą spektrofotometru (Infinite M200; Tecan; Grodig, Austria) przy długości fali λ = 540 nm, z korekcją tła przy długości fali λ = 640 nm. Żywotność komórek wyliczono z następującego wzoru:

Żywotność komórek wyliczono z następującego wzoru:

$$\text{Żywotność komórek} = (A-D)/(CM-D) \times 100$$

Gdzie:

A – absorbancja dla próbek

D- absorbancja DMSO

CM- absorbancja kontroli pozytywnej

Stężenie chitozanu powodujące 50% inhibicji aktywności komórek (IC_{50}) zostało obliczone dla stężeń chitozanu, które spowodowały spadek żywotności komórek poniżej 50%. Zostało określone metodą dopasowania sigmoidalnego danych, w programie statystycznym GraphPad Prism *GraphPad Software, wersja 6.01, Boston, MA, USA). Zarówno dla próbek chitozanu z raków (**publikacja 2**) jak i dla próbek chitozanu z *D. villosus* i *N. integer* (**publikacja 3**) zaobserwowano brak cytotoksyczności dla stężeń poniżej 250 µg/ mL. Zgodnie z oczekiwaniami, rosła ona wraz z czasem ekspozycji.

3.8.2.3. Analiza statystyczna

Wyniki analizowano za pomocą dwuczynnikowej analizy wariancji (ANOVA). Korzystano z programu R (pakiety: emmeans 1.8.5, car 3.1-2, best Normalize 1.9.0 i DHARMa 0.4.6), różnicę uznawano za istotną na poziomie $p < 0.05$.

4. Omówienie uzyskanych wyników

Chityna uzyskana z *P. leptodactylus* i *F. limosus* **(Publikacja 2)** metodą konwencjonalną stanowiła około 20 % suchej masy pancerzy, natomiast z *D. villosus* i *N. integer* **(Publikacja 3)** stanowiła kolejno 8 % i 7 %. Wyniki uzyskane z analiz FTIR Widma dla próbek chityny i chitozanu otrzymanych metodą konwencjonalną **(Publikacja 2, Figure 1.: b, c, Figure 2.: b, c ; Publikacja 3., Figure 1.:a, b.:, Figure 2.: a, b.:)**, pokazały charakterystyczne dla nich pasma amidowe I i II oraz pasma odniesienia -CH- i -C-O-C (Mucha, 2010), wskazując na prawidłowy przebieg ekstrakcji i deacetylacji. Widma dla

próbek chityny uzyskanej w wyniku ekstrakcji z użyciem mieszaniny NADES z gatunków *F. limosus*, *N. integer* i *D. villosus* **(Publikacja 2, Figure 1.: e; Publikacja 3, Figure 1.: c, d.:)** pokazały dodatkowe pasmo w okolicach 1414 cm^{-1} co może wskazywać pozostałości białek (Zhu i in., 2017). Dla gatunku *P. leptodactylus* **(Publikacja 2, Figure 1.: d)** udało się przeprowadzić kompletną ekstrakcję z mieszaniną NADES po wprowadzeniu dodatkowego etapu, przed ekstrakcją. W niniejszych badaniach korzystano z metody opisanej w 2017 r. (Zhu i in.), w których testowano różne systemy NADES do ekstrakcji chityny z pancerzy homara. Możliwe, że ekstrakcja dla *F. limosus*, *N. integer* i *D. villosus* nie powiodła się, ze względu na inny skład lub proporcje białek do soli mineralnych i do chityny w kompleksach budujących ich pancerze (Kaya i in., 2014). Efektywność mieszanin NADES zależy od ich odczynu, proporcji między HBD, HBA a materiałem, lepkości oraz temperatury, w jakiej przeprowadza się ekstrakcję (która również wpływa na lepkość mieszaniny) (Hong i in., 2018; Huang i in. 2018; Zhou i in., 2019). W niniejszych badaniach przeprowadzono ekstrakcję z mieszaniną NADES w kilku wariantach. Najpierw podwyższono temperaturę utrzymywaną w trakcie ekstrakcji, jednak zmianę w widmie FTIR zaobserwowano tylko dla chityny uzyskanej z pancerzy gatunku *P. leptodactylus.* Następnie dodano etap przed ekstrakcją, w którym materiał mieszano z kwasem cytrynowym, aby osłabić wiązania między chityną a białkami (Zhu i in., 2017), jednak dalej nie przyniosło to widocznych zmian w widmach uzyskanych dla badanych gatunków.

W celu potwierdzenia efektywności ekstrakcji i deacetylacji metodą konwencjonalną materiału z *P. leptodactylus*, *F. limosus* **(Publikacja 2, Figure 3.: b, c, Figure 4.: b, c)**, *D. villosus* i *N. integer* **(Publikacja 3., Figure 3.: c, d; Figure 4.: a, b)** przeprowadzono analizę TGA. Dla każdej próbki zaobserwowano dwa główne etapy degradacji, objawiające się spadkiem masy próbki. Pierwszy spadek związany jest z parowaniem wody zawartej w materiale. Wśród próbek otrzymanych za pomocą ekstrakcji konwencjonalnej, najmniej wody zawierała próbka otrzymana z *N. integer*, natomiast najwięcej chityna z *P. leptodactylus*, co może wskazywać na jej lepsze właściwości higroskopijne. Wszystkie otrzymane w niniejszych badaniach próbki chityny miały niższą zawartość wody niż materiał referencyjny. Dla chitozanu również zaobserwowano niższe wartości zawartości wody w próbkach niż dla materiału referencyjnego. Kolejny spadek masy próbki związany jest z jej degradacją i zazwyczaj zaczyna się powyżej 200 °C. Najpierw degradacji ulega 2-amino-2-deoksy-d-

glokopyranoza (GlcN), a następnie 2-acetamido-2-deoksy-d-glokopyranoza (GlcNAc) (Nam i in., 2010). Wszystkie próbki uzyskanej chityny miały wyższą temperaturę degradacji niż materiał referencyjny. Analiza TGA może wskazać, które próbki chitozanu mają wyższą, a które niższą wartość stopnia deacetylacji (DD), jednak nie są one jednoznaczne. Niższa temperatura rozkładu próbki może z jednej strony wskazywać na jej wyższą wartość DD, a z drugiej jej zanieczyszczenie (Nam i in., 2010). Zgodnie z tym założeniem, analiza potwierdza skuteczność deacetylacji dla próbek chitozanu otrzymanych z *P. leptodactylus*, *F. limosus* i *D. villosus.* Dla próbki uzyskanej dla *N. integer*, temperatura degradacji materiału przed deacetylacją była niższa niż po, co może wskazywać na jej zanieczyszczenie. Tylko dla *P. leptodactylus* temperatura rozpoczęcia degradacji chityny była niższa dla próbki otrzymanej metodą konwencjonalną niż z mieszaniną NADES. W przypadku *P. leptodactylus* różnica ta może wskazywać na większą czystość chityny uzyskanej za pomocą mieszaniny NADES, lub na częściową deacetylację chityny uzyskanej metodą konwencjonalną. W przypadku chityny z pozostałych gatunków różnica najprawdopodobniej jest spowodowana obecnością białek, widocznych na widmach uzyskanych za pomocą FTIR.

Mikrostruktura powierzchni chityny i chitozanu może być różna w zależności od źródła, z którego pochodzi. W 2014 r. (Kaya i in.) został zaproponowany podział na 3 kategorie: strukturę mikrowłóknistą i porowatę, strukturę tylko porowatą i strukturę tylko włóknistą. Dzięki analizie SEM zaobserwowano, że chitozan uzyskany z pancerzy raków **(Publikacja 2: Figure 6.)** ma strukturę charakteryzującą się dużymi porami i włóknistą strukturą. Na powierzchni próbek chitozanu z *D. villosus* i *N. integer* **(Publikacja 3: Figure 5.)** również zaobserwowano pory, jednak mniejsze i bardziej regularne i nie zaobserwowano struktur włóknistych. Mikrostruktura powierzchni chitozanu może być jedną z cech decydujących o potencjalnym zastosowaniu badanego polimeru (Kaya i in., 2015). W inżynierii tkankowej chitozan jest uznawany za atrakcyjny biopolimer, między innymi ze względu na mikrowłókna, z których można tworzyć odpowiednie struktury. Porowatość materiału również ma zalety, ze względu na umożliwianie namnażanie komórek tkanek, a także transport substancji odżywczych (Sikora i in., 2021)

Jedną z cech decydujących o aktywności biologicznej chitozanu jest masa cząsteczkowa (M_w). Wartości M_w dla chitozanu uzyskanego z *P. leptodactulus* i *F. limosus* **(Publikacja 2)** wyniosły kolejno 236 kDa i 229 kDa, natomiast dla *D. villosus* i *N. integer* **(Publikacja 3)** wyniosły kolejno 347 kDa i 361 kDa. Zespół Hsu i in., (2004)

zaklasyfikował w swoich badaniach chitozan o Mw 181 kDa i 204 kDa jako chitozan o średniej M_w, natomiast powyżej 300 kDa jako chitozan o dużej Mw. Biorąc pod uwagę podane zakresy, chitozan uzyskany z *P. leptodactulus* i *F. limosus* można przypisać do kategorii chitozanu o średniej M_w, natomiast z *D. villosus* i *N. integer* do kategorii chitozanu o dużej M_w. Badania przeprowadzone w 2014 r. (Hajji i in.) wykazały, że wraz ze wzrostem M_w chitozanu wzrasta jego aktywność przeciwbakteryjna, w stosunku do bakterii Gram dodatnich, natomiast w stosunku bakterii Gram ujemnych spada. Zazwyczaj w badaniach na aktywność przeciwbakteryjną chitozanu testy przeprowadza się na bakteriach *E. coli* (bakterii Gram ujemnej) i *S. aureus* (bakterii Gram dodatniej), które uznane są jako bakterie antybiotykooporne (Fleckenstein i in., 2019; Ford i in., 2021). Zgodnie z oczekiwaniami, aktywność przeciwbakteryjna badanych próbek chitozanu była niższa niż ampicyliny. Chitozan uzyskany z *F. limosus* **(Publikacja 2: Table 4.)** miał najwyższe wartości MIC i MIB spośród badanych próbek, dla obu szczepów bakterii. Jego wartości MIC i MIB były również wyższe dla *S. aureus*, niż dla *E. coli*, w przeciwieństwie do chitozanu uzyskanego z *P. leptodactylus* **(Publikacja 2: Table 4.)** i z *D. villosus* **(Publikacja 3)**. Większość uzyskanych wartości MIC i MBC dla badanych próbek było niższych niż wartości dla materiału referencyjnego, z wyjątkiem wartości MIC dla *E. coli* próbek *P. leptodactylus* i *F. limosus*. Najniższymi wartościami MIC i MBC, czyli najwyższą aktywnością przeciwbakteryjną charakteryzował się chitozan uzyskany z *D. villosus*.

W celu określenia cytotoksyczności materiału uzyskanego w wyniku deacetylacji, prowadzono testy MTT - jeden z najpowszechniej stosowanych testów do określenia wpływu wybranych czynników na żywotność komórek. Jest to analiza kolorymetryczna, która wykazuje liniową zależność między żywotnością komórek, a wytwarzanym kolorem (Van de Loosdrecht i in., 1994). Żywe komórki, ze sprawnie działającymi mitochondriami, wykazują szybkie tempo redukcji soli tetrazolowej (MTT, kolor żółty), za pomocą dehydrogenazy mitochondrialnej, do kryształów formazanu (kolor fioletowy). Im większa intensywność koloru fioletowego w próbkach, tym większa aktywność mitochondrialna komórek (Bahuguna i in., 2017).Testy MTT, wpływu badanych próbek chitozanu na komórki nabłonkowe A549, wykazały, zgodnie z oczekiwaniem, spadek żywotności komórek wraz z zwiększeniem czasu inkubacji. Założono, że spadek żywotności komórek poniżej wartości 70 % oznacza aktywność cytotoksyczną badanej próbki. Chitozan uzyskany z *F. limosus* **(Publikacja 2: Figure 8.: b)** wykazał się

najniższą cytotoksycznością, a wartości żywotności komórek spadły poniżej 70 % przy stężeniach powyżej 250 µg/mL. Próbki chitozanu z *D. villosus* i *N. integer* **(Publikacja 3: Figure 7.: a, b)** również wykazały działanie cytotoksyczne dopiero powyżej stężenia 250 µg/mL. Dla chitozanu z *P. leptodactylus* **(Publikacja 2: Figure 8.: a)** wartości żywotności komórek A549 przy 48 h ekspozycji spadły poniżej 70 % dla wszystkich testowanych stężeń, jednak przy czasie inkubacji 24 h, tak jak w pozostałych przypadkach, działanie cytotoksyczne wystąpiło dla stężeń powyżej 250 ug/mL. Jednocześnie, najniższą wartość IC_{50} dla 24 h i 48 h inkubacji odnotowano dla chitozanu uzyskanego z *N. integer,* podczas gdy dla chitozanu uzyskanego z *P. leptodactylus* wartości IC_{50} były najwyższe.

Weryfikacja hipotez i wnioski

Wyniki uzyskane w ramach niniejszej pracy umożliwiły weryfikację postawionych hipotez:

i. Wybrane gatunki skorupiaków mogą stanowić alternatywne źródło chityny oraz chitozanu z nakierowaniem na zastosowanie medyczne

Zawartość chityny w pancerzach gatunków *P. leptodactylus* i *F. limosus* **(Publikacja 2)** wynosiła około 20 %. Wartość ta jest zbliżona do wartościu uzyskanych dla innych gatunków raków, które rozpatruje się pozytywnie jako źródło chityny (Meyers i in., 1990; Abdou, 2008; Kaya i in., 2015). Dla *N. integer* i *D. villosus* **(Publikacja 3)** wartości te były znacznie niższe, jednak przygotowanie materiału do ekstrakcji było szybsze i łatwiejsze.

Wyniki dla chitozanu uzyskanego z *P. leptodactylus* i *F. limosus* pod względem aktywności przeciwbakteryjnej i mikrostruktury powierzchni wskazują na możliwość wykorzystania tych polimerów w medycynie. Oba polimery wykazały aktywność przeciwbakteryjną w niższych stężeniach niż chitozan będący materiałem kontrolnym, jednak wyższych niż ampicylina. Natomiast ich mikrostruktura powierzchni charakteryzowała się porowatością i włóknistością. Włóknista struktura może być przydatna przy tworzeniu opatrunków (Mucha, 2010; Ishihara i in., 2015). Jest również cechą pożądaną w inżynierii tkankowej, ze względu na możliwość tworzenia odpowiednich struktur (Sikora i in., 2021). Porowatość również jest pozytywnie rozpatrywana w inżynierii tkankowej ze względu na możliwość przepływu substancji odżywczych do komórek oraz wspomaganie ich wzrostu w tworzonych strukturach

(Rodríguez-Vázquez i in., 2015). Mikrostruktura powierzchni chitozanu uzyskanego z *D. villosus* i *N. integer* **(Publikacja 3)** również charakteryzowała się porowatością, jednak pory były znacznie mniejsze. Jeżeli dany polimer chitozanu ma mieć zastosowanie medyczne, musi zostać poddany testom na cytotoksyczność, biorąc pod uwagę odpowiedni typ komórek, w zależności od planowane aplikacji. W niniejszej pracy zbadano cytotoksycznośc chitozanu względem komórek nabłonkowych A549, stosowanych przy badań substancji o potencjalnym zastosowaniu w lekach wziewnych, ze względu na ich podobieństwo do komórek pęcherzykowych płucnych typu II. Testy MTT przeprowadzone w niniejszych badaniach wykazały, że komórki nabłonkowe nie powinny być wystawione na działanie chitozanu uzyskanego z *P. leptodactylus* na okres czasu dłuższy niż 24 h. Dodatkowo, stosowane stężenie wszystkich zbadanych w niniejszych badaniach próbek chitozanu nie powinno przekraczać 250 µl/ mL.

Na podstawie uzyskanych wyników **Hipoteza 1** została zweryfikowana pozytywnie. *P. leptodactylus*, *F. limosus*, *D. villosus N. integer*, i mają potencjał jako alternatywne źródło chityny i chitozanu.

ii. Za pomocą metody „zielonej" z użyciem NADES można wyizolować chitynę z wybranych gatunków skorupiaków

Zastosowana w niniejszych badaniach mieszanina NADES okazała się być efektywna tylko dla ekstrakcji chityny z pancerzy *P. leptodactylus* **(Publikacja 2)**. Dla materiału uzyskanego z *F. limosus* **(Publikacja 2)**, *D. villosus* i *N. integer* **(Publikacja 3)** widma FTIR i wyniki uzyskane z TGA wskazały na obecność białek w próbkach. W związku z tym uznano, że **Hipoteza 2** została zweryfikowana częściowo pozytywnie.

Na podstawie niniejszych badań wyciągnięto następujące wnioski:

- Zastosowana w niniejszych badaniach konwencjonalna metoda eksytrakcji pozwoliła na otrzymanie chityny z wybranych gatunków skorupiaków.

- Mikrostruktura powierzchni chitozanu uzyskanego z *P. leptodactylus* i *F. limosus* była włóknista i porowata.

- Mikrostruktura powierzchni chitozanu uzyskanego z D. villosus i N. integer była gładka z drobnymi porami.

- Chitozan uzyskany z pancerzy *F. limosus* wykazał wyższą aktywność przeciwbakteryjną wobec szczepu modelowego bakterii Gram dodatnich niż wobec szczepu modelowego bakterii Gram ujemnych

- Chitozan uzyskany z gatunków *P. leptodactylus* i *D. villosus* wykazały wyższą aktywność przeciwbakteryjną wobec szczepu modelowego bakterii Gram ujemnych niż Gram dodatnich

- Przy 24 h inkubacji chitozan pozyskany z gatunków *P. leptodactylus*, *F. limosus, D. villosus* i *N. integer* wykazał aktywność cytotoksyczną przy stężeniach powyżej 250 μg/mL

- Zastosowana w niniejszych badaniach mieszanina NADES okazała się być efektywna tylko dla ekstrakcji chityny z pancerzy *P. leptodactylus*. W pozostałych przypadkach nie udało się całkowicie odseparować chityny od białek.

Dalszy rozwój badań

Niniejsze badania wzbogacają wiedzę w zakresie źródeł alternatywnych chityny i chitozanu. Mogą być przydatne przy utylizacji biomasy powstałej w wyniku konsumpcji raków gatunku *P. leptodactylus*, oraz przy utylizacji biomasy powstałej w wyniku walki z IAS. Dodatkowo przyczyniają się do lepszego zrozumienia ekstrakcji z użyciem mieszanin NADES.

Warto dalej studiować temat wykorzystania mieszanin NADES, lub innych bardzie przyjaznych środowisku metod. W przypadku gatunków będących przedmiotem niniejszej pracy, warto sprawdzić czy inne proporcje wykorzystanych rozpuszczalników NADES, lub inne systemy NADES nie dałyby lepszego rezultatu ekstrakcji.

Dla dalszego zrozumienia aktywności biologicznej otrzymanych związków warto określić dla nich stopień deacetylacji i krystaliczności, a także kontynuować nadania dotyczące kompatybilności danych polimerów.

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From chitin to chitosan–a potential natural antimicrobial agent.

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FROM CHITIN TO CHITOSAN – A POTENTIAL NATURAL ANTIMICROBIAL AGENT

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Abstract

Chitin is a naturally occurring polymer. Together with its derivatives such as chitosan, it has a wide spectrum of application possibilities, and many properties not yet exploited. Chitosan possesses many features desirable in an ideal antimicrobial polymer. It shows activity against multidrug-resistant bacterial and fungal strains that pose a challenge to modern medicine. Chitosan also shows activity against certain viruses, such as SARS-CoV-2. It might be used as a drug or a vaccine delivery system, is biodegradable, bioavailable and considered safe for medical use.

It is important to continue exploring the potential of chitosan, as well as to investigate its sources. Indeed, many sources of this polymer are still not or have been poorly described. In this paper, we compile the current state of knowledge on the antimicrobial properties of chitosan, list alternative sources of chitin to highlight the potential of these two polymers and encourage further research.

