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Research Article

Bioactive compounds in mucus and shell microstructure of the European ear snail (*Radix auricularia*)

Kutluyer Kocabaş F.[1*], Kocabaş M.[2], Kizak V.[1], Korkmaz B.[3]

1Munzur University, Fisheries Faculty, 62000, Tunceli, Türkiye
2Karadeniz Technical University Faculty of Forestry, Department of Wildlife Ecology and Management 61080, Trabzon, Türkiye
3Department of Pharmacognosy, Faculty of Pharmacy, Karadeniz Technical University, 61100, Trabzon, Türkiye
*Correspondence: mkocabas@hotmail.com; mkocabas@ktu.edu.tr

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Abstract

Radix auricularia is a non-native and invasive freshwater snail species and it may have destructive impacts on biodiversity and ecosystem structure. In recent years, snail mucus, as a sticky substance, has garnered significant attention in the medical and cosmetic industries due to its content of numerous bioactive compounds. Despite frequently being discarded as waste due to the build-up of calcium carbonate crystals through biomineralization, shells of freshwater snail serve multiple functions in aquatic environments and have potential industrial applications. Hence, this study aims to identify bioactive compounds in the mucus and analyze the shell microstructure and mineralogy of the European ear snail (*Radix auricularia*). Bioactive constituents of mucus were characterized by Gas Chromatography-Mass Spectrometry (GC-FID-MS) analysis. We also conducted analyses on the organic matrix of shells from *R. auricularia* using X-ray diffraction (XRD) and Scanning Electron Microscope-Energy Dispersive Spectroscopy (SEM-EDS). Our results indicated that ten compounds are characterized by Eicosane (34.16%), 9-Octadecen-1-ol, (Z)- (11.86%) and *N,N*-Dimetylpalmitamide (5.55%) as the main components of mucus. The antioxidant potential, antifungal and anti-inflammatory properties of Eicosane was in notable quantity in the mucus. In addition, the elemental composition of shell powder comprised of carbon, aluminium, oxygen, calcium, iron, magnesium, silicium, manganese, sodium, sulphur and phosphorus. Aragonite emerged as the dominant mineralogy in the inorganic layer of their shells, as confirmed by XRD. Overall, individual compounds of mucus could be isolated and purified mucus compounds could have applications in pharmaceuticals and health sectors. The shells of *R. auricularia* offer potential for diverse applications and contribute to environmental sustainability as valuable biomaterials.

Introduction

The mucus produced by the glands in the footplate, enveloping the entire exterior of the organism, serves to minimize friction, safeguard the snail's foot during movement, retain moisture, and facilitate hunting and mating activities, arraying metabolic homeostasis (Richter, 1980; Ballance *et al.*, 2004; Phrompanya *et al.*, 2023; Deng *et al.*, 2023; Liegertova and Malý, 2023). Mucus has received great attention due to its usage in pain relief and treatment of various injuries and diseases, wound healing, aging, inflammation, tumor since ancient times (Rashad *et al.*, 2023). Moreover, mucus with a cosmetic ingredient has been applied in the cosmetic and skincare industry due to its multifaceted properties (Rizzi *et al.,* 2021). On the other hand, early studies on mucus characterization involved in gastropod species about extraction, structure, biochemical composition, enzymatic activities, beneficial and therapeutic qualities (Nantarat *et al.,* 2019; Phrompanya *et al.,* 2023). Even so, research on the mucus composition of freshwater snails remains inadequate. Shells of mollusks consist of calcium carbonate in different forms (crystal, calcite, and aragonite, vaterite) and organic components and primarily proteins (Chakraborty *et al.,* 2020; Yarra *et al*., 2021; Kutluyer Kocabaş and Kocabaş, 2024). To the best of our knowledge, the microstructure and composition of shells in *R. auricularia* have not yet been documented. According to the briefly mentioned reasons, the current study aimed to identify active ingredients in *R. auricularia* mucus by GC-FID-MS analysis and to assess the elemental composition and microstructure of shells by Energy Dispersive X-ray spectroscopy coupled to Scanning Electron Microscopy (SEM-EDX) and XRD**.**

Material and methods

Study species and mucus collection

R. auricularia (n: 20) was collected from Demirköprü Dam Lake (Manisa, Türkiye) using rakes for raking, metal-framed scoops, and shovels, and transferred to the laboratory. They were adapted to culture medium in 20-liter glass aquariums within a temperature-controlled room set at 20±1°C, under a 16:8 hour light-dark photoperiod. In order to prevent contamination, the snails were deprived of food for three days prior to mucus collection. Mucus was extracted from snails' bodies using a spray stimulation method with water jets (3% NaCl) (Jayashankar *et al*., 2014), and the mucus secretion from individuals was collected and stored at 4°C.