***Keywords**: chitin extraction, chitosan, antimicrobial activity, sources of chitin*

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1. Introduction

Chitin (β-1-4-linked *N*-acetylglucosamine) is the second most abundant biopolymer on the planet. It has existed since the first fungi evolved, which was about 810-715 million years ago [1]. Its first extractions were performed independently by Hachett, Braconnot and Odier, at the turn of the nineteenth century [2], and it was first deacetylated in 1859 by Rouget. Since then, chitin and its derivatives have been of great interest to scientists. They are desirable biomaterials due to their properties like biocompatibility, biodegradability, low toxicity and high reactivity [3, 4]. Chitosan, one of the chitin derivatives, is already used in beverage production for microbial control, sulphite reduction and heavy metal trapping; as a diet supplement for weight loss; in cosmetics; and in wound dressings. However, there are many more possible applications that have not been implemented yet [5]. Since the beginning of the twenty-first century, the global population has experiences pandemics caused by SARS (2003), H5N1 (2003), H1N1 (2009), Cholera in Haiti (2010), MERS-CoV (2012) and Ebola in West Africa (2014). Currently, the world is struggling not only with a novel coronavirus, SARS-CoV-2 [6], but also with drug-resistant bacterial and fungal strains that can cause severe and dangerous infections [7, 8]. The development of new diseases might induce an increase in the number of medicaments in the environment. Chitosan constitutes a promising source for pharmaceuticals based on natural products. It is considered the most promising, new generation antimicrobial polymer [9]. Its antifungal, antibacterial and antiviral activities have attracted the attention of many scientists [10-13]. It shows inhibition against some multidrug-resistant fungi and bacteria, and might be useful to prevent biofilm development [14, 15]. It might also help in developing solutions for some currently challenging pathogenic viruses like SARS-CoV-2, MERS-CoV and HIV-1, for which there are limited or no effective medicines [16, 17].

In 1999, Muzzarelli [18] estimated chitin production in the biosphere to be 10^{10} gigatons per year; however, we are still learning about new, alternative sources of this polymer that, in some cases, have not been taken into account [19, 20]. Its source determines its properties and is an important criterion in choosing the chitin extraction method [21-25]. Although seafood waste and fungi constitute the main sources of chitin, and thus chitosan, there are many alternative sources of this polymer [26].

This review summarises the current state of knowledge of the chitosan manufacturing process, the characteristics of this polymer and its potential as an antimicrobial agent. It also highlights alternative sources of chitin, from which chitosan is derived. The aim of this review is to highlight the potential of chitosan as a natural polymer with antimicrobial activity, and to encourage continued research on this polymer, its preparation and application.

2. Chitin

Chitin is a high-molecular-weight polysaccharide that forms light, rigid and hardly soluble structures [27]. It contains reactive amide groups that define its cationic nature, high adsorption capacities and biological activity [28, 29]. It is made of chains that range from 1000 to 3000 units [30], arranged into sheets linked by N-H···O=C hydrogen bonds between amide and carbonyl groups. It has three polymorphic crystalline structures: α, β and γ.

α-chitin is characterised by antiparallel chains with strong inter-sheet and intra-sheet hydrogen bonding, alongside highest molecular weight and decomposition temperature compared with the other allomorphs. It is considered the most stable chitin form. On the contrary, β-chitin has parallel chains with only intra-sheet bonding and weak intermolecular forces. It is more flexible and reactive and has greater affinity for solvents than α-chitin.

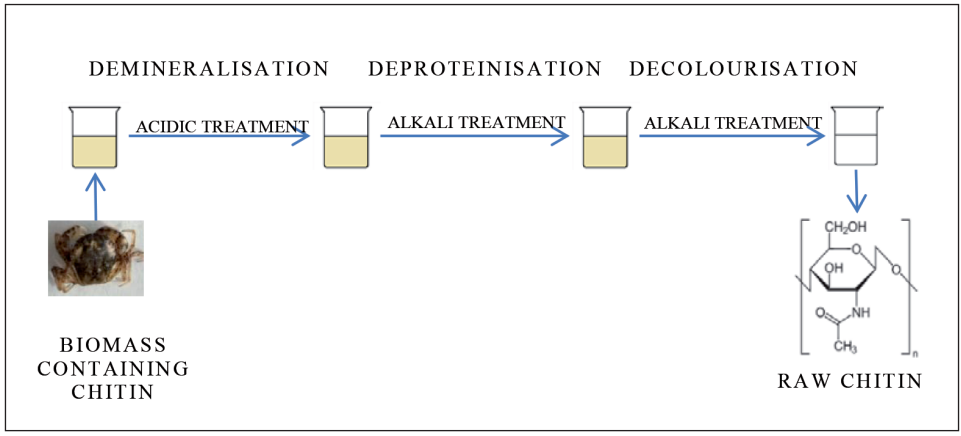

Figure 1. The process of chitin extraction

γ-chitin is the rarest form, characterised by one antiparallel chain for every three chains [31]. Chitin is mainly obtained from crustaceans [28, 21]. It usually occurs combined with proteins, minerals, lipids and pigments [32]. The process of chitin extraction usually consists of three steps: demineralisation, deproteinisation and, optionally, decolourisation (Figure 1).

Methods used to obtain chitin can be divided into chemical and biological [26]. In chemical extraction, to remove mineral salts like calcium carbonate or calcium phosphate, the sample is treated with a strong acid: hydrochloric acid (HCl), nitric acid (HNO_3), sulphuric acid (H_2SO_3), acetic acid (CH_3COOH) or formic acid (HCOOH). The next step, protein separation, is performed with a strong alkaline treatment, usually sodium hydroxide (NaOH) [2]. The most common chemical method of chitin extraction, used for industrial production, is performed with HCl, NaOH and a hydrogen peroxide (H_2O_2) treatment for purification [32, 33]. According to studies performed by Hoqani and colleagues [34] on shrimp waste, the optimal conditions for this method are: 3% HCl at 25°C for 1 h, 50% NaOH at 110°C for 3 h and then 30% H_2O_2 treatment for 3 h, for its decolourisation [34]. This method is considered economically viable and efficient, but on a commercial scale, it is not neutral for the environment. It consumes a lot of energy, and a huge amount of water, resulting in high carbon dioxide (CO_2) and wastewater production [26]. Moreover, the harsh reagents used in this method can negatively affect the properties of the extracted chitin [2]. These harsh reagents might be replaced with ionic liquids (ILs, organic salts with melting points below 100°C), like [C2mim]OAc (1-ethyl-methylimidazolium acetate). The use of ILs enables obtaining chitin with higher purity and molecular weight than that obtained during the industrial production [35, 36]. Other alternative reagents are natural deep eutectic solvents, called NADES [37-39]. NADES are a mixture of two or more primary metabolites that becomes liquid at room temperature [40]. They are considered safe for the environment and non-toxic [41]. The advantages of using NADES, like malic acid or choline chloride, is the short extraction time, efficiency and possibility to retrieve and to reuse them [39]. Another potential way to obtain chitin is electrochemically assisted extraction. This procedure, performed on black coral *Cirrhipathes* sp., lasts 12 h, requires fewer chemicals than other methods and results in pure, colourless chitin scaffolds in its original membranous formation [42]. Meanwhile, hybrid chitin extraction with use of dielectric barrier discharge (DBD) for deproteinisation

and lactic acid for demineralisation, performed on *Pandalus borealis* shells, allowed the removal of about 90% of protein, and almost all mineral content. The deproteinisation process lasted about 6 min, and the mineral content removal was performed for 15 or 30 min. The only waste produced in this process was gaseous products (CO_2N_2 and H_2CO) [43, 44].

The biological method of chitin extraction involves bacterial fermentation and allows reuse of the obtained proteins and minerals as nutrients for humans or animals [2, 32, 45]. Fermentation performed on *Penaeus vannamei* waste with *Lactobacillus rhamnoides* and *Bacillus amyloliquefaciens* yielded chitin comparable to commercially produced chitin. The demineralisation and deproteinisation efficiency were 97.5% and 96.8%, respectively. Furthermore, the broth resulting from fermentation is suggested to have a high nutritional value could be used as food for animals [45].

3. Chitosan

Chitin can be dissolved in some anhydrous carboxylic acids and concentrated mineral acids, like phosphoric acid. A research team led by Biniaś [46] developed a method for the fabrication of biomaterials from chitin with use of concentrated phosphoric acid; it yields chitin fibres and spheres. However, chitin deacetylated derivatives like chitosan (Figure 2) have more applications than chitin by itself [20]. Chitosan is more reactive and more soluble than chitin [30]. It might be modified in various ways and is considered a promising antitumour, antifungal, antibacterial, antioxidant and antiviral agent [10-13]. Chitosan is considered a relatively non-toxic and safe polymer [3]. To deacetylate chitin, it is treated with NaOH at a high temperature (90-140°C). The duration and applied temperature in this procedure affect the chitosan properties. However, there is also an enzymatic method that is more stable and does not affect the structure of the chitosan obtained [30].

Chitosan refers to a group of polymers comprising long chains of *N*-acetylglucosamine. It can be divided into four groups based on its deacetylation degree: low (55%-70%), medium (70%-85%), high (85%-95%) and ultrahigh (95%-100%) [47]. Its molecular weight varies between 50 and 2000 kDa. It is insoluble in water and alkaline solutions but it can be dissolved in almost all aqueous acids [11, 48]. Its activity depends on the position

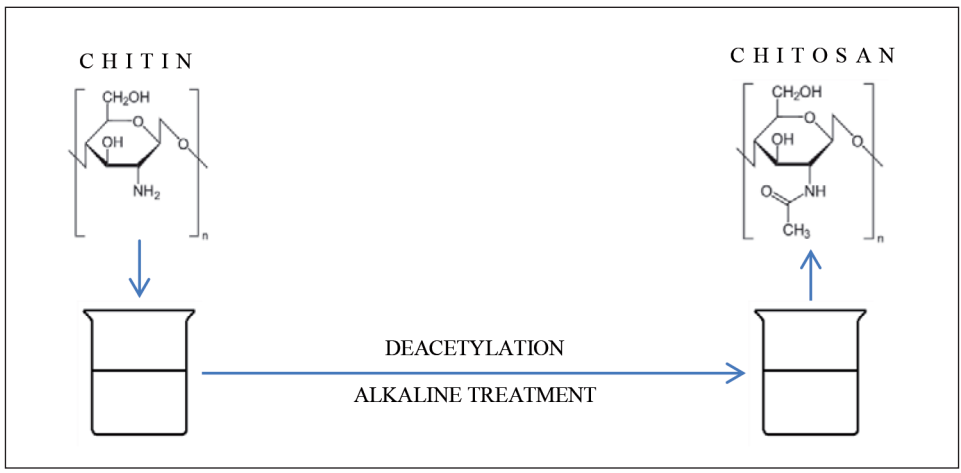

Figure 2. The process used toobtain chitosan

of sulphate groups in glucosamine residues [49]. The presence of polar groups provides good hygroscopicity, moisture retention [50] and electrostatic attraction that provides its mucoadhesion [51]. The positively charged amino groups allow chitosan to bind negatively charged surfaces. It can bind to heavy metals, and its sorption ability depends on its form and temperature [52]. Chitosan also reacts with aldehydes and acyl chlorides, and it can be also modified via hydrolysis or sulfation [11]. Its degradation products also show biological activities [53]. These features present the possibility to create chitosan derivatives with a broad spectrum of biological activities.

4. Antimicrobial Activity of Chitosan

There are many factors that can affect chitosan's antimicrobial activity, like conditions of the chitin extraction method, which impact its molecular weight and deacetylation degree, conditions and time of chitosan storage, experimental conditions and, finally, the type of the target [9, 12, 54, 55].

4.1. Antibacterial Activity of Chitosan

There are a few proposed mechanisms of chitosan's antibacterial activity. One of them relies on the electrostatic interaction of chitosan with bacterial cell wall. The positively charged chitosan interacts with negatively charged bacterial cell wall components, causing disruption of the cell wall and its leakage. This mechanism has been observed during assays performed with chitosan acetate solutions against *Escherichia coli* and *Staphylococcus aureus* [56], and during studies on cationic chitosan derivatives such as N-(2-hydroxypropyl)-3-trimethylammonium chitosan chloride (HTCC) performed on drug resistant *Klebsiella pneumoniae*, *S. aureus* and *Enterococcus faecium* [57]. The other mechanism concerns the ability of low-molecular-weight chitosan to penetrate the bacterial membrane, combine with bacterial DNA and inhibit messenger RNA (mRNA) synthesis. Meanwhile, high-molecular-weight chitosan inhibits the growth of aerobic bacteria. It forms a dense film on the cell surface and prevents the uptake of nutrients and oxygen [9, 54]. Chitosan chelates ions like Mg^{2+} and Ca^{2+}, which maintain enzymatic functions and integrity of the cell membrane [9, 58]. The chitosan antibacterial activity has also been observed against *K. pneumoniae* and *S. aureus*, which are drug resistant and cause severe infections [8, 59, 60]. There are also studies showing that chitosan ascorbate exhibits antibacterial activity, most notably in terms of inhibition of *Corynebacterium matruchotii* and *Aggregatibacter actinomycetemcomitans* [61]. Furthermore, studies on graphene/chitosan nanocomposites have shown its inhibitory activity against *Pseudomonas aeruginosa* and *K. pneumoniae* biofilm development, which is a favourable factor involved in bacterial drug resistance [14, 62]. Chitosan might also be useful for wound healing. Besides its antimicrobial activity, it helps in collagen deposition, induces hyaluronic acid synthesis at the wound site and activates macrophages to prevent tissue overgrowth [63]. Nanosystems based on hyaluronic acid and chitosan are considered a promising therapeutic agents in wound healing [58]. Chitosan is already used in various forms as a wound dressing [9].

4.2. Antifungal Activity of Chitosan

The fungal cell wall is composed mainly of glucan, mannan and chitin. The structure of the cell wall is unique for each species [64]. The majority of studies on fungal chitosan have focused on its activity against fungi considered to be pests, because they cause substantial damages to crops, and the pesticides currently in use are a threat to the environment, especially when their use is poorly managed [65]. Chitosan can stimulate the chitinase activity of plants and is considered to be very effective against spore

germination, germ tube elongation and radial growth of fungi [66]. There have also been a few studies concerning the activity of chitosan against fungal strains that are pathogenic to humans. Some fungal infections might cause systemic infections and are becoming a major problem [7]. Furthermore, some of them are resistant to drugs [67]. It is difficult to fight pathogenic fungi because the same drug used to fight fungal pathogens might be toxic to the host. Chitosan nanoparticles can cause morphological, structural and molecular changes in fungal cells [68, 69]. The assays performed on fungal strains of *Candida* sp. and *Cryptococcus* sp. with HTCC showed its inhibitory activity, with low cytotoxicity against human erythrocytes and mammalian cells. It disrupted the integrity of fungal cell membrane, causing its death [56].

4.3. Antiviral Activity of Chitosan

Viral particles consist of nucleic acid genome (RNA or DNA), a capsid (a protein coat) and in some cases an envelope (lipid membrane). Due to the lack of the elements crucial for growth and replication, viruses rely on living cells to provide all of the enzyme systems, energy, ribosomes and molecular building blocks needed for replication [70]. Chitosan can cause structural changes in viral particles and damage their integrity or bind to viral receptor proteins [71]. Some chitosan sulfonic acid derivatives show inhibitory activity against the replication of retroviruses and members of the *Herpesviridae* family [72]. Sulphated chitosan derivatives – (1-4)-2-deoxy-2-sulfoamido-3-O-sulfo-β-d-glucopyranan – show potent and specific inhibition against HIV-1 infection. They block the interaction between gp120 viral protein and its cellular receptor [16]. Modified chitosan is also considered a potential agent against SARS-CoV-2, the novel coronavirus responsible for the current global COVID-19 pandemic. The HTCC and hydrophobically modified HTCC showed activity against HCoV-NL-63 and murine hepatitis virus (both from coronavirus family) [73]. It interacts with the coronaviral spike protein (S) of HCoV-NL63, blocking its interaction with its cellular receptor angiotensin-converting enzyme 2 (ACE-2) [73, 74]. Experiments performed *in vitro* and *in vivo* with HTCC showed successful inhibition of SARS-CoV-2 and MERS-CoV replication [17]. Moreover, chitosan nanoparticles can synergise curcumin activity against HCV-4 replication and entry into a hepatic cells [75]. Chitosan might also be used for pulmonary delivery of drugs and for vaccine delivery system [49, 76, 77]. It is degraded by lysozymes into non-toxic products and releases drug at a slow rate. Chitosan can absorb proteins, amino acids and nucleic acids via complexation and ion exchange [50]. It addition, all positively charged chitosan species shows mucoadhesive properties, and their hydrophobic derivatives aggregate between cells in a pattern that allows for local drug release and improves its biodegradation [78].

5. Sources of Chitin

The properties and features of chitin depend on its source; however, the ecology or even the gender of the investigated specimens also might be relevant [22]. It might differ in structure, molecular weight and deacetylation degree, which affects the solubility and chemical reactivity [79]. The main source of chitin used for industrial production is seafood waste, not only because of its availability, but also because it is important from the environmental point of view to recycle waste [80-84]. Yet, there are many other sources of this polymer in the biosphere [18, 85].

Fungi from class Zygomycetes have a cell wall mostly composed of chitin. It is considered a promising source of chitin [86, 87]. This chitin source does not require the harsh chemical treatment, does not need to be demineralised, the material for extraction is available all year long (fermenting plants produce tons of fungal mycelia waste) and it is not contaminated with heavy metals [9, 87]. Extracted chitosan polymers

might be bound to glucan and inorganic compounds, but it is possible to purify them during an additional step of acid treatment [88]. Nwe et al. [87] showed that fungal chitosan stimulates plant growth better than chitosan obtained from shrimp and might be an excellent scaffolding material for construction of a template for tissue regeneration [87]. The deacetylation degree of chitosan extracted from *Absidia coerulea*, *Rhizopus microsporus* var. *oligosporus*, and *Mucor circinelloides* is apparently higher than the deacetylation degree of standard crustacean chitosan [25].

The research performed on mushrooms has mostly focused on their nutritional values, but some researchers have confirmed the presence of chitin in their cell wall [23, 89-93]. Research on two mushroom species, *Lactarius vellereus* and *Phyllophora ribis*, showed that the chitin content in both cases is higher than in some insect species. The chitin obtained from these mushrooms might be useful as an antimicrobial and antioxidant agent. Interestingly, chitin obtained from the aforementioned mushrooms, in addition to *Lentinula edodes*, has a structure with no nanofibres or nanopores [23, 94].

The first evidence of chitin in skeletal fibres of marine sponges was published in 2007 [95]. The three-dimensional structure of this chitin makes it an promising candidate for application in biomedicine [96]. Mutsenko and colleagues [97] suggest that chitinous scaffolds derived from the demosponge *Aplysina aerophoba* might be an attractive model for further human mesenchymal stromal cell (hMSC)-based tissue engineering. The *in vitro* studies showed it supports adhesion, viability, growth and proliferation of hMSCs [97].

Chitin is also abundant in the cuticle of insects. It occurs in complexes with proteins and it is localised in the cuticle proper [98]. Chitin obtained from the dragonflies *Sympetrum fonscolombii* eye was characterised as α-chitin fibrils varying in length, and with a high crystallinity index [99]. Cockroach (*Periplaneta americana*) wings are made with α-chitin that has a nanoporous structure, yet it has no microfibres. Moreover, it has a different structure than chitin obtained from other body parts of cockroaches [100]. α-Chitin was also isolated from beetle larva cuticle and silkworm (*Bombyx mori*) pupa exuvia [101]. Whereas most studies have reported α- or β-chitin forms, chitin obtained from cocoon of the moth *Orgyia dubia* represents the γ-form [102]. Research has also been performed using honeybees (*Apis mellifera*), as their corpses constitute an apiculture waste. While the chitin content was estimated as 8.8%, honeybee bodies are also considered a potential source of proteins and lipids [103]. Researchers have also focused on a host of other insects as a source of chitin: bumblebees *Bombus terrestris* [104]; cicadas [105]; black soldier flies [106]; Coleoptera species *Lucanus cervus* and *Polyphylla fullo*; Orthoptera species *Bradyporus (Callimenus) sureyai* and *Gryllotalpa gryllotalpa* [107]; grasshoppers [108]; larvae and adults of Colorado potato beetles (*Leptinotarsa decemlineata*) [109]; superworm (*Zophobas morio*) larvae [110]; and an invasive Asian hornet *Vespa velutina*, which is suggested to be a promising alternative source of chitin [111].