Analysis of mucus by GC-FID-MS

Mucus of *R. auricularia* (125 mg each) were extracted (x3 times) with HPLC grade *n*-hexane (1 mL, each) at room temperature to avoid contaminants that could interfere with the results. The initial n-hexane solutions underwent filtration using a 0.45 µm filter to remove particulate matter and were then concentrated under ambient atmospheric conditions. The analysis of *n*-hexane extracts of mucus was conducted using a Shimadzu QP2010 ultra-GC-FID/MS and a Shimadzu 2010 plus FID, equipped with a PAL AOC-5000 plus autosampler and Shimadzu Class-5000 Chromatography Workstation software. The separation was carried out using a

Restek Rxi-5MS capillary column (30 mm × 0.25 mm × 0.25 μm) (USA). The GC-FID-MS analysis of the n-hexane extracts was performed in split mode (1:30) at 230°C. The n-hexane extract solutions (1 μL, HPLC grade) were injected and analyzed with the column initially held at 60°C for 2 minutes, then increased to 240°C with a 3°C/min heating ramp. The oven program was as follows: the initial temperature was 60°C for 2 minutes, increased to 240°C over 3 minutes, and then held at the final temperature of 250°C for 4 minutes. Helium (99.999%) was used as the carrier gas at a constant flow rate of 1 mL/min. Detection was performed in electronic impact mode (EI) with an ionization voltage of 70 eV, using scan mode (40-450 m/z) for mass acquisition. The Kovats method using *n*-alkanes (C_6-C_{32}) as standards was used for determination of retention indices of the *n*-hexane extracts of mucus. The NIST (National Institute of Standards and Technology) database was used for analyzing the mass spectrum. The fragmentation patterns of the unidentified components were compared with those of known substances in the NIST, Wiley7NL, FFNSC1.2, and W9N11 library (Yayli *et al*., 2022). The relative percentage of each bio-component was determined by comparing its average peak area to the total peak area. The average peak area is derived from multiple analyses. The names, molecular weights, and structures of the components in the test materials were identified.

Microstructure observation and elemental composition analysis of shell

The soft tissue of the snails was removed. Shells were washed and purified under running water. After washing, the shells were dried at room temperature. The shells were crushed to powder using a mortar and pestle. For accessing the inner layers, shells were immersed in 0.1 M EDTA for 10 min as previously described by Mu *et al.* (2018). Samples were coated with Gold after chemical treatment. Hitachi SU3500 scanning electron microscope was used for SEM observations. Energy-dispersive X-ray spectroscopy (EDS, Oxford INCA X-ray spectrometer) was used with AZtec and INCA software (EDS). The acceleration voltage for elemental distribution maps was 20 kV and count rates were between 1,000 and 2,000 s^{-1}.

Phase composition tests

For identification of phase composition, XRD analysis was realized in powdered shells by a Rigaku miniflex600 X-ray diffractometer (0.02º at scattering scope (2θ) ranging from 10 to 90º by using monochromatic Cu-Ka radiation [1.5406 (λ)] at 40 kV and 15 mA). To assess the aragonite transformation, $CaCO_3$ (ref JCPDS 9013800, 4001361, 4001361, 9013800, Rigaku) peaks were used as a reference.

Results

GC-FID-MS chromatogram for the *n*-hexane extracts of the mucus indicated 17 peaks (2 unknown) and 13 (2 unknown) peaks, respectively. The mass spectrum and structure of the individual components of *R. auricularia* are presented in Figure 1. The mucus consisted mainly of alkanes (34.4%) followed by unsaturated fatty alcohol (11.86%), organooxygen (4.42%) and phthalate ester (4.27%).