Spiders like *Geolycosa vultuosa* or *Hogna radiata* might constitute another source of chitin [112]. Both species contain α-chitin. It has been estimated that spiders could produce 2-6 million tons of cuticle per year globally. Their cuticle (in most cases) does not contain mineral phases; hence, the demineralisation step could be skipped during extraction. The isolated chitin from *Caribena versicolor* is almost 'ready-to-use'. The structure is tubular and porous, and it is suggested that it might be used in tissue engineering [113].

Crustaceans such as lobsters, krill and crayfish might also be an important source of chitin because their shells constitute a significant part of crustal wastes [32, 114, 115]. The α-chitin extracted from the lobster *Homarus americanus* has a strong, fibrous crystalline structure [116]. The data regarding crustaceans that are not commercially

fished are very limited. However, gammarids they produce α-chitin. The chitin content of *Gammarus argaeus* species is about 11%-12%. It has nanofibrils and pores [117].

In nudibranch from the eolid group, chitin occurs in three different organs: radula teeth, cuticle of the head alimentary tract and in intracellular granules. These chitinous granules are placed in epidermal cells of the skin and in gut epithelium and serve as self-defence [118]. *Conus inscriptus* has a shell consisting of α-chitin with a lower molecular weight than commercial chitin. It has regularly arranged dense pores with irregularly arranged dense nanofibres and a low molecular weight. It could perhaps be used for tissue engineering, textiles or wound dressing [24].

Chitin has also been extracted from shell and operculum of *Nerita (Dostia) crepidula* [119] and the prosmatic layer of the shell of the Japanese pearl oyster *Pinctada fucata* [120], and has also been found in shells of *Tympanotonus fusatus* and *Lissachatina filica* [121]. There were also studies performed on chitons, a group of molluscs. It is suggested, that the chiton chitosan has higher antioxidant activity than commercially available chitosan [122].

The literature about chitin in bryozoans is very limited. However, research conducted by Kaya and colleagues [123] revealed that *Plumatella repens* contains chitin composed of nanofibres with nanopores. Furthermore, the chitin content in dry mass of the bryozoan was higher than in comparatively studied species of insect (*Palomena prasina*) and fungus (*Fomes fomentarius*) [123].

Barnacles (*Austromegabalanus psittacus* and *Chelonibia patula*) produce hard shells consisting of parallel α-chitin fibres [124, 125]. Interestingly, studies conducted by Kaya and colleagues [125] showed that chitin contained in *C. patula* shells can be obtained in a shorter time compared with common chitin sources like shrimp, crayfish or crab.

Squids bone, called gladius or pen, contain β-chitin. The pen of *Loligo chinensis* has a higher chitin content than in shrimp and crab shells, and a low mineral content. Hence, demineralisation of chitin obtained from this species could likely be skipped [126]. Chitosan obtained from a commercially fished *Loligo vulgaris* is considered an effective antioxidant and antimicrobial agent [127]. β-Chitin has also obtained from a squid *Uroteuthis duvauceli*, and the cuttlefish species *Sepia prashadi* [128, 129] and *Sepia officinalis* [79]. Greven and colleagues [130] isolated α-chitin from cuticle of velvet worms, *Peripatoides novazealandia*. Its structure has no nanopores, yet shows distinct nanofibres. Chitin is also found in fish scales [20, 131]. Obtaining chitin from fish scales could solve the problem of fish waste utilisation. α-Chitin was recently isolated from a fast growing plant *Leucaena leucocephala* pods [19]. This tree was originally found in Central America and Mexico and has spread throughout the world including Asia and Malysia [132].

6. Conclusions

There are a few methods of chitin extraction. Considering the impact on the environment and time of extraction, as well as the purity of the obtained polymer, the electrochemically assisted methods and the ones performed with NADES seem to be worth further examination and, if possible, application in industrial chitin production. Selection of the method and its conditions depends on the source of chitin, and determines its quality as well as its reactivity. Chitosan is a promising biomaterial as an antimicrobial agent. It has many desirable features like reactivity and low toxicity. It can be used as antimicrobial agent alone or as an enhancer for other molecules with biological activity. It may be used in medicine to create safer drugs and more effective vaccines. It shows activity against bacterial, fungal and viral pathogens with which modern medicine is currently struggling. Chitosan constitutes an attractive replacement for current drugs not only due to its

biological activity, but also because of its biodegradability and bioavailability. It is mainly obtained from crustaceans, and it creates a great opportunity to reduce seafood wastes residing in the environment. However, there are many other organisms producing chitin, and they might constitute an attractive source of this polymer due to ease of cultivation, amount of produced chitin per year, its quality and properties, or because they may require less harsh and less time-consuming methods of its extraction. It seems that besides seafood waste and fungi, insects and spiders may also be a promising source of chitin. Hence, it might be worth studying them more thoroughly.

7. References

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Oświadczenia współautorów

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Involved:

- First author, corresponding author
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- Conceptualization
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Publikacja 2

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Article

Pontastacus leptodactylus (Eschscholtz, 1823) and *Faxonius limosus* (Rafinesque, 1817) as New, Alternative Sources of Chitin and Chitosan

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Abstract: The growing demand for chitin and chitosan makes it necessary to look for new sources of these polymers and to develop more environmentally friendly methods for their isolation. The subjects of the current study were chitin and chitosan extracted from shells of two crayfish species: *P. leptodactylus* and *F. limosus*. The obtained polymers were characterized by physicochemical properties (molecular weight, thermal stability, and structure). The obtained chitosan was evaluated regarding biocompatibility and antimicrobial activity. The yield of chitin obtained from *P. leptodactylus* and *F. limosus* with a standard method was 22 ± 2.7% and 20 ± 3.6% (*w*/*w*), respectively (a preliminary extraction with a natural deep eutectic solvent was performed successfully only for *P. leptodactylus*). The yield of chitosan production was 15 ± 0.3% and 14 ± 4.2%, respectively. Both chitosan samples showed antimicrobial activity against *E. coli* and *S. aureus*. Cytotoxicity assays revealed a time- and concentration-dependent effect, with a milder impact at concentrations up to 250 µg/mL. A more favourable profile was observed for chitosan from *F. limosus* shells.

Keywords: chitin; chitosan; antibacterial activity; cytotoxicity; NADES

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1. Introduction

Chitin (poly-(1→4)-β-*N*-acetyl-D-glucosamine) is a commonly occurring polymer in the environment. It might be found in fungi cell walls, cuticles of insects and crustacean shells [1]. Commercially, chitin is mainly obtained from seafood wastes. However, the demand for chitin is growing [2], so it is essential to look for new sources of this polymer. In this study, we chose two crayfish species, *Pontastacus leptodactylus* (Eschscholtz, 1823) and *Faxonius limosus* (Rafinesque, 1817) shells, as a source of this polymer. They are both widespread, euryhaline [3–6], omnivorous, benthic crustaceans [7–9].

P. leptodactylus, also known as Turkish, Galician, swamp, narrow-clawed or pond crayfish [10], is native to Europe [11]. According to the Food and Agriculture Organization of the United Nations (FAO) [12], *P. leptodactylus* aquaculture ranks fifth in producing crayfish species in global aquaculture. It is cultivated among others in Turkey [13] and Poland due to its nutritious meat and to reduce the overfishing of natural resources [10].

F. limosus (spiny-cheek crayfish) is native to North America and was introduced to Europe for cultivation. Currently, it is considered one of the most invasive crayfish species in Europe and a carrier of crayfish plague [14]. Invasive alien species (IAS) are one of the major environmental problems globally [15]. According to European Regulation 1143/2014 on IAS, all European Member States must take measures to combat IAS, including prevention, early detection, eradication, and management. Studies already conducted on *F. limosus* meat indicate that it is good quality and might be used in the food industry [16], but there was no research on this species regarding chitin contained in its shells. We hypothesize that shell wastes obtained from both crayfish species might constitute an additional source of chitin.

Chitin in crustacean shells occurs in complexes with mineral salts and proteins. In industrial processing, chitin isolation consists of acidic demineralization and alkali deproteinization, usually carried out with hydrochloric acid (HCl) and sodium hydroxide (NaOH). This process produces a huge amount of wastewater and energy consumption [17]. Strong reagents can also induce chitosan degradation [18]. One of the most promising alternative extraction methods is those based on natural deep eutectic solvents (NADES) mixtures. NADES are mixtures of at least two primary metabolites: one of them constitutes a hydrogen bond donor (HBD) and the other a hydrogen bond acceptor (HBA). After being combined, they become a mixture with a lower melting point than the melting points of each component [19]. They are considered safe for the environment and non-toxic [20]. Extractions with NADES mixtures consume less water, energy, and time. Another advantage is the higher extraction efficiency and better quality of the obtained polymer [21]. However, chitin quality varies depending on the source, and its extraction might require different working conditions [22].

Chitin can be used in wound dressings or tissue engineering [23,24]. However, deacetylation (usually carried out with NaOH [17]) enhances solubility and significantly broadens application possibilities. Chitin polymers with a degree of deacetylation > 55% are called "chitosan" [25]. The free amino groups give chitosan a positive charge, thus, many desirable features, like mucoadhesivity or antimicrobial activity [26,27]. The antimicrobial activity is a desirable feature in medical applications, like wound dressing or for an application as an antibiotic [28]. It is considered a promising agent as a drug carrier. Chitosan was approved by the American Food and Drug Administration (FDA) as a wound dressing [29] and can be found on the market in cosmetics and dietary supplements. However, depending on many factors, such as the source and extraction method, the properties of chitosan might vary. Thus, the biocompatibility of these polymers will be different and should be investigated separately in terms of the planned application and target [30].

This paper aimed to assess if *P. leptodactylus* and *F. limosus* shells are economically viable as alternative sources of chitin and chitosan to isolate chitin from *P. leptodactylus* and *F. limosus* shells using the method proposed by Zhu et al. [31] and to assess the antibacterial activity and cytotoxicity of the obtained chitosan samples.

2. Materials and Methods

2.1. Materials

2.1.1. Biomass

F. limosus specimens were collected from Jamno Lake (Gospodarstwo Rybackie "Mielno" sp. z o.o., Mielno, Poland) in the summer of 2015, and *P. leptodactylus* specimens were taken from aquaculture (Gospodarstwo Rybackie Bytów sp. z o.o., Bytów, Poland) during the summer and autumn of 2020. They were treated following the method described by Rodriguez-Veiga et al. [32] and were washed and later boiled for 8 h, for easier tissue separation. The water was changed five times, and after this, the specimens were dried at 50 °C for 24 h. Then, the shells were separated from the remains of other tissues and milled to powder.

2.1.2. Reagents

Commercial chitin and chitosan standards were purchased from (Sigma-Aldrich, Milan, Italy), and used as a control in this study. Hydrochloride (HCl), sodium hydroxide (NaOH), choline chloride ($(CH_3)_3N(Cl)CH_2CH_2OH$), malonic acid ($CH_2(COOH)_2$) and citric acid ($C_6H_8O_7$) were purchased from (Sigma-Aldrich, Milan, Italy), Tryptone Soya Broth (TSB) and Iso-Sensitest broth (ISB) were purchased from Oxoid (Basingstoke, UK). Dulbecco's Modified Eagle's (DMEM), L-glutamine, non-essential amino acids and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Burghausen, Germany). PBS tablets pH 7.4 were purchased from VWR (Rosny-sous-Bois, France). Dimethyl sulfoxide (DMSO) was acquired from Carlo Erba (Sabadell, Spain), and penicillin and bovine serum (FBS) from Gibco (Life Technologies, Carlsband, CA, USA). A lactate dehydrogenase (LDH) kit was provided by Takara Bio (Tokyo, Japan).

2.2. Chitin and Chitosan Isolation

2.2.1. Standard Chitin Extraction

Demineralization

The material demineralization was carried out at room temperature, under magnetic stirring (30 min), with an aqueous solution of HCl (3.6%, *w*/*v*) added to the sample at a solid/liquid ratio of 1 g/15 mL. The obtained material was centrifuged, the supernatant liquid was removed, and the remaining material was thoroughly washed with 25 mL of distilled water in a centrifuge (Eppendorf 5804R, Eppendorf, Milano, Italy) at 4000 rpm (6 times for 5 min) until neutral pH was achieved. The obtained material was dried in the oven at 80 °C (48 h).

Deproteinization

The sample deproteinization was performed with an aqueous solution of NaOH (8%, *w*/*v*) added to the material obtained earlier at a solid/liquid ratio of 1 g/15 mL, at 100 °C, under stirring for 1 h. Then, again, it was centrifuged, the supernatant liquid was removed, and the remaining material was thoroughly washed with 25 mL of distilled water in a centrifuge at 4000 rpm (3 times for 5 min) until neutral pH was achieved and dried in the oven at 80 °C (48 h).

2.2.2. Standard Chitosan Extraction

Deacetylation

The process of deacetylation was carried out in a reflux apparatus (round bottom flask equipped with a refrigerator and heated in an oil bath) with an aqueous solution of NaOH (60%, *w*/*v*) that was added to the sample at a solid/liquid ratio 1 g/20 mL at 180 °C under stirring for 2 h. The obtained material was centrifuged, the supernatant liquid was removed, and the remaining material was thoroughly washed with 25 mL of distilled water in a centrifuge at 4000 rpm (4 times for 5 min) to obtain neutral pH and dried in the oven at 80 °C (48 h) afterwards.

2.2.3. NADES Chitin Extraction

NADES mixture was prepared following the modified procedure of Zhu et al. [31]. In a two-necked round-bottom flask, 8.59 g of choline chloride and 12.8 g of malonic acid were heated in an oil bath, at 100 °C, under mechanical stirring until a homogenous and colourless solution was formed. Then 1.5 g of crayfish shell powder was added, constituting 7% of the solution. The extraction was performed in two variants:

- The material was kept with NADES at 100 °C under mechanical stirring for 2 h. After filtration under vacuum, the obtained material was washed seven times with 25 mL of distilled water in a centrifuge at 4000 rpm (7 times for 5 min) until the neutral pH was obtained and dried in the oven at 80 °C for 48 h. Then, the extracted material was additionally treated with 20 mL of H_2O_2 at 10% for 2 h at 80 °C, washed with 25 mL

of distilled water in a centrifuge at 4000 rpm (7 times for 5 min) until the neutral pH was obtained, and dried in the oven at 80 °C for 48 h.

- The material was pre-treated with 10 mL of $C_6H_8O_7$ (10%, *w*/*v*) for 1 h at room temperature and then treated following the point above.

Chitin and chitosan yields were calculated with the following equations:

$$\text{Yield}_{\text{chitin}}\ [\%] = (W_{\text{chitin}}/W_{\text{shell}}) \times 100 \quad (1)$$

$$\text{Yield}_{\text{chitosan from chitin}}\ [\%] = (W_{\text{chitosan}}/W_{\text{chitin}}) \times 100 \quad (2)$$

$$\text{Yield}_{\text{chitosan from shell}}\ [\%] = (W_{\text{chitosan}}/W_{\text{shell}}) \times 100 \quad (3)$$

where W_{chitin} is the weight of the obtained chitin, W_{shell} is the weight of the raw material, and W_{chitosan} is the weight of the obtained chitosan.

2.3. Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR analysis was carried out with a Spectrum One FTIR spectrophotometer (Perkin Elmer, Wellesley, MA, USA) equipped with a MIRacle^{TM} ATR device (Pike Technologies, Madison, WI, USA) over the frequency range of 4000–650 cm^{-1} at a resolution of 4 cm^{-1} (64 scans). The spectra were collected at least in triplicate.

2.4. Thermogravimetric Analysis (TG)

Thermogravimetric (TG) analysis was conducted with Mettler Toledo Star^{e} System (Mettler Toledo, Milan, Italy) equipped with a TGA/DSC 1 module. To perform the analysis, about 5 mg of each sample was put in alumina crucibles with lids (scanning rate of 10 K min^{-1} from 30 °C to 500 °C) under a nitrogen atmosphere (flow rate 50 mL min^{-1}). The instrument was previously calibrated with indium as a standard reference, and the measurements were done at least in triplicate.

2.5. Scanning Electron Microscopy (SEM)

The surface of the obtained chitins and chitosan samples was analysed with scanning electron microscopy (SEM). The samples were sputtered with Au and examined using an EVO MA10instrument (Carl Zeiss, Oberkochen, Germany). The measurements were performed under an ultra-high vacuum with an electron generation voltage of 5 kV and a working distance of 8.5 mm.

2.6. Molar Mass Distribution

The molar mass distribution of the obtained polymers was estimated by High-Performance Size Exclusion Chromatography (HPSEC) and carried out in a modular system comprised of a Biotech Degasi GPC degasser (Biotech, Onsala, Sweden), a Waters 515 HPLC pump (Waters, Milford, MA, USA), and a Knauer K-2300 refractive index detector (Knauer, Berlin, Germany). Two Shodex columns OHpak SB-806M HQ (Showa Denko K.K., Tokyo, Japan) of 300 mm × 8.0 mm were used in series with a 50 mm × 6.0 mm OHpak SB-G 6B guard column (Showa Denko K.K., Tokyo, Japan).

A 0.2 M $NaNO_3$ + 0.5 M CH_3COOH aqueous solution (0.02% NaN_3) was used as a mobile phase at 1 mL/min. Chitosan samples were dissolved in the eluent at 5 mg/mL overnight, centrifuged at 4000 rpm for 10 min, and filtered through 0.45 µm filters. A conventional calibration curve was set using polystyrene sulfonate standards dissolved at 5 mg/mL in the former eluent.

2.7. Antimicrobial Assays

2.7.1. Chitosan Salification

Both chitosan samples were dissolved in HCl (3.6%, *w*/*v*) at a solid/liquid ratio of 1 g/6.2 mL, with distilled water in proportion to 100 mL per 1 g of the sample. Each solution

was dialyzed for 24 h, under stirring, at room temperature. The water was changed every 2 h. The dialyzed samples were frozen in a diagonal position and then lyophilized.

2.7.2. Bacteria Culture

Escherichia coli (ATCC 10536) and *Staphylococcus aureus* (ATCC 6538) strains were cultured in TSB at 37 °C. The bacteria cultures were centrifuged at 3000 rpm for 20 min to separate cells from broth and then suspended in PBS (pH 7.3). The suspension was diluted to adjust the number of cells to 1×10^7–1×10^8 CFU/mL.

2.7.3. Evaluation of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The antimicrobial activity of the obtained chitosan samples was tested against *Escherichia coli* (ATCC 10536) and *Staphylococcus aureus* (ATCC 6538) as models for Gram-negative and Gram-positive bacteria, respectively. The density of the used suspension was 1×10^7–1×10^8 CFU/mL. All the extracts were dissolved in a 10% DMSO aqueous solution at different concentrations. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined by the two-fold serial broth dilution method in ISB according to Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) procedures [32]. The starting inoculum was 1.0×10^7 CFU/mL. Concentrations of extracts were tested in the range of 0.1–9.2 mg/mL. The MIC was the lowest extract concentration inhibiting observable microbial growth after 24 h incubation at 37 °C. The MBC was the lowest concentration resulting in >99.9% reduction of the initial inoculum after 24 h of incubation at 37 °C. All experiments were performed in triplicate [33]. Solvent blanks were included. The positive control was stock standard solutions of ampicillin.

2.8. Cytotoxicity Assay (MTT)

2.8.1. Cell Culture

Human lung epithelial adenocarcinoma cells (A549), provided by American Type Culture Collection (ATCC, Manassas, VA, USA), were used in passages 84–94. They were cultured in flasks (75 cm^2) at 37 °C in an incubator with 5% CO_2/95% humidified atmospheric air. The CCM used for this cell line was (DMEM) supplemented with L-glutamine at 1% (v/v), penicillin/streptomycin at 1% (v/v), non-essential amino acids at 1% (v/v), and FBS at 10% (v/v). CCM was exchanged twice a week.

2.8.2. Assessment of metabolic activity

The metabolic activity of the A549 cells after exposure to the chitosan samples was evaluated with an MTT assay following the International Organization for Standardization indications [34]. Chitosan samples were solubilized in acetic acid (1%, v/v) at a concentration of 4 mg/mL, then dispersed in CCM at concentrations ranging between 15.62 and 1000 µg/mL. To perform the assays, cells were seeded in 96-well plates at 1×10^4 cells/well the day before the exposure. After the overnight incubation, the CCM was discarded and replaced with chitosan samples. CCM and SDS solution at 2% (w/v) were used as a positive and negative control of cell viability, respectively. After 3, 24, and 48 h of exposure, the samples were replaced with 30 µL of MTT (5 mg/mL in PBS pH 7.4), which remained in contact with the cells for 2 h at 37 °C. Then, 50 µL of DMSO was added to each well to solubilise the formed formazan crystals. The absorbance was read by spectrophotometry (Infinite M200; Tecan, Grödig, Austria) at 540 nm, with background correction at 640 nm. All experiments were performed in triplicate. The cell viability was calculated from the equation:

$$\text{Cell viability}(\%) = \frac{\text{A} - \text{D}}{\text{CM} - \text{D}} \times 100 \quad (4)$$

where A is an absorbance obtained upon exposure for each sample, D is an absorbance measured for DMSO, and CM is an absorbance read for the cells incubated in CCM. The half maximal inhibitory concentration (IC_{50}) was calculated for samples with cell viability

reaching less than 50%. IC_{50} values were determined by sigmoidal fitting of the data in the GraphPad Prism statistical program (GraphPad Software, Version 6.01, Boston, MA, USA).

2.9. Statistical Analysis

The results were analysed by two-way analysis of variance (ANOVA). The used software was R (packages: emmeans 1.8.5, car 3.1-2, bestNormalize 1.9.0 and DHARMa 0.4.6), and differences were considered significant at a level of $p < 0.05$.

3. Results

3.1. Chitin and Chitosan Recovery

3.1.1. Chitin and Chitosan Obtained Using Standard Chemical Procedures

The average chitin yield in *P. leptodactylus* and *F. limosus* shells was 22 ± 2.7% (n = 5) and 20 ± 3.4% (n = 4) (*w*/*w*), respectively. The media of chitosan yield obtained from chitin was 70 ± 13.0% (n = 2) and 76 ± 9.0% (n = 2) (*w*/*w*) respectively. Therefore the content of chitosan obtained from shells was 15 ± 0.3% and 14 ± 4.2% (*w*/*w*) (Table 1). All the obtained chitin and chitosan polymers were slightly brownish.

Table 1. Chitin and chitosan recovery [%] in shells of *P. leptodactylus* and *F. limosus* obtained with a standard extraction, NADES, and NADES with citric acid pre-treatment.

Species	Treatment	Chitin Recovery [%]	Chitosan Recovery from Chitin [%]	Chitosan Recovery from Shells [%]
P. leptodactylus	standard extraction	22 ± 2.7 [a]	70 ± 13.0 [b]	15 ± 0.3 [b]
P. leptodactylus	NADES	24 [c]	N/D	N/D
P. leptodactylus	NADES with citric acid pretreatment	29 [c]	N/D	N/D
F. limosus	standard extraction	20 ± 3.4 [d]	76 ± 9.0 [b]	14 ± 4.2 [b]
F. limosus	NADES	31 [c]	N/D	N/D
F. limosus	NADES with citric acid pretreatment	25 [c]	N/D	N/D

[a] n = 5; [b] n = 2; [c] n = 1; [d] n = 4; N/D—not determined.

3.1.2. Preliminary Extraction with NADES Mixture

The extraction with NADES allowed us to obtain chitin from P. leptodactylus shells successfully. The yield was higher compared to the standard method. The NADES extraction performed on *F. limosus* shells did not complete chitin purification. Further FTIR analysis showed the remains of protein. The extraction with citric acid pre-treatment resulted in a higher chitin yield from *P. leptodactylus*, while for *F. limosus* shells, the yield of the obtained material was lower (about 25%). However, no changes in further analysis were observed. In the current study, each extraction with NADES was performed only once.

3.2. Fourier Transform Infrared Spectroscopy (FTIR)

Chitin and Chitosan Obtained Using Standard Extraction Characterisation

FTIR spectra of *P. leptodactylus* and *F. limosus* samples corresponded to the spectrum of the chitin standard (Figure 1, spectrum a). The Chitin standard used as reference was characterized by amide I band at 1622 cm^{-1}, amide II at 1551 cm^{-1}, -C-H stretching at 2875 cm^{-1}, and -C-O-C- stretching at 1070 cm^{-1} and 1026 cm^{-1} (Table 2). For the material obtained with standard extraction performed on *P. leptodactylus* (spectrum b) and *F. limosus* (spectrum c) shells, amide I bands were observed at 1633 cm^{-1} and 1628 cm^{-1}, amide II bands at 1553 cm^{-1} and 1552 cm^{-1}, -C-H stretching at 2879 cm^{-1} and at 2890 cm^{-1}, and -C-O-C- stretching at 1063 cm^{-1} and 1026 cm^{-1}, and at 1067 cm^{-1} and 1026 cm^{-1} respectively. Similarly, for the material obtained with NADES from *P. leptodactylus* (spectrum c) and *F. limosus* (spectrum e) shells, the amide I band was observed at 1622 cm^{-1} and 1635 cm^{-1}, the amide II at 1551 cm^{-1} and 1549 cm^{-1}, -C-H stretching at 2883 cm^{-1} and 2874 cm^{-1}, and -C-O-C- stretching at 1071 cm^{-1} and 1011 cm^{-1}, and 1068 cm^{-1} and 1027 cm^{-1} respectively.

For the *F. limosus* shells, the extraction wasn't complete, and proteins remained in the sample, as appeared by the presence of the bands at 1414 cm^{-1}.

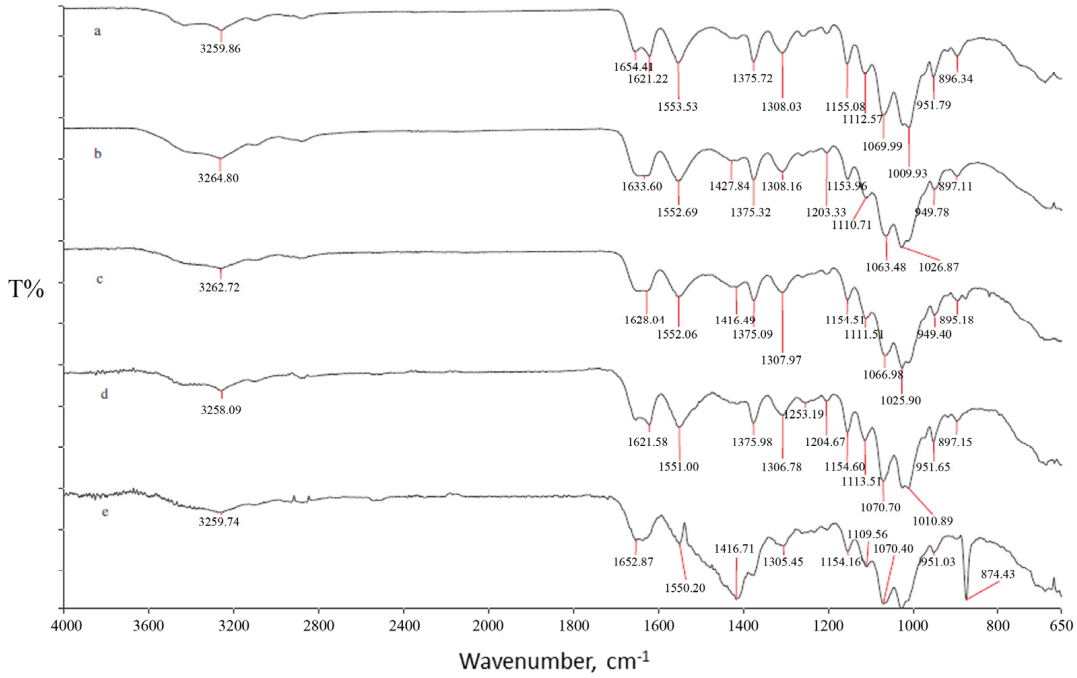


Figure 1. FTIR spectra of chitin standard (a), and materials obtained from: *P. leptodactylus* (b), and *F. limosus* shells (c) with standard method and materials obtained from *P. leptodactylus* (d) and *F. limosus* (e) shells with NADES.

Table 2. FTIR spectra assignments of the relevant bands for chitin standard and material obtained from *P. leptodactylus* and *F. limosus* shells.

Functional Group	Commercial Chitin Wavenumber [cm^{-1}]	*P. leptodactylus* Wavenumber [cm^{-1}]	*F. limosus* Wavenumber [cm^{-1}]	*P. leptodactylus* (NADES) Wavenumber [cm^{-1}]	*F. limosus* (NADES) Wavenumber [cm^{-1}]
Amide I	1622	1633	1628	1622	1635
Amide II	1551	1553	1552	1551	1549
-C-H	2875	2879	2890	2883	2874
-C-O-C-	1070	1063	1067	1071	1068
	1026	1026	1026	1011	1027

Recorded FTIR spectra for both *P. leptodactylus* and *F. limosus* deacetylated samples were like the FTIR spectrum of chitosan standard (Figure 2), with small differences in wavenumbers and adsorption intensity (Table 3). Chitosan standard (spectrum a), used as a reference, was characterized by amide I band at 1647 cm^{-1}, amide II at 1564 cm^{-1}, -C-H stretching at 2872 cm^{-1}, and -C-O-C- stretching at 1063 cm^{-1} and 102m^{-1} (Table 2). For the deacetylated material from *P. leptodactylus* (spectrum b) and *F. limosus* (spectrum c) shells, amide I bands were observed at around 1650 cm^{-1} and the amide II bands at around 1590 cm^{-1} for both species. The -C-H stretching was observed at 2874 cm^{-1} and 2864 cm^{-1}, respectively. The -C-O-C- stretching at 1054 cm^{-1} and 1027 cm^{-1} for *P. leptodactylus* and at 1059 cm^{-1} and 1026 cm^{-1} for *F. limosus*.

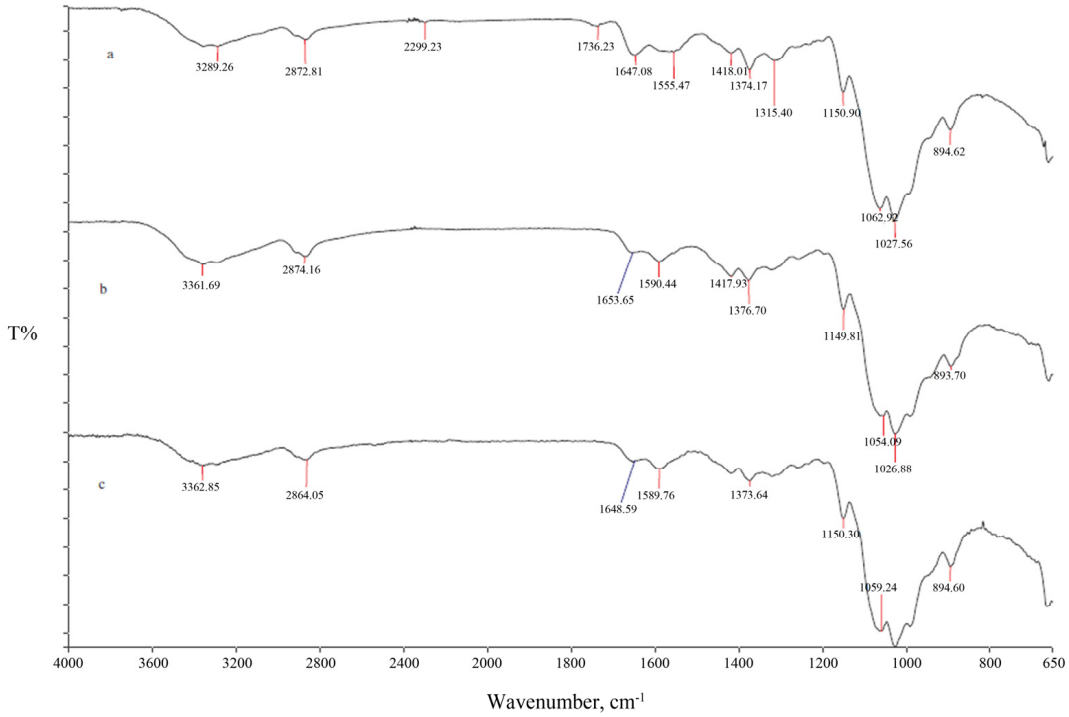


Figure 2. FTIR spectra of chitosan standard (a) and deacetylated materials obtained from *P. leptodactylus* (b) and *F. limosus* (c) shells using the standard method.

Table 3. FTIR spectra assignments of the relevant bands for chitosan standard and material obtained after deacetylation of the material obtained from *P. leptodactylus* and *F. limosus* shells.

Functional Group	Commercial Chitosan Wavenumber [cm^{-1}]	*P. leptodactylus* Wavenumber [cm^{-1}]	*F. limosus* Wavenumber [cm^{-1}]
Amide I	1647	1654	1650
Amide II	1564	1590	1590
-C-H	2872	2874	2864
-C-O-C-	1063 1027	1054 1027	1059 1026

The lower adsorption intensity for amide I and II in the spectra of chitosan samples compared to the spectra of chitin samples corresponds to decreased residual N-acetyl units, indicating the deacetylation process went successfully.

3.3. Thermogravimetric Analysis (TG) of Chitin and Chitosan Obtained Using Standard Extraction Characterisation

The thermogravimetric analyses performed on chitin extracted from *P. leptodactylus* and *F. limosus* shells using both standard and NADES methods are reported in Figure 3. All TG curves obtained are like the TG curve of the chitin standard (curve a). Their thermal profiles are composed of two main steps: the first corresponds to water evaporation, and the second to amine group degradation [35]. For the chitin standard, water evaporation lasted until around 100 °C with a mass loss of 7.7 ± 0.2%. The beginning degradation of amine groups was observed at around 164 °C with a mass loss of 75.0 ± 0.3%. This second step could be divided into two events relative to polymeric chain degradation and acetylated and deacetylated unit decomposition, respectively, and was also observed for *P. leptodactylus* (curve b) and *F. limosus* (curve c) samples. For the *P. leptodactylus* sample, the first step of sample dehydration lasted until 94 °C with an initial mass loss of 5.7 ± 0.5%. For the *F. limosus* sample, the initial mass loss was 5.4 ± 0.7% and lasted until 95 °C. For the *P. leptodactylus* sample, the second step was observed at 202 °C with

a total mass loss of 78.5 ± 0.6%, while for the *F. limosus* sample, the starting degradation temperature was 206 °C with a total mass loss of 77.6 ± 0.2%. The TG profiles registered on chitin samples extracted with NADES mixture from shells of the two crayfish species showed similar thermal profiles. For the sample obtained with the NADES method, from *P. leptodactylus* (curve d), the water evaporation lasted until 148 °C, and the mass loss was about 6.5 ± 0.6%.

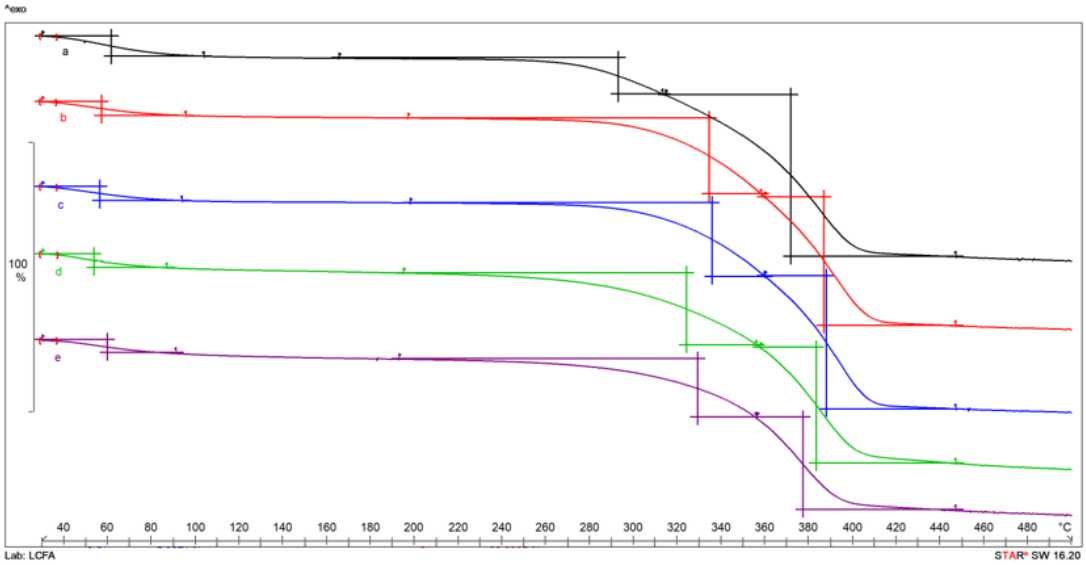


Figure 3. TG curves of chitin standard (a), samples obtained from standard extraction performed on *P. leptodactylus* (b) and *F. limosus* (c) shells and after extraction with NADES mixture performed on *P. leptodactylus* (d) and *F. limosus* (e) shells.

In comparison, due to the decomposition, the second step started at 230 °C with a mass loss of 70.1 ± 0.8%. The sample obtained from *F. limosus* shells also showed two steps: the first one lasted until 182 °C with a mass loss of 6.6 ± 0.5%, and the second one was initiated at 221 °C with a mass loss of 54.4 ± 0.3%. The different decomposition temperatures of the samples obtained with the NADES extraction method compared to those obtained with the chemical method are probably due to a greater impurity of the former.

Figure 4 shows the profiles of the recorded curves from TG analysis of chitosan from *P. leptodactylus* (curve b) and *F. limosus* (curve c) shells, which were like to the curve obtained for chitosan standard (curve a). All of them decomposed in two significant steps related to successfully performed deacetylation. Similarly, as for chitin, the first step of mass loss corresponds to water evaporation and the second to sample degradation. The first step was observed for commercial chitosan with a mass loss of 10.8 ± 0.3% and lasted until 170 °C. Sample degradation was observed at 214 °C with a mass loss of 52.4 ± 0.4%. The first step, corresponding to water evaporation in the *P. leptodactylus* sample, lasted until 146 °C with a mass loss of 7.9 ± 0.8%, and the second one, corresponding to the sample decomposition, started from 196 °C with a mass loss of 54.5 ± 0.6%. Analogously, for the chitosan from *F. limosus* shells, the first step lasted until 195 °C with a mass loss 7.2 ± 0.4%, and the sample decomposition started from 211 °C with a mass loss of 53.5 ± 0.7%.

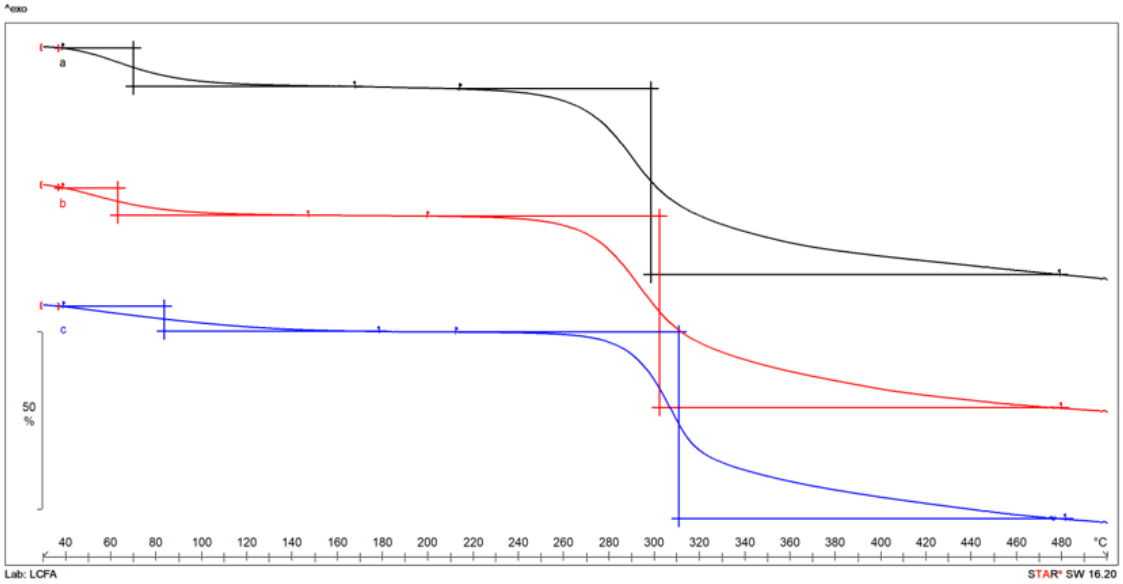
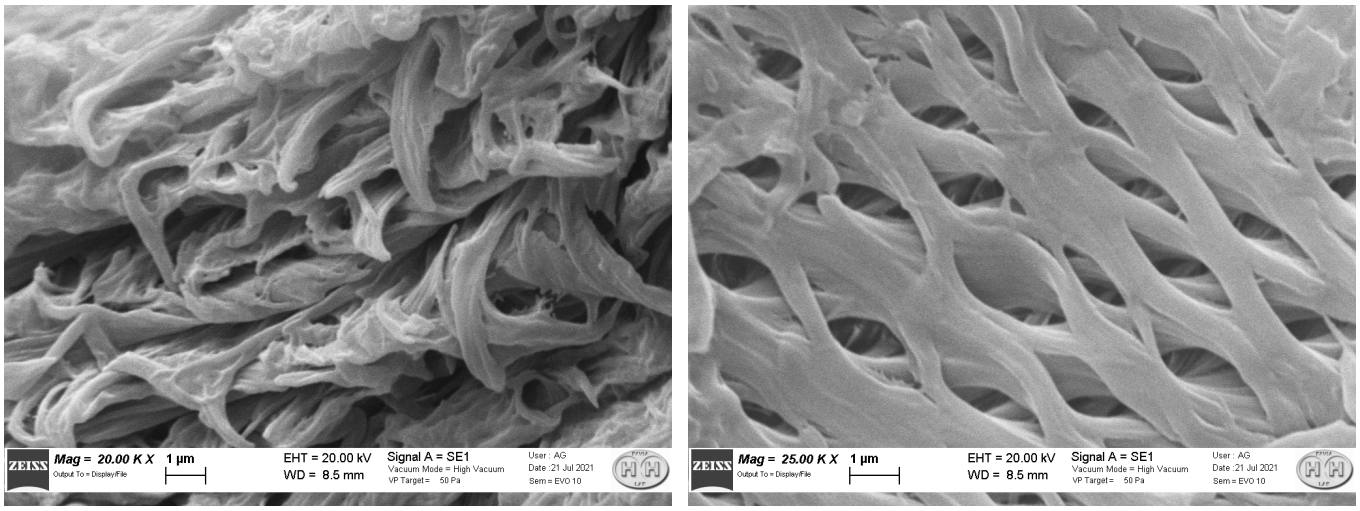


Figure 4. TG curves of chitosan standard (a), material obtained after *P. leptodactylus* (b), and *F. limosus* (c) samples deacetylation.

3.4. Scanning Electron Microscopy (SEM)

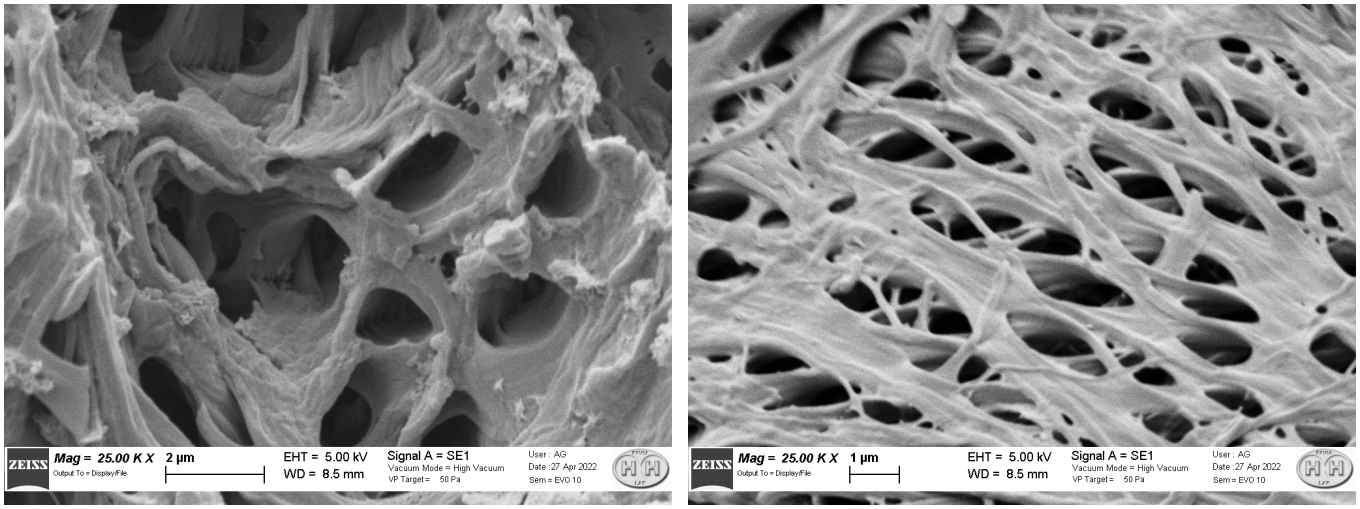
The structure of chitin from *P. leptodactylus* shells obtained with NADES is arranged in visible layers (Figure 5a) and has a fibre-like, well-organised porous structure (Figure 5b). The pores section is elongated in shape with the major and minor axes up to 2 µm and 1 µm in length, respectively.



(**a**) (**b**)

Figure 5. SEM images of chitin obtained from *P. leptodactylus* shells with NADES ((**a**) 20.00K×, (**b**) 25.00K×).

For both species, chitosan has a fibre-like structure with pores (Figure 6). Chitosan obtained from *F. limosus* shells (Figure 6b) seems to have a better-organised and more porous structure with thinner layers and interconnected pores than chitosan from *P. leptodactylus* (Figure 6a). Also, the pores shape and size are less homogeneous.



(**a**) (**b**)

Figure 6. SEM images of chitosan were obtained from *P. leptodactylus* (**a**) and *F. limosus* (**b**) using standard extraction.

3.5. Molecular Weight (MW)

Both chitosans were characterized by HPSEC (Figure 7) to determine their molecular weight distribution. Chitosan obtained from *P. leptodactylus* shells presented a number average molecular weight (M_n) of 24 kDa, a weight average molecular weight (M_W) of 236 kDa and a molecular dispersity ($Đ$m) of 9.8. In contrast, chitosan from *F. limosus* shells presented a M_n of 19 kDa, a M_W of 229 kDa and a $Đ$m of 12.3.

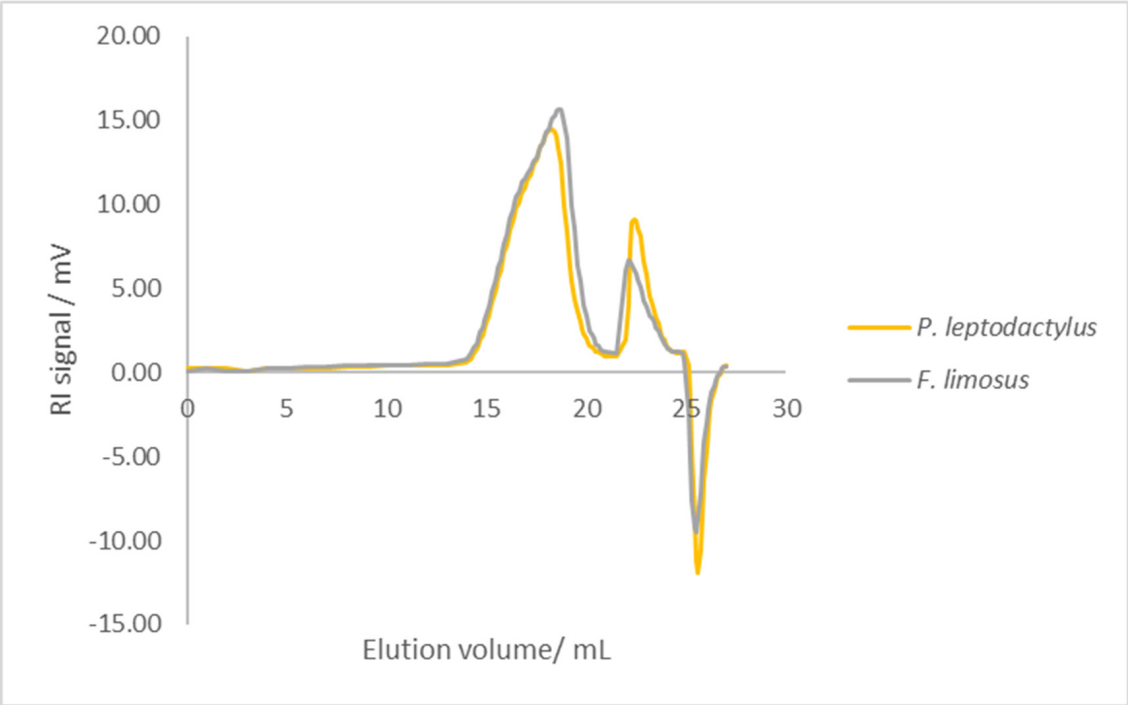


Figure 7. Size exclusion chromatography elution profiles of chitosan samples from *P. leptodactylus* and *F. limosus*.

3.6. Antimicrobial Activity

The MIC and MBC of the obtained chitosan polymers were determined against *E. coli* (gram-negative) and *S. aureus* (gram-positive) bacteria. These are humans' most common

pathogenic bacteria, showing resistance to some currently used antibiotics, e.g., ampicillin [36–38]. The antimicrobial activity is presented in Table 4. The MIC against *E. coli* for both chitosan samples was higher than the concentration of chitosan standard (commercially available). However, MBC against *E. coli* and MIC and MBC against *S. aureus* values were lower for the chitosan sample than for the chitosan standard. Finally, all the samples tested have an antibacterial activity lower than the ampicillin, used as a positive control. The activity of chitosan from *P. leptodactylus* shells was stronger against *S. aureus* than against *E. coli*.

Table 4. The minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations [mg/mL] of chitosan was obtained from *P. leptodactylus* and *F. limosus* shells and of ampicillin against *E. coli* and *S. aureus*.

Species	MIC (*E. coli*)	MIC (*S. aureus*)	MBC (*E. coli*)	MBC (*S. aureus*)
P. leptodactylus	1.19	1.59	1.58	2.48
F. limosus	2.48	1.96	4.90	4.90
Chitosan s.*	1.15	2.30	>9.20	>9.20
Ampicilin	5×10^{-3}	0.5×10^{-3}	10×10^{-3}	2×10^{-3}

* Chitosan standard, commercially available.

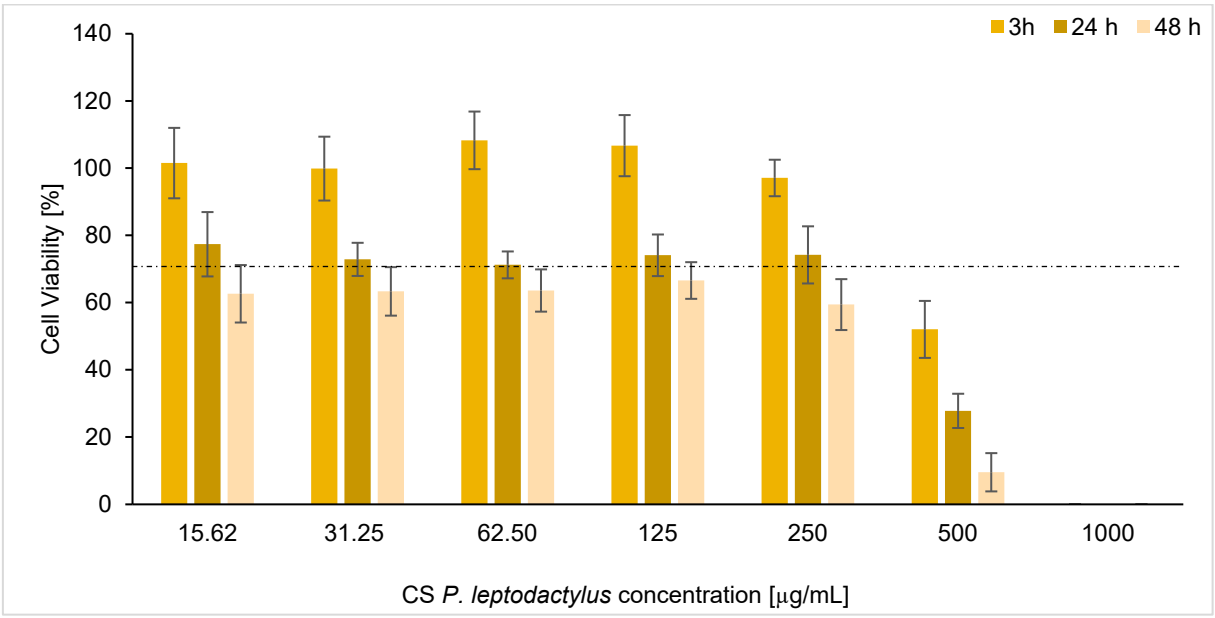
3.7. Determination of Cytotoxicity

The chitosan samples under study were tested at concentrations up to 1000 µg/mL regarding their effect on the metabolic activity of human epithelial cells. The metabolic activity was considered an indicator of cell viability, and a decrease in this parameter to values below 70% of the control (cells incubated with cell culture medium) were interpreted as a toxic effect, as indicated by ISO 10993 [34]. The results are depicted in Figure 8, showing a milder effect from the chitosan obtained from *F. limosus* as compared to that obtained from *P. leptodactylus*. In fact, for the former, a cytotoxic effect was only found when the concentration reached 500 µg/mL ($p < 0.05$), where cell viability decreased beyond 70%. At that concentration, a time-dependent effect was seen ($p < 0.05$), with cell viability varying within 10% and 65% for the period between 3 h and 48 h of exposure. On the contrary, for chitosan from *P. leptodactylus*, cell viability below 70% was determined at all the concentrations when the exposure was extended to 48 h ($p < 0.05$). The other two exposure times generally not eliciting a toxic effect. As observed for chitosan from *F. limosus*, the 500 µg/mL concentration also resulted in a time-dependent effect ($p < 0.05$) with cell viability between 10% and 52% for exposures between 3 h and 48 h. The concentration of 1000 µg/mL is massively toxic in both chitosan samples. Acetic acid, used to prepare the initial chitosan stock solution, could have a contribution, but this was observed to be minor. A control testing the effect of acetic acid in concentrations corresponding to those present in each chitosan sample revealed that only the higher amount of acetic acid (corresponding to chitosan 1000 µg/mL) decreased cell viability to values below 70% (around 65% independently of the exposure time). Therefore, the toxic effect observed in chitosan at concentrations of 500 µg/mL or higher could not be ascribed to acetic acid and should be considered an effect of the proper chitosan.

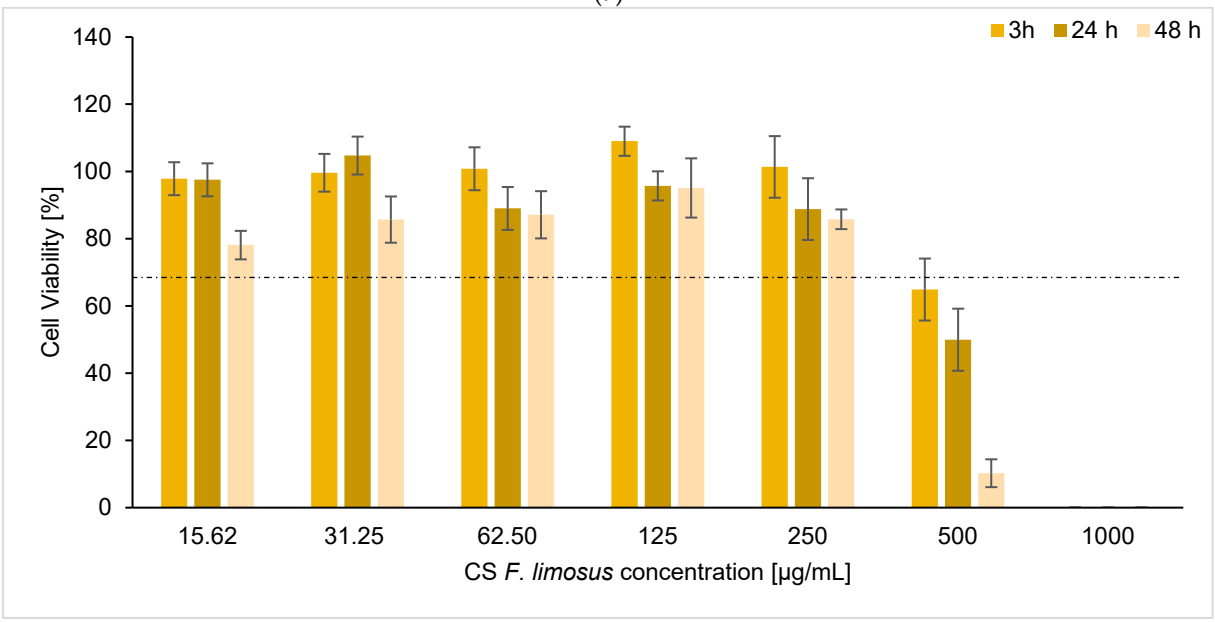
The half-maximal inhibitory concentration (IC_{50}) was calculated for both chitosan samples (Table 5). In both cases, the IC_{50} value decreased with the increase in the exposure time, as expected.

Table 5. IC_{50} values [mg/mL] calculated for chitosan obtained from *P. leptodactylus* and *F. limosus* for epithelial cell line (A459) after 3, 24 and 48 h.

Species	IC_{50} (3 h)	IC_{50} (24 h)	IC_{50} (48 h)
P. leptodactylus	0.51	0.24	0.14
F. limosus	0.52	0.49	0.34



(**a**)



(**b**)

Figure 8. A549 cell viability was determined upon 3 h, 24 h, and 48 h exposure to chitosan from *P. leptodactylus* (**a**) and *F. limosus* (**b**). Data represent mean ± SEM (n = 3).

4. Discussion

Crayfish shells might contain about 15% to 26% of chitin [39–43]. Chitin yield isolated from *P. leptodactylus* and *F. limosus* shells is within this range. The chitin yield obtained from *P. leptodactylus* shells was lower than the yield reported for the same species by [43]. The observed differences may be attributed to different extraction methods [44].

The most common method used for chitin isolation is carried out with HCl and NaOH [17]. According to Gadgey and Bahekar [44], strong acid and alkali treatment can negatively affect the obtained polymer. Using this method on a commercial scale is also considered hazardous to the environment. One of the most promising methods for chitin extraction is based on NADES mixtures, as they are considered environmentally friendly and efficient [17,45]. Furthermore, some NADES might be reused [46–48]. Studies performed by Zhu et al. [31], Hong et al. (2018) on lobster shells, and Saravana et al. [49,50]

on shrimp shells showed that it is possible to obtain chitin with high purity with the use of NADES mixture, composed of choline chloride as HBA, and malonic acid as HBD. In the study described herein, chitin isolation was performed following the method described in the work of Zhu et al. [31] but at higher temperatures during extraction. The FTIR and TG analysis indicated that the extraction performed on *P. leptodactylus* shells resulted in the obtention of chitin.

In contrast, extraction performed on *F. limosus* shells resulted in chitin with protein residues observed on FTIR spectra. The purity of chitin depends on many factors, such as reaction time, solvents, and their concentration, material/solvent ratio, pre-treatment, preparation of material, and its source [51]. Crustacean shell composition varies between species and might affect the success of chitin isolation [52,53]. The extraction resulted in a product with a higher yield, and the SEM analysis showed a better-organised microfibrillar structure than the one obtained with the standard method.

According to that reported by Kaya et al. [54], chitin and chitosan surface morphologies might be classified as (1) with porosity and microfibrillar structure, (2) without porosity or microfibrillar structure or (3) only with microfibrillar structure. Chitin obtained from *P. leptodacylus* and *F. limosus* shells showed microfibrillar structure with porosity, which might indicate good adsorption properties. The nano-fibre-like structures might suit wound dressings, plastics, or protection coats [55]. The structure of chitin extracted with NADES mixture looks well organised and smooth, suggesting promising properties of the polymer.

Molecular weight (MW) is one of the most critical factors that determines polymers biological properties. Ribeiro et al. [56] classified chitosan with M_w of about 571 kDa as high MW and chitosan with M_w of about 158 kDa as low MW, meanwhile Hsu et al. [57] classified chitosan with M_w ranging from 181 kDa to 204 kDa as medium MW and from M_w 300 kDa onwards as high MW. Chitosan samples obtained in the current study from both species, with M_w around 230 kDa, may be classified as medium MW. Chitosan has higher mechanical strength and a slower degradation rate with increased viscosity average molecular weight (M_V) [58]. Still, with this increase, the viscosity of the polymer solutions also increases, and the encapsulation efficiency decreases [59]. Medium *MW* chitosan might be used in bioplastics [40]. Another important factor is the MW distribution related to molecular dispersity. For monodisperse polymers (all chains of the same length), D_m is 1. The best-controlled synthetic polymers (e.g., low molecular dispersity polymers used for calibration) have a D_m of 1.02–1.10 [60]. In our study, both chitosan samples presented high D_m values, around 10.

Chitosan, in general, shows antibacterial activity, but it varies between polymers of this group and depends on many factors, such as MW, DD, source, and the chosen target [61]. The preliminary antibacterial assessment usually concerns activity against *E. coli* and *S. aureus*, considered common, pathogenic, drug-resistant bacteria [37,38]. Chitosan, by itself, has lower activity than clinical antimicrobial drugs [28,62]. It was observed for both chitosan samples investigated in the current study that antibacterial activity was much lower than that of ampicillin. According to Zheng and Zhu [63], chitosan with higher MW inhibits bacterial growth by blocking nutrient adsorption by forming a film. The studies performed by Liu et al. [64] evidenced that when the antibacterial activity of chitosan samples was analysed with varied MW and concentration, chitosan of low MW had higher activity than chitosan of high MW. In the current study, both chitosan samples showed antibacterial activity. Chitosan from *P. leptodactylus* shells was characterised by a slightly higher M_W and higher antibacterial activity against *E. coli* and *S. aureus* than chitosan obtained from *F. limosus*.

Moreover, these results correlate with the works of Burgos-Diaz et al. [65] on chitosan with two-fold higher M_W (about 589 kDa) from *Parastacus pugnax* shells, that was characterised by much higher antibacterial activity against both *E. coli* (MBC = 0.31 mg/mL), and *S. aureus* (MBC = 0.04 mg/mL) [65]. On the other hand, the MIC for chitosan from isopod *Saduria entomon* with medium M_W 313 kDa against *S. aureus* was determined at 3.34 mg/mL, which is higher than MIC for both *P. leptodactylus* and *F. limosus* samples.

Furthermore, the activity for *S. entomon* sample against *E. coli* was not observed at all [32]. Chitosan obtained from *P. leptodactylus* shells showed stronger antibacterial activity against gram-positive bacteria than against gram-negative bacteria. The same tendency was also observed for chitosan from *S. entomon* [32], *P. pugnax* [65], and commercial chitosan with M_V 224 kDa in studies conducted by No et al. [66]. On the contrary, chitosan from *F. limosus* shells showed stronger inhibition activity against *S. aureus*, whereas the MBC values were determined at the same concentration against both strains.

Chitosan is generally characterized by biodegradability, low toxicity and bioadhesive properties, and it has been described to provide controlled release when used as matrix material of drug carriers [67,68]. In this regard, it has been used to produce nanoparticles, microparticles and films, which thus benefit from the described properties, showing great potential as drug carriers [69,70]. However, the features of each chitosan vary depending on many factors such as its source, DD and MW, and the cytotoxicity of these polymers also differs. Cytotoxicity assays are the first step in the biocompatibility assessment, and it is essential to investigate each candidate in specific conditions appropriate for the chosen application and target [30]. In this study, both chitosan samples showed time- and concentration-dependent toxicity against A549 cells. The dose-dependent cytotoxicity of chitosan observed herein was previously reported [70], attributed to the medium M_W of the tested chitosan and the related diffusional limitations. The slight time-dependent cytotoxicity was also observed for medium MW chitosan by Franca et al. [71]. In that study, a reduction in cell viability to 70% was observed for a concentration of about 1000 μg/mL. In another study [72], cytotoxicity for medium MW chitosan was observed at 250 μg/mL after 24 h of exposure to the samples, with reduced cell viability to <60%. The same study showed even stronger cytotoxicity for chitosan obtained from Mayfly and low MW chitosan. The authors suggested that chitosan's toxicity might be dependent on its source. Cytotoxicity observed for *P. leptodactylus* and *F. limosus* chitosan samples was mild when concentrations up to 250 μg/mL were tested, which could be adequate in many applications [73,74].

5. Conclusions

It is possible to obtain 22% and 20% of chitin yield from *P. leptodactylus* and *F. limosus* shells, respectively. Furthermore, it is possible to obtain chitin from *P. leptodactylus* shells with a NADES mixture composed of choline chloride and malonic acid. However, the chosen extraction conditions are too mild to separate the chitin-protein complex in *F. limosus* shells. The deacetylation of each chitin was successful, indicating that *P. leptodactylus* and *F. limosus* shells might also be used as chitosan sources. The chitosan obtained from both crustaceans revealed antimicrobial activity against *E. coli* and *S. aureus* bacteria. Chitosan from *P. leptodactylus* showed stronger antibacterial activity against gram-positive bacteria than against gram-negative bacteria. Contrarily, the MIC values for *F. limosus* chitosan were lower for gram-negative bacteria, whereas the MBC values were equal. Both chitosan samples showed cytotoxicity against A549 cells, particularly for concentrations above 250 μg/mL, with a more favourable profile for chitosan from *F. limosus* shells.

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Oświadczenia współautorów

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Autor contribution statement

I declare that my contribution in publication:

Nuc Z., Brusotti G., Catenacci L., Grenha A., Pontes J.F., Pinto da Silva J., Ana da Costa A.M., Moro P., Milanese C., Grisoli P., Sorrenti M., Dobrzycka-Krahel A., Bonferoni M.C., Caramella C.M. 2023. *Astacus leptodactylus* (Eschscholtz, 1823) and *Faxonius limosus* (Rafinesque, 1817) as new, alternative sources of chitin and chitosan. Water, 15(17), 3024. DOI: 10.3390/w15173024.

Included in my doctoral dissertation entitled: Chosen crustacean species from Polish waters as an alternative source of chitin and chitosan

Involved:

- First author, corresponding author
- Software
- Formal analysis
- Investigation
- Data curation
- Writing, editing
- Visualization

_____

Gdynia, September 18, 2025

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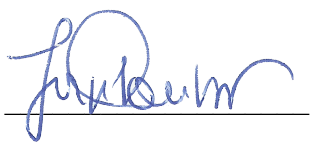

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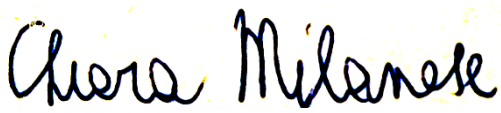

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Green extraction of chitin from two crustacean species: *Dikerogammarus villosus* (Sowinsky, 1894) and *Neomysis integer* (Leach, 1814) and characterization of chitin and chitosan obtained from them with standard method

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Abstract

Chitin is a highly demanded biopolymer, found in arthropods exoskeleton. Its properties might differ depending on the source and isolation method. Due to the negative impact of the industrially used method of extraction, it is important to explore more green methods. In current studies chitin was extracted from *Neomysis integer* (mysid) and *Dikerogammarus villosus* (gammarid). It was carried out with mixture made with malonic acid and choline chloride, and with standard method based on HCl and NaOH, for a reference. Conventionally obtained chitin was further deacetylated, in order to obtain chitosan. Obtained polymers were characterized in terms of their physicochemical properties, and antimicrobial and cytotoxic activity. Chitin yield isolated from D. villosus and N. integer with the standard method was 8 ± 0.8 and 4 ± 0.3, and chitosan was 4.8 ± 2.0 and 2 ± 1.0, respectively. Extraction with NADES did not result in a full chitin isolation. Chitosan obtained from D. villosus presented a weight average molecular weight (Mw) of 347 kDa, and chitosan from *N. integer* was characterized with Mw of 361 kDa. Both chitosan polymers showed antibacterial activity against E. coli and S. aureus, and cytotoxicity at concentrarions higher than 250 μg/mL.

Keywords: crustaceans, chitin, chitosan, Natural Deep Eutectic Solvents, physicochemical properties, antibacterial activity, cytotoxicity, A549 cells

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1. Introduction

Chitin ($C_8H_{13}O_5N$), a group of polymers made with N-acetyl-2-amino-2-deoxy-d-glucopyranose and 2-amino 2-deoxy-d-glucose by 1–4 glycosidic bonds, is found in a wide group of organisms, including crustacean, insect or fungi species [1].

It is used in supplements and biomaterials, however, due to the low solubility, it is mostly used as a chitosan source. Free amino and hydroxyl groups of chitosan enhance its solubility and biological activity. Its properties vary between species and might be affected by the process of chitin isolation and deacetylation [2].

Chitin polymers produced by crustaceans appear in complexes with proteins and minerals, therefore, its isolation consists of demineralization and deproteinization. The commercially used method, is carried out with HCl and NaOH. However, this method uses high amount of water and energy, and results in high waste production [3].Following the goals of the green chemistry, in recent years, few alternative methods have been proposed. The y are based on enzyme-assisted extraction (EAE), electrochemical extraction (ECE), microbial fermentation, ionic liquid (ILs) or natural deep eutectic solvents (NADES) [4]. NADES mixtures consist of 2 or more primary metabolites in which one of them serve as hydrogen bond acceptor (HBA), and the other as hydrogen bond donor (HBD). Each component has higher melting point than the resulting mixture[5]. Some of them, like choline chloride (ChCl) or Citric acid are Generally Recognized As Safe (GRAS) by U.S. Food and Drug Administration (FDA)[6]. ChCl is commonly used as HBA, and it was also used in current study with malonic acid as HBD. According of Zhu at all (2015)[7] this mixture has a >70% biodegradability after 28 days.

The subject of the current study was chitin isolated from two species *Dikerogammarus villosus* (Sowinsky, 1894), *Neomysis integer* (Leach, 1814). Both are crustacean species, living in brackish waters, and occur in the Baltic Sea. *D. villosus* is a nonindigenous gammarid species in this region, with Ponto-Caspian origin. It is considered an invasive species (IAS) in many settled areas, and needs to be monitored, and in case it became invasive there must be taken special measures imposed by European Union, to minimize its possible negative impact on the ecosystem. *N. integer*, on the other hand, is a commonly occurring mysid species native for Atlantic Ocean. Just as D. villosus it is a benthic species, however it mostly lives in the water column [8,9].

Chitin and chitosan properties vary between species, therefore each of them must be studied separately, to better understand, how and if it can be used. In this study we performed the chitin extraction on *D. villosus* and *N. integer* using deep eutectic solvents, compared it with the standard method, and characterized the physicochemical properties of the obtained materials.

2. Materials and Methods

2.1. Material and reagents

D. villosus and *N. integer* specimens were collected from the seashore of the Gulf of Gdansk (Gdynia, Poland), during summer 2020 and 2021. They were collected with a sieve, with a mesh size of 1 mm. Collected samples were analyzed under a binocular, and each individual was classified into the appropriate taxonomic group. All specimens were dried at 50 °C for 24 h, and then milled to powder.

Reagents used in experiments

Commercial chitin and chitosan standards that were used as control, were purchased from Sigma-Aldrich, (Milan, Italy).

Reagents used for chitin extractions and deacetylation: hydrochloride (HCl) and sodium hydroxide (NaOH) acquired from Sigma-Aldrich, (Milan, Italy), choline chloride ($(CH_3)_3N(Cl)CH_2CH_2OH$), malonic acid ($CH_2(COOH)_2$), and citric acid ($C_6H_8O_7$) purchased from Sigma-Aldrich (Milan, Italy).

Material and reagents used for antibacterial and cytotoxicity assays: Escherichia coli (ATCC 10536) and Staphylococcus aureus (ATCC 6538) strains, and human lung epithelial adenocarcinoma cells (A549) were acquired from American Type Culture Collection (ATCC, Manassas, VA, USA), Tryptone Soya Broth (TSB) and Iso-Sensitest broth (ISB) were purchased from Oxoid (Basingstoke, UK), Dulbecco's Modified Eagle's Medium (DMEM), L-glutamine, non-essential aminoacids and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Burghausen, Germany), PBS tablets pH 7.4 were acquired from VWR (Rosny-sous-Bois, France), dimethyl sulfoxide (DMSO) was acquired from Carlo Erba (Sabadell, Spain), penicillin and bovine serum (FBS) from Gibco (Life Technologies, Carlsband, CA, USA), lactate dehydrogenase (LDH) kit was provided by Takara Bio (Tokyo, Japan).

2.2. Isolation of chitin and chitosan

2.2.1. Standard isolation of chitin

Demineralization

The crustacean powder was demineralized with an aqueous solution of HCl at 3.6% (w/v), at room temperature, under magnetic stirring (30 min), at solid/liquid ratio of 1g/15 mL. It was centrifuged, the supernatant liquid was removed, and the remaining material was thoroughly washed with 25 mL of distilled water in a centrifuge (Eppendorf 5804R) at 4000 rpm (6 times for 5 min) until neutral pH was achieved. The obtained material was dried in the oven at 80 °C (48 h).

Deproteinization

The demineralized sample was treated with an aqueous solution of NaOH at 8% (w/v) at solid/liquid ratio of 1g/15 mL, at 100 °C, under stirring for 1 h. Then, it was centrifuged, the supernatant liquid was removed, and the remaining material was thoroughly washed with 25 mL of distilled water in a centrifuge at 4000 rpm (3 times for 5 min) until neutral pH was achieved, and dried in the oven at 80 °C (48 h).

2.2.2. Standard isolation of chitosan

Deacetylation

Obtained chitin was deacetylated with an aqueous solution of NaOH at 60% (w/v) which was added to the sample at solid/liquid ratio 1g/20 mL, at 180 °C under stirring for 2 h. The obtained material was centrifuged, the supernatant liquid was removed, and the remaining material was thoroughly washed with 25 mL of distilled water in a centrifuge at 4000 rpm (4 times for 5 min), until neutral pH was reached, and then it as dried in the oven at 80 °C (48 h).

2.2.4. Preliminary NADES chitin extraction

NADES mixture was prepared as described in Zhu et al. [10] paper. 8.59 g of choline chloride and 12.8 g of malonic acid were mixed together under mechanical stirring in a

two-necked round-bottom flask, and kept heated in an oil bath, at 100 °C, until the colorless, homogenous solution was formed

Extraction was performed in following variants:

a) 1.5 g of crustacean dry mass was added to the earlier prepared mixture, so it constituted 7% of the solution. It was under mechanical stirring at 80 °C for the next 2 h. Then it was filtered under vacuum and thoroughly washed with 25 mL of distilled water in a centrifuge (7 times, at 4000 rpm, for 5 min), until the neutral pH was reached. Then it was dried at the oven for 48 h at 80°C, and treated with H2O2 at 10% for another 2 h at 80 °C, and then again, washed until the sample pH was neutral (it was washed 7 times with 25 mL of distilled water in centrifuge for 5 min each, at 4000rpm), and dried in the oven at 80 °C for 48 h.

b) 1.5 g of crustacean dry mass was added to the earlier prepared mixture, so it constituted 7% of the solution. It was under mechanical stirring at 100 °C for the next 2 h. Then it was filtered under vacuum and thoroughly washed with 25 mL of distilled water in a centrifuge (7 times, at 4000 rpm, for 5 min), until the neutral pH was reached. Then it was dried at the oven for 48 h at 80°C. Afterward, it was treated with boiling water, and then again dried at the oven (80 °C, 48 h).

c) 1.5 g of crustacean dry mass pre-treated with 10 ml of C6H8O7 (10%, w/v) for 1 h at room temperature, and then added to the earlier prepared mixture at the same ratio as before. It was under mechanical stirring at 100 °C for the next 2 h. Then it was filtered under vacuum and thoroughly washed with 25 mL of distilled water in a centrifuge (7 times, at 4000 rpm, for 5 min), until the neutral pH was reached. Then it was dried at the oven for 48 h at 80°C.

Chitin and chitosan yields were calculated basing on samples weight before, and after process of extraction, and deacetylation.

2.3. Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR analysis was carried out with a Spectrum One FTIR spectrophotometer (Perkin Elmer, Wellesley, MA USA) equipped with a MIRacleTM ATR device (Pike Technologies, Madison, Wisconsin, USA) over the frequency range of 4000 - 650 cm-1 at a resolution of 4 cm-1 (64 scans). The spectra were collected at least in triplicate.

2.4. Thermogravimetric analysis (TG)

Thermogravimetric (TG) analysis was carried out with Mettler Toledo Stare System (Mettler Toledo, Milan, Italy) equipped with a TGA/DSC 1 module. To perform the analysis, about 5 mg of each sample was put in alumina crucibles with lids (scanning rate of 10 K min-1 from 30 °C to 500 °C) under a nitrogen atmosphere (flow rate 50 mL min−1). The instrument was previously calibrated with indium as a standard reference and the measurements were done at least in triplicate.

2.5. Scanning Electron Microscopy (SEM)

The surface of the obtained chitins and chitosan samples was analysed with scanning electron microscopy (SEM). The samples were sputtered with Au and examined using an EVO MA10 instrument. The measurements were performed under ultra-high vacuum with an electron generation voltage of 5 kV and a working distance of 8.5 mm.

2.6. Molar mass distribution

The molar mass distribution of the obtained polymers was estimated by High-Performance Size Exclusion Chromatography (HPSEC), and carried out in a modular system comprised of a Biotech Degasi GPC degasser, a Waters 515 HPLC pump, and a Knauer K-2300 refractive index detector. Two Shodex columns OHpak SB-806M HQ of 300 × 8.0 mm were used in series with a 50 × 6.0 mm OHpak SB-G 6B guard column.

A 0.2 M $NaNO_3$ + 0.5 M CH_3COOH aqueous solution (0.02% NaN_3) was used as a mobile phase at, 1 mL/min. Chitosan samples were dissolved in the eluent at 5 mg/mL

overnight, centrifuged at 4000 rpm for 10 min, and filtered through 0.45 μm filters. A conventional calibration curve was set using polystyrene sulfonate standards dissolved at 5 mg/mL in the former eluent.

2.7. Antimicrobial assays

2.7.1. Material preparation

Chitosan samples extracted from *D. villosus* and *N. integer* shells was treated with 3,6% HCl and distilled water in ratio 1 g/6.2 mL/100 mL. Each solution was put into an earlier prepared dialysis membrane, which was and in left in distilled water, under magnetic stirring, at a room temperature, for about 24 h.

2.7.2. Bacteria culture

Escherichia coli (ATCC 10536) and Staphylococcus aureus (ATCC 6538) strains were cultured in TSB at 37 °C. The bacteria cultures were centrifuged at 3000 rpm for 20 min to separate cells from broth and then suspended in PBS (pH 7.3). The suspension was diluted to adjust the number of cells to 1 × 107–1 ×108 CFU/mL.

2.7.2. Evaluation of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

The antimicrobial activity of the obtained chitosan samples was tested against *E. coli* (ATCC 10536) and *S. aureus* (ATCC 6538). The density of the used suspension was 1 × 107 – 1 × 108 CFU/mL. All the extracts were dissolved in 10% DMSO aqueous solution at different concentrations. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined by two-fold serial broth dilution method in ISB according to Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) procedures [11]. The starting inoculum was 1.0 × 107 CFU/mL. Concentrations of extracts were tested in the range 0.1 – 9.2 mg/mL. The MIC was the lowest extract concentration inhibiting observable microbial growth after 24 h incubation at 37 °C. The MBC was the lowest concentration resulting in >99.9% reduction of the initial inoculum after 24 h of incubation at 37 °C. All experiments were performed in triplicate. Solvent blanks were included. The positive control was stock standard solutions of ampicillin.

2.8. Cytotoxicity assay (MTT)

2.8.1. Cell Culture

Human lung epithelial adenocarcinoma cells (A549) were used in passages 84-94. They were cultured at 37 °C in an incubator with 5% CO_2/95% humidified atmospheric air, in flasks (75 cm^2). The CCM used for this cell line (DMEM) was supplemented with non-essential amino acids 1% (v/v), L-glutamine at 1% (v/v), FBS at 10% (v/v), and penicillin/streptomycin at 1% (v/v), and it was exchanged twice a week.

2.8.2. Assessment of metabolic activity

The effect of chitosan samples on A549 cells metabolic activity was evaluated with MTT assays, according to indications of International Organization for Standardization [12]. Chitosan samples were solubilized in acetic acid (1%, v/v) at concentration 4 mg/mL, and dispersed in CCM at concentrations ranging between 15.62 and 1000 μg/mL. The A549 cells were seeded in 96-well plates at 1x104 cells/well the day before the assay. They were incubated overnight, and after, the CCM was replaced with chitosan samples. CCM was used as the positive control, and the SDS solution at 2% (w/v) was used as a negative control. After the time of exposure (3, 24, and 48 h) passed, chitosan samples were replaced with 30 μL of MTT solution (5 mg/mL in PBS, pH 7.4). After 2 h of incubation at 37 ºC, in order to solubilize the formed formazon, 50 μL of DMSO was added to each well. The absorbance was read by spectrophotometry (Infinite M200; Tecan, Grödig, Austria) at 540 nm, with background correction at 640 nm. All experiments were performed in triplicate. The cell viability was calculated from the equation:

$$\text{Cell viability}(\%)=(A-D)/(CM-D)\times 100$$

Where A is an absorbance obtained upon exposure for each sample, D is an absorbance measured for DMSO, and CM is an absorbance read for the cells incubated in CCM. The half maximal inhibitory concentration (IC_{50}) was calculated for samples with cell viability reaching less than 50%. IC_{50} values were determined by sigmoidal fitting of the data in the GraphPad Prism statistical program (GraphPad Software, Version 6.01, USA).

2.9. Statistical analysis

The results were analysed by two-way analysis of variance (ANOVA). The used software was R, and differences were considered significant at a level of $p < 0.05$.

3. Results

This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn.

3.1. Chitin and chitosan content

3.1.1. Chitin and chitosan obtained using standard chemical procedures

The media of chitin yield obtained with standard method, from *D. villosus* was 8 ± 0.8 % (n=4) and for *N. integer* was 4 ± 0.3 % (w/w)(n=4) respectively (Table 1). The media of chitosan yield obtained from chitin was 63 ± 13.0 % (n=3) and 58 ± 9.0 % (w/w)(n=3) respectively (Table 1).

3.1.2. Preliminary extraction with NADES mixture

In current study, all approaches to chitin extraction with NADES mixture from D. villosus and *N. integer* resulted in incomplete isolation. Each of them was performed singularly. The first approach of chitin extraction with NADES mixture resulted in Extraction with NADES performed on *D. villosus* and *N. integer* resulted in incomplete chitin isolation. The further FTIR analysis showed remains of protein. The extraction with citric acid pre-treatment resulted in higher chitin yield from *D. villosus*, while for *N. integer* shells the yield of the obtained material was lower (about 25 %). However, no changes in further analysis were observed.

Table 1. Chitin and chitosan yield [%] in shells of *D. villosus* and *N. integer* obtained with a-standard method, b-NADES and H2O2 aftertreatment, c-NADES with increased temperature during extraction, and boiling water aftertreatment, d- NADES with citric acid pre-treatment.

Species	Treatment	Chitin yield [%]	Chitosan yield from chitin [%]	Chitosan yield from dry mass [%]
D. villosus [a]	standard	8.0 ± 0.8	63.0± 13.0	4.8 ± 2.0
D. villosus [b]	NADES	21	-	-
D. villosus [c]	NADES	27	-	-
D. villosus [d]	NADES	19	-	-
N. integer [a]	standard	4.0 ± 0.3	58.0 ± 9.0	2.0 ± 1.0
N. integer [b]	NADES	24	-	-
N. integer [c]	NADES	26	-	-

3.2. Fourier Transform Infrared Spectroscopy (FTIR)

3.2.1. Chitin and chitosan obtained using standard extraction characterization

Recorded FTIR spectra of *D. villosus* and *N. integer* samples corresponded to the FTIR spectrum of chitin used as a reference material (Figure 1, a) The reference material was characterized with amide I band at 1622 cm^{-1}, amide II at 1551 cm^{-1}, -C-H stretching at 2875 cm^{-1}, and -C-O-C- stretching at 1070 cm^{-1} and at 1028 cm^{-1}. The material obtained with standard extraction method, from *D. villosus* (Figure 1, b)) was characterized with an amide I band at 1643 cm^{-1}, amide II at 1643 cm^{-1}, -C-H stretching at 2874 cm^{-1}, and -C-O-C-

stretching at 1062 cm^{-1} and at 1028 cm^{-1}. The *N. integer* sample (spectrum…) was characterized with an amide I band at 1647 cm^{-1}, amide II at 1554 cm^{-1}, -C-H stretching at 2875 cm^{-1}, and -C-O-C- stretching at 1060 cm^{-1} and at 1028 cm^{-1}.

Similarly, for the material obtained with NADES from *D. villosus* (Figure 1, c) and *N. integer* (Figure 1., d)), the amide I band was observed at 1634 cm-1 and 1626 cm-1, the amide II at 1538 cm-1 and 1536 cm-1, -C-H stretching at 2916 cm-1 and 2920 cm-1, and -C-O-C- stretching at 1070 cm-1 and 1026 cm-1, and 1068 cm-1 and 1028 cm-1 respectively. FTIR spectra for both samples extracted with NADES mixture indicated incomplete extraction.

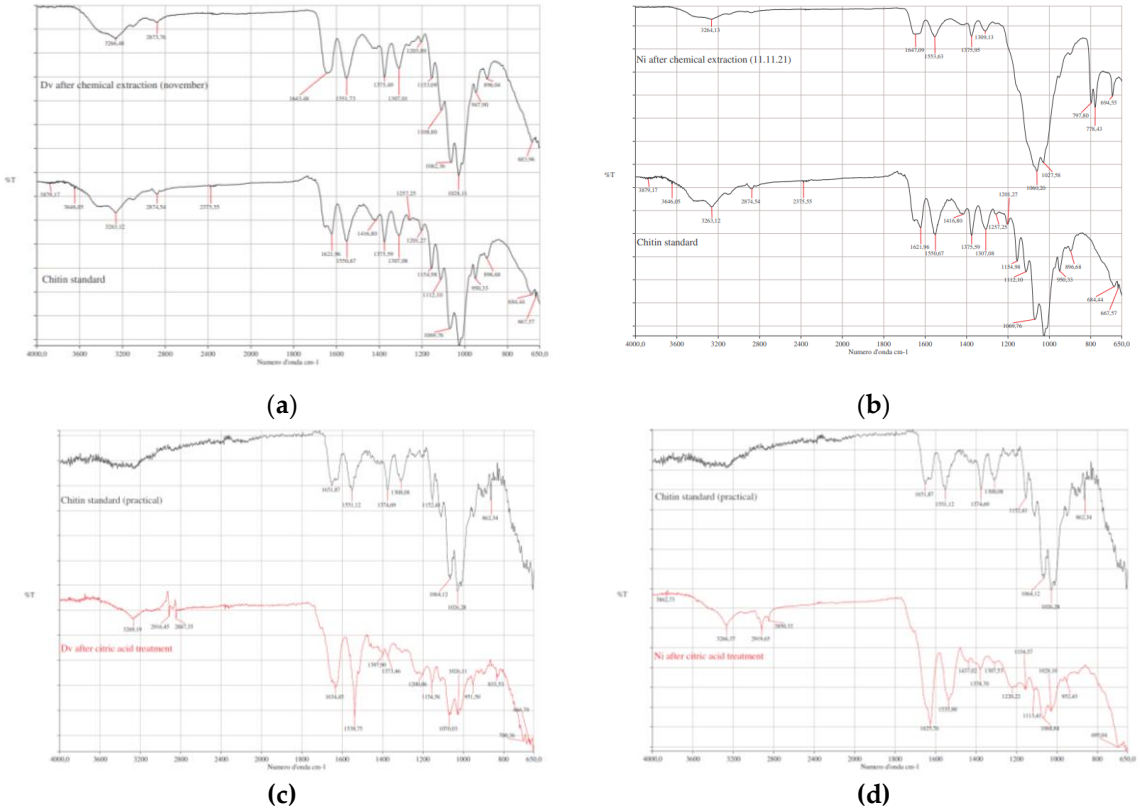
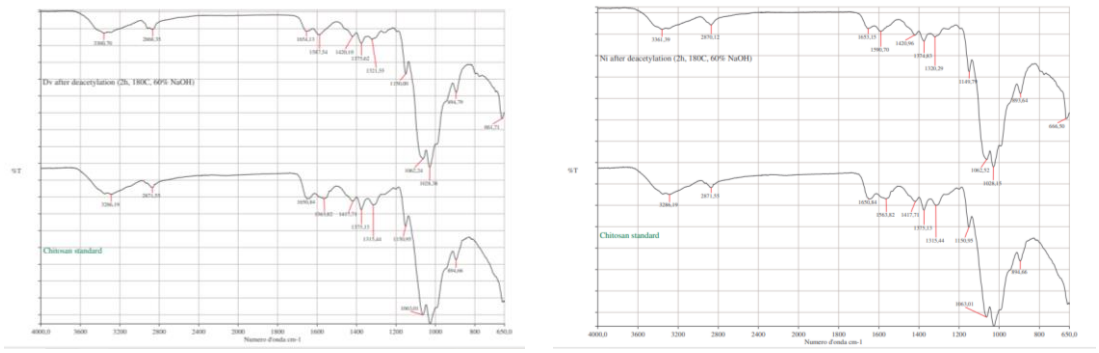


Figure 1. FTIR spectra of chitin isolated from *D. villosus* and *N. integer* with standard extraction (a and b)and NADES (c and d).

As it is shown at the Figure 2, the recorded FTIR spectra for both deacetylated samples were similar to chitosan standard, differing in adsorption intensity and wavenumbers. Chitosan standard was characterized by amide I band at 1650cm^{-1}, amide II at 1562cm-1, -C-H stretching at 2872 cm^{-1} and -C-O-C- stretching at 1063 cm-1 and 1028 cm-1. The same bands and stretchings were observed for *D. villosus* (Figure 1., a) samples at 1654 cm^{-1}, 1588 cm^{-1}, 28768 cm^{-1}, 1062 and 1028, respectively, and *N. integer* (Figure 1., b) samples were characterized by amide I bands at 1653 cm^{-1}, amide II at 1591 cm^{-1} -C-H stretching at 2870 cm^{-1} and -C-O-C- stretching at 1063 cm^{-1} and 1028 cm^{-1}.



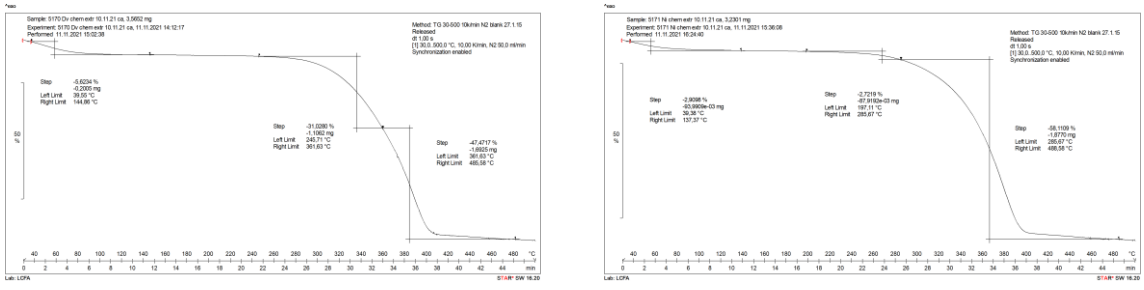
(**a**) (**b**)

Figure 2. FTIR spectra of chitosan samples obtained from *D. villosus* (a), and *N. integer* (b)compared to a reference material.

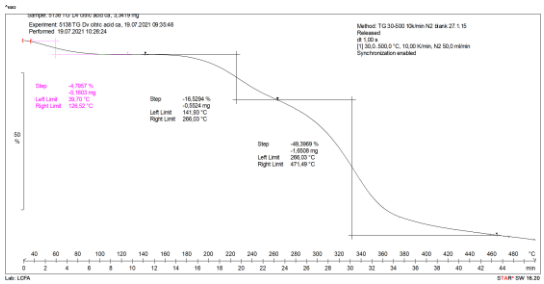
Thermogravimetric Analysis (TG) of Chitin and Chitosan Obtained Using Standard Extraction Characterisation

Results obtained from thermogravimetric analysis of the material obtained from *D. villosus* and *N. integer* correspond to TGA curve of chitin reference material (Figure 3). According to their thermal profiles, the process of the samples thermal degradation might be divided into two main steps. The first one corresponds to the water evaporation and the second to the chitin degradation. For the chitin sample obtained from *D. villosus* (Figure 3, a) water content was about 5.6%, and its evaporation lasted until 145 °C , for *N. integer* (Figure 3., b) sample it lasted until 137 °C, and the mass loss was 2.9%. The mass loss of the *D. villosus* sample during the second step was 78.5 % and started at 246 °C, meanwhile for the *N. integer* sample it started at 197 °C with a mass loss of 60.4%.

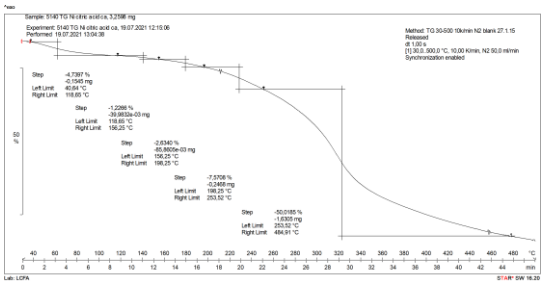
Samples obtained with NADES corresponded to the reference material (Figure 3). The dehydration of *D. villosus* sample (Figure 3., c) lasted until 126 °C and resulted in mass loss of 4.8%. For *N. integer* (Figure 3.,d) sample this step lasted until 118 °C and the mass loss was 4.8%. The *D. villosus* sample degradation started at 141 °C and with a mass loss of 65.9 %, and for *N. integer* at 118 °C with a mass loss of 61.4 %. The first step of mass loss for the chitin reference material (Figure 3., e) lasted until around 153 °C. Water constituted around 7.7% of the sample. The second step corresponding to the sample degradation was observed around 164 °C with mass loss of 75.0%.



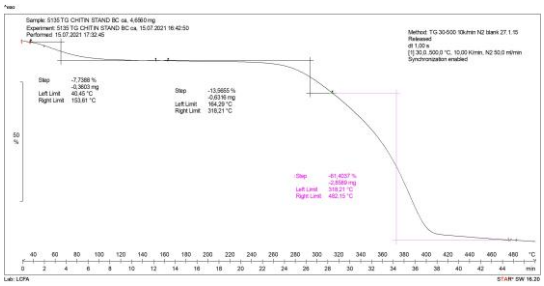
(**a**) (**b**)



(c)



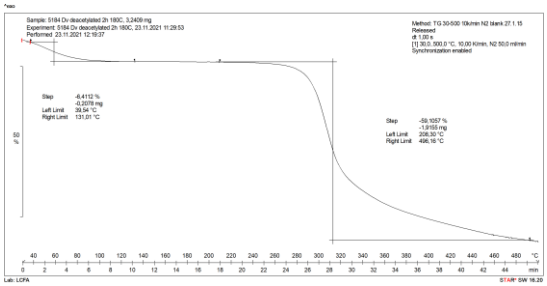
(d)



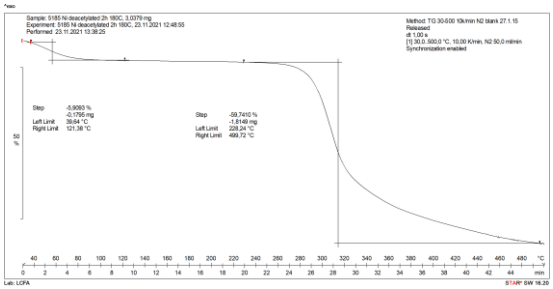
(e)

Figure 3. TGA profile of chitin from *D. villosus* (a) and *N. integer* (b) obtained with conventional method, and from *D. villosus* (c) *N. integer* (d) with NADES mixture with a reference material (e).

As it is shown on the Figure 4., chitosan samples from *D. villosus* (a) and *N. integer* (b) decomposed in two steps, similarly to the chitin. The dehydratation of *D. villosus* sample lasted until 131 °C with a mass loss of 6.4 %, and for *N. integer* until 121 °C with a mass loss of 5.1 %. The second step for *D. villosus* and *N. integer* sample it was observed at 208 °C and 228 °C respectively. The mass loss for *D. villosus* sample was 59.1 % and for *N. integer* sample was 59.7 %.



(a)

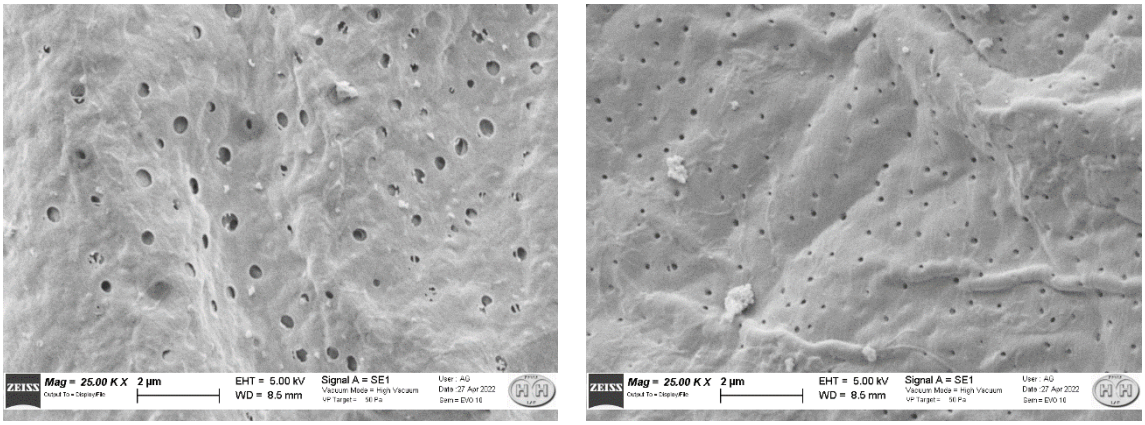


(b)

Figure 4. TGA profile of chitosan obtained from *D. villosus* (a) and *N.integer* (b) and of the chitosan reference material(c).

SEM

The microstructure of both *D. villosus* (a) and *N. integer* (b) chitin polymers was smooth with little, rather regular pores (Figure 5.).



(**a**) (**b**)

Figure 5. SEM pictures of chitin obtained from a) *D. villosus* and b) *N.integer*.

Molecular Weight (MW)

The molecular weight distribution of the obtained chitosan samples determined with HPSEC is shown in the Figure 6. Chitosan obtained from *D. villosus* presented a numer average molecular weight (Mn) of 36 kDa, a weight average molecular weight (Mw) of 347 kDa, and molecular dispersity (Dm) of 9.73. Chitosan from *N. integer* was characterized with Mn of 13 kDa, Mw of 361 kDa and Dm of 27.

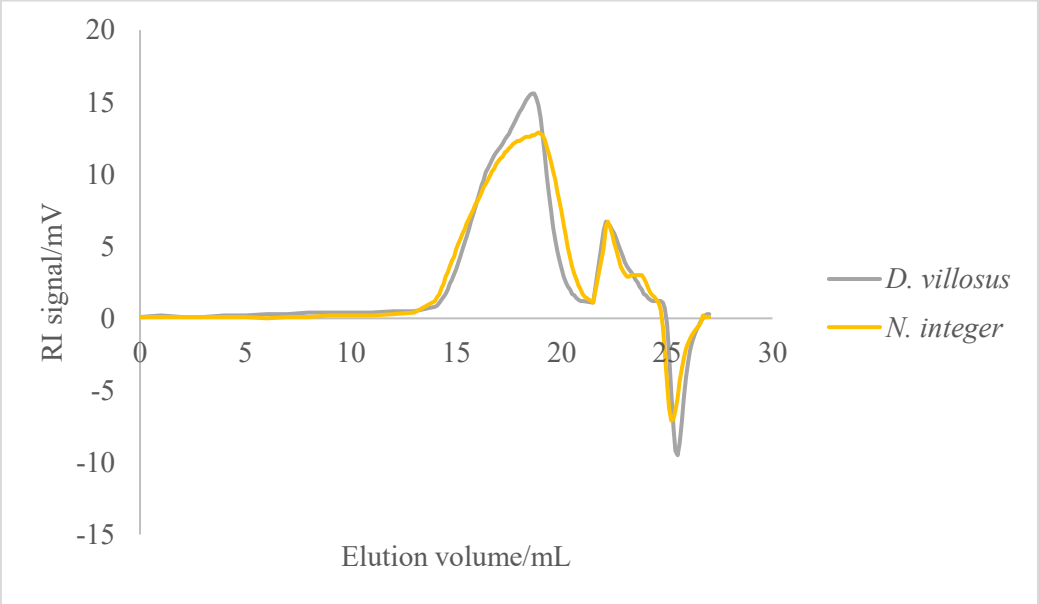


Figure 6. Size exclusion chromatography elusion profiles of *D. villosus* and *N. integer* chitosan samples.

Antibacterial activity

Chitosan obtained from *D. villosus* showed stronger antibacterial activity against *E. coli* and *S. aureus* than chitosan used as a reference material. The activity was stronger towards *E. coli*. The *D. villosus* chitosan sample showed much weaker antibacterial activity in comparison to ampicillin (Table 2).

Table 2. The minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations [mg/mL] of chitosan was obtained from D. villosus and of ampicillin against E. coli and S. aureus.

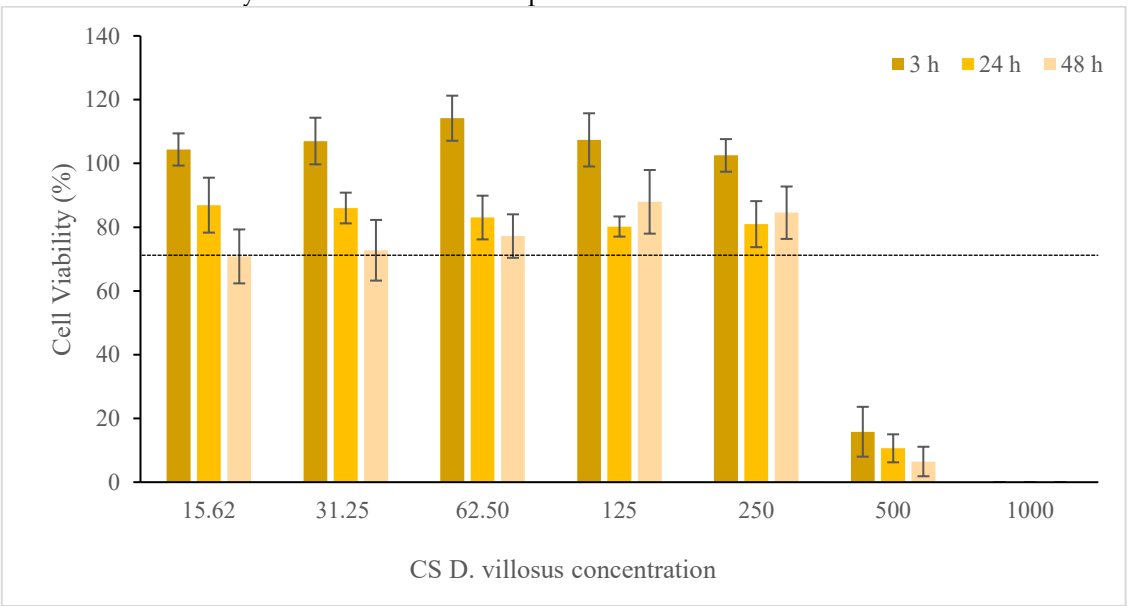
Species	**MIC**	**MIC**	**MBC**	**MBC**

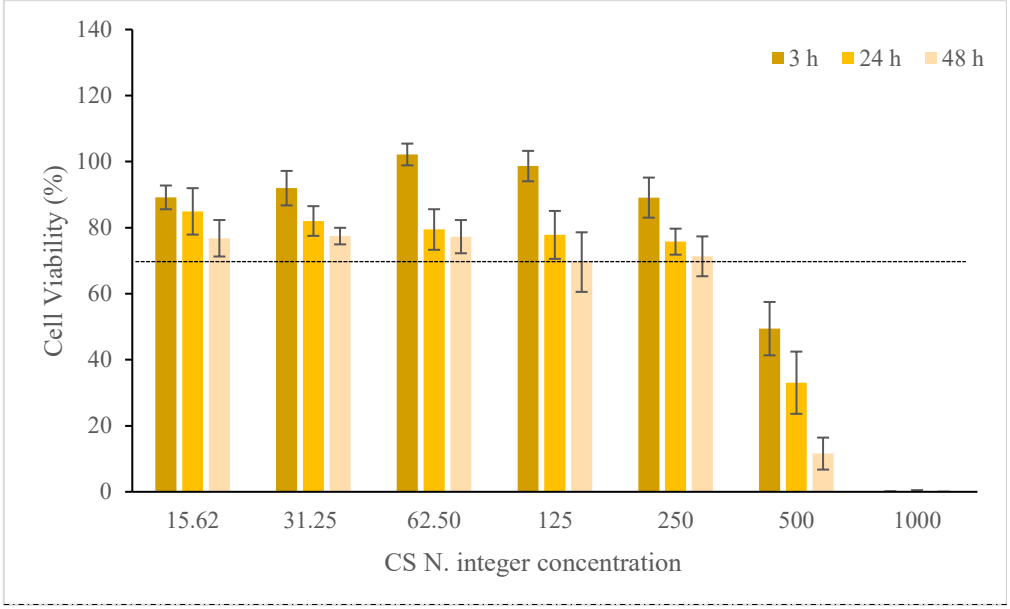
	(*E. coli*)	(*S. aureus*)	(*E. coli*)	(*S. aureus*)
D. villosus	0.10	0.10	0.42	0.84
Chitosan*	1.15	2.30	>9.20	>9.20
Ampicilin	5 x 10-3	0.5 x 10-3	10 x 10-3	2 x 10-3

Determination of cytotoxicity

In order to determine cytotoxicity of both, *N. integer* and *D. villosus* chitosan samples, cell viability was assessed. It was determined based on chitosan samples the effect on metabolic activity of human epithelial cells. Each of them was tested at 7 different concentrations, up to 1000 μg / mL, and compared with control. According to ISO 10993 [11], cell viability values below 70% of the control indicate a cytotoxic effect of the tested agent. Cytotoxic effect was observed at the concentration of 500 μg/mL for both chitosan samples under study ($p<0.05$), with cell viability ranging from 5% to 50%, depending on exposure time (Figure x). At all tested concentrations of *N. integer* chitosan time-dependent effect was seen ($p<0.05$). Meanwhile for *D. villosus* sample, at concentrations 125 μg / mL and 250 μg / mL cell viability values were lower for 24 h and 48 h than 3 h of exposure, however, values for time of exposure 48 h were slightly higher than for 24 h. The concentration of 1000 μg / mL was observed to be massively cytotoxic for both chitosan samples. There is a possibility that acetic acid, used to prepare the initial stock contributed to the cytotoxicity. However, the control prepared to test its effect on the epithelial cells showed cytotoxicity only at higher concentrations. Therefore it was assumed that the acetic acid effect was minor.

The half-maximal inhibitory concentration (IC_{50}) calculated for both chitosan samples is shown in a Table 3. As expected, for both samples, IC_{50} value decreased with the time of exposure. However, for *D. villosus* chitosan sample IC_{50} value was same for 24 h and 48 h of exposure time. Meanwhile for *N. integer* chitosan sample IC_{50} value decrease gradually with the increase of exposure time.





(b)

Figure 7. SEM pictures of chitin obtained from a) *D. villosus* and b) *N.integer*.

Table 3. IC_{50} values [mg/mL] for chitosan samples obtained from *D. villosus* and *N. integer* for epithelial cells A549, after 3 h, 24 h and 48 h of incubation.

Species	**IC_{50}(3h)**	**IC_{50}(24h)**	**IC_{50}(48h)**
D. villosus	0.46	0.33	0.33
N. integer	0.53	0.32	0.22

4. Discussion

In gammarid species chitin might constitute about 6.6-7.8% of their mass [13], and in current study the obtained chitin yield from D. villosus species fit within this range. As for mysid species this range is 0.48-7.0% , and chitin yield obtained from N. integer was within this range [14, 15]. The obtention was possible with the conventional method of chitin extraction. The NADES system composed with choline chloride and malonic acid wasn't efficient enough to separate completely chitin from proteins. Temperature change, hot water treatment and citric acid pretreatment didn't improve the extraction. The effectiveness of NADES systems depends on their molar ratio, viscosity, temperature, pH and ratio NADES:material [16-18]. Perhaps, changing some of these factors could improve results.

Deacetylation of D. villosus and N. integer chitin resulted in obtention of high Mw chitosan [19], with high polydispersity index, indicating low stability of the obtained polymers [20]. These features are important in understanding the biological activity of the obtained polymers. Both chitosan samples showed higher MIC and MBC activity greatest than standard chitosan used as a reference towards E. coli and S. aureus bacteria. These strains are often used as models for Gram-negative and Gram-positive bacteria, respectively. They are considered drug- resistant bacteria [21,22], therefore it is important to search for new antibacterial agents , or for agents that can enhance antibacterial activity of other antibiotics. Chitosan is known for its antibacterial activity, however this activity varies depending on its DD and Mw (Zheng I in., 2003).

The cytotoxicity of the obtained chitosan samples was time dependent. The results showed, that it could be used in concentrations lower than 250 ug/mL in further steps of the biocompatibility assessment [24].

The microstructure of biopolymers may differ regarding the source. In this case, the microstructure of both D. villosus and N. integer chitin polymers was smooth with little, rather regular pores. In tissue engineering, chitosan is considered an attractive biopolymer, because of the microfibers from which suitable structures can be created. The porosity of the material has advantages, as it allows for the proliferation of tissue cells as well as the transport of nutrients [25](Sikora et al., 2021).

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Abbreviations

The following abbreviations are used in this manuscript:

FTIR	fourier – transform infrated spectroscopy
HBA	hydrogen bond acceptor
HBD	hydrogen bond donor
IAS	invasive alien species
IC	inhibitory concentration
MBC	minimum bactericidal concentration
MIC	minimum inhibitory concentration
Mw	molecular weight
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolinum bromide)
NADES	natural deep eutectic solvents
SEM	scanning electron microscopy
TG	thermogravimetric analysis
FTIR	fourier – transform infrated spectroscopy

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Autor contribution statement

I declare that my contribution in publication:

Nuc Z., Brusotti G., Catenacci L., Grenha A., Pontes J.F., Pinto da Silva J., Ana da Costa A.M., Moro P., Milanese C., Grisoli P., Sorrenti M., Dobrzycka-Krahel A., Bonferoni M.C., Caramella C.M. 2023. Astacus leptodactylus (Eschscholtz, 1823) and *Faxonius limosus* (Rafinesque, 1817) as new, alternative sources of chitin and chitosan. Water, 15(17), 3024. DOI: 10.3390/w15173024.

Included in my doctoral dissertation entitled: Chosen crustacean species from Polish waters as an alternative source of chitin and chitosan

Involved:

- First author, corresponding author
- Software
- Formal analysis
- Investigation
- Data curation
- Writing, editing
- Visualization

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Gdynia, September 18, 2025

dr hab. Aldona Dobrzycka-Krahel, prof. UWSB Merito

WSB Merito University,

Business Faculty

Autor contribution statement

I declare that my contribution in publication:

Nuc Z., Brusotti G., Catenacci L., Grenha A., Pontes J.F., Pinto da Silva J., Ana da Costa A.M., Moro P., Milanese C., Grisoli P., Sorrenti M., **Dobrzycka-Krahel A.,** Bonferoni M.C., Caramella C.M. Green extraction of chitin from two crustacean species: *Dikerogammarus villosus* (Sowinsky, 1894) and *Neomysis integer* (Leach, 1814) and characterization of chitin and chitosan obtained from them with standard method

Included in doctoral dissertation by Zofia Nuc entitled: Chosen crustacean species from Polish waters as an alternative source of chitin and chitosan

Involved:

- Conceptualization
- Validation
- Writing, review, editing
- Supervision
- Project administration

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Autor contribution statement

I declare that my contribution in publication:

Nuc Z., Brusotti G., Catenacci L., **Grenha A**., Pontes J.F., Pinto da Silva J., Ana da Costa A.M., Moro P., Milanese C., Grisoli P., Sorrenti M., Dobrzycka-Krahel A., Bonferoni M.C., Caramella C.M. *Dikerogammarus villosus* (Sowinsky, 1894) and *Neomysis integer* (Leach, 1814) and characterization of chitin and chitosan obtained from them with standard method

Included in the doctoral dissertation by Zofia Nuc entitled: Chosen crustacean species from Polish waters as an alternative source of chitin and chitosan

Involved:

- Conceptualization
- Methodology
- Validation
- Investigation
- Writing, review and editing
- Supervision

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Nuc Z., Brusotti G., Catenacci L., Grenha A., **Pontes J.F.,** Pinto da Silva J., Ana da Costa A.M., Moro P., Milanese C., Grisoli P., Sorrenti M., Dobrzycka-Krahel A., Bonferoni M.C., Caramella C.M. *Dikerogammarus villosus* (Sowinsky, 1894) and *Neomysis integer* (Leach, 1814) and characterization of chitin and chitosan obtained from them with standard method

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Involved:

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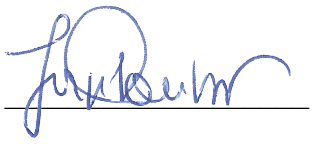

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Autor contribution statement

I declare that my contribution in publication:

Nuc Z., Brusotti G., Catenacci L., Grenha A., Pontes J.F., **Pinto da Silva J.,** Ana da Costa A.M., Moro P., Milanese C., Grisoli P., Sorrenti M., Dobrzycka-Krahel A., Bonferoni M.C., Caramella C.M. *Dikerogammarus villosus* (Sowinsky, 1894) and *Neomysis integer* (Leach, 1814) and characterization of chitin and chitosan obtained from them with standard method

Included in my doctoral dissertation by Zofia Nuc entitled: Chosen crustacean species from Polish waters as an alternative source of chitin and chitosan

Involved:

- Software
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- Writing, review and editing

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Autor contribution statement

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Nuc Z., Brusotti G., Catenacci L., Grenha A., Pontes J.F., Pinto da Silva J., **Ana da Costa A.M.,** Moro P., Milanese C., Grisoli P., Sorrenti M., Dobrzycka-Krahel A., Bonferoni M.C., Caramella C.M. *Dikerogammarus villosus* (Sowinsky, 1894) and *Neomysis integer* (Leach, 1814) and characterization of chitin and chitosan obtained from them with standard method

Included in the doctoral dissertation by Zofia Nuc entitled: Chosen crustacean species from Polish waters as an alternative source of chitin and chitosan

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- Methodology
- Validation
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Pavia, September 18, 2025

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Autor contribution statement

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Nuc Z., Brusotti G., Catenacci L., Grenha A., Pontes J.F., Pinto da Silva J., Ana da Costa A.M., Moro P., **Milanese C.,** Grisoli P., Sorrenti M., Dobrzycka-Krahel A., Bonferoni M.C., Caramella C.M. *Dikerogammarus villosus* (Sowinsky, 1894) and *Neomysis integer* (Leach, 1814) and characterization of chitin and chitosan obtained from them with standard method

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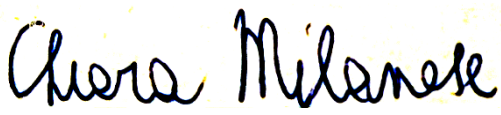

Pavia, September 18, 2025

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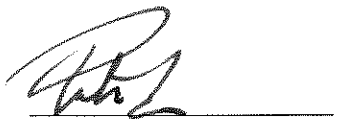

Author's academic resume

Education

- **PhD studies (2019-2025)**

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Doctoral School of Natural Science (DSNS)

- **MS studies (2017-2019)**

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- **BC studies (2014-2017)**

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Publications

(2023) **Nuc Z.,** Brusotti G., Catenacci L., Grenha A., Pontes J.F., Pinto da Silva J., Ana da Costa A.M., Moro P., Milanese C., Grisoli P., Sorrenti M., Dobrzycka-Krahel A., Bonferoni M.C., Caramella C.M. 2023. Astacus leptodactylus (Eschscholtz, 1823) and Faxonius limosus (Rafinesque, 1817) as new, alternative sources of chitin and chitosan. Water, 15(17), 3024

(2023) Dobrzycka-Krahel, A. Stepien, C. A., **Nuc Z.** Neocosmopolitan distributions of invertebrate aquatic invasive species due to euryhaline geographic history and human-mediated dispersal: Ponto-Caspian versus other geographic origins. Ecological Processes, 12(1), 1-16.

(2021) **Nuc Z.**, Dobrzycka-Krahel A. From chitin to chitosan–a potential natural antimicrobial agent. Progress on Chemistry and Application of Chitin and its Derivatives, 26, 23-40.

Interships

05 - 07.2021 "Chitin extraction and characterization of the gammarid species *Dikerogammarus villosus*" at the Universita di Pavia, in Italy.

10 - 12.2021 „Deacetylation of the chitin from *P. leptodactylus, F. limosus, N. integer, and D. villosus* using green methods and its characterisation" at the Universita di Pavia, in Italy.

01 - 03.2023 "Biocompatibility of chitosan obtained from selected crustaceans from Poland" at Centro de Ciencias do Mar do Algarve (CCMAR), in Portugal.

Conferences

- Oral presentation:

(2023) **Nuc Z.**, Brusotti G., Moro P., Grenha A., Pontes J. F., Pinto da Silva J., Rosa da Costa A.M., Catenacci L., Milanese C., Grisoli P.,Sorrenti M., Dobrzycka-Krahel A., Bonferoni .C., Caramella C., Crustaceans from polish waters as alternative sources of chitin and chitosan. The 14th International Conference of the European Chitin Society (EUCHIS 2023), Siglufjörður, Islandia

- Poster presentation:

(2022) **Nuc Z.**, Brusotti G., Moro P., Catenacci L., Milanese C., Sorrenti M., Dobrzycka-Krahel A., Bonferoni M.C., Caramella C.M. Faxonius limosus (Rafinesque, 1817) as an alternative source of chitin and chitosan. VII Simposio Científico de Alumnos de la Facultad de Ciencias del Mar y Ambientales, Kadyks, Hiszpania

(2022) **Nuc Z.,** Brusotti G., Moro P., Catenacci L., Milanese C., Sorrenti M., Dobrzycka-Krahel A., Bonferoni M.C., Caramella C.M. Astacus leptodactylus (Eschscholtz, 1823) in circular economy. XXVII Conference of Polish Chitin Society "New aspects on chemistry and application of chitin and its derivatives", Poznań, Polska

(2021) Błaszczyk A., **Nuc Z.** *Anticoagulant activity of Baltic cyanobacteria belonging to Nostocales order – preliminary study.* 39th International Conference of the Polish Phycological Society, Gdynia i Łeba, Polska (poster)