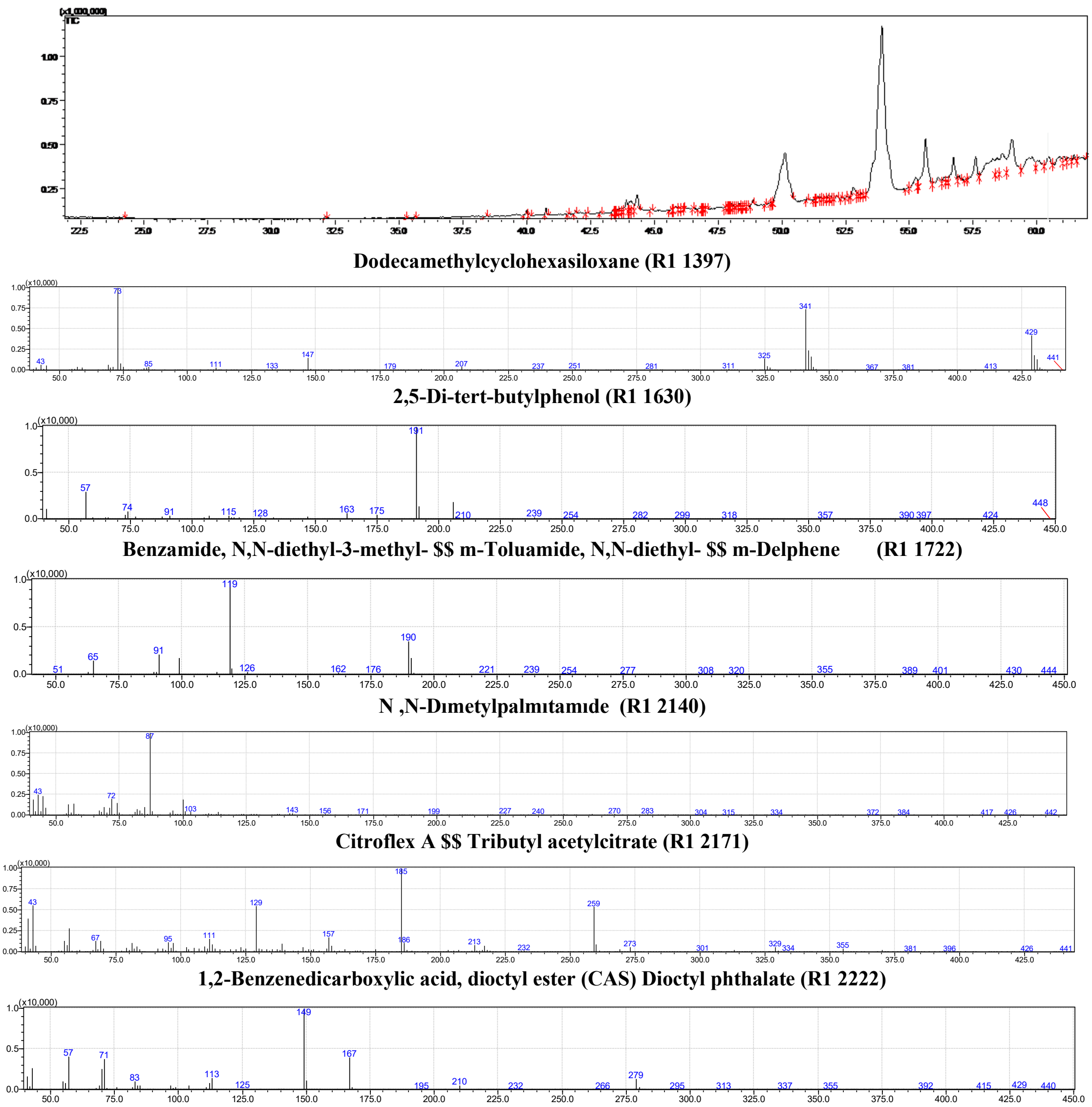

Figure 1: GC-FID-MS chromatogram and mass spectra of compounds obtained from *n*-hexane extract of *Radix auricularia* mucus.

The classifications, activities, and uses of the identified components in mucus are summarized in Table 1.

In of the mineralogy of shells analysed in this study, aragonite form of $CaCO_3$ was dominant in an inorganic layer of shells as shown by XRD analysis (Fig. 2).

Figure 3 demonstrated structural aspect of the organic matrix of *R. auricularia* shells (SEM) at different magnifications with organic matrix fibers forming parallel layers of different widths and densities observed after surface treatment.

Table 1: Bio-active components of *Radix auricularia* mucus determined by GC-FID-MS analysis.

Peak no	Retention Time (RT) (s)	Compound name	Nature of compound	Molecular Formula (MF)	MW (g/mol)	Peak area (%)	Activities/usage
1	32.100	Dodecamethylcyclohexasiloxane	organosilicon	$C_{12}H_{36}O_6Si_6$	444.92	0.1486	cosmetics and personal care products, polishes and waxes, washing & cleaning products and semiconductors, health services and formulation of mixtures and/or re-packaging
2	35.620	Pentadecane	alkane	$C_{15}H_{32}$	212.41	0.2460	
3	40.754	2,5-Di-tert-butylphenol	alkylbenzene	$C_{14}H_{22}O$	206.32	0.9523	antioxidant
4	43.899	Benzamide	monocarboxylic acid amide	$C_{12}H_{17}NO$	191.27	0.8347	therapeutic
5	50.127	Eicosane	alkane	$C_{20}H_{42}$	282.5	34.1603	cosmetics, lubricants, plasticizers, petrochemical industry, antifungal, antioxidant, wound healing (Larranaga *et al.*, 2016; Bhat *et al.*, 2024)
6	52.794	Palmityl acetate	acetate ester	$C_{18}H_{36}O_2$	284.5	2.8421	emollient, masking agent and skin conditioner, pheromone, epitope.
7	55.634	9-Octadecen-1-ol, (Z)-	unsaturated fatty alcohol	$C_{18}H_{36}O$	268.5	11.8627	cosmetics and skin care products, lubricant, permeation and penetration enhancers to facilitate topical drug delivery (Kováčik *et al.*, 2023)
8	56.727	N ,N-Dımetylpalmıtamıde		$C_{21}H_{44}N_2O$	340.6	5.5505	liquids, lotions, sprays, impregnated materials, insecticide
9	57.589	Citroflex A	organooxygen	$C_{20}H_{34}O_8$	402.5	4.4280	versatile plasticizer, toys and food-contact applications, perfumery, cosmetics
10	59.012	1,2-Benzenedicarboxylic acid	phthalate ester	$C_{24}H_{38}O_4$	390.6	4.2722	manufacture of a variety of plastics and coating products, cosmetics and pesticides.

* Dr. Duke‟s Phytochemical and Ethnobotanical Databases, 2014.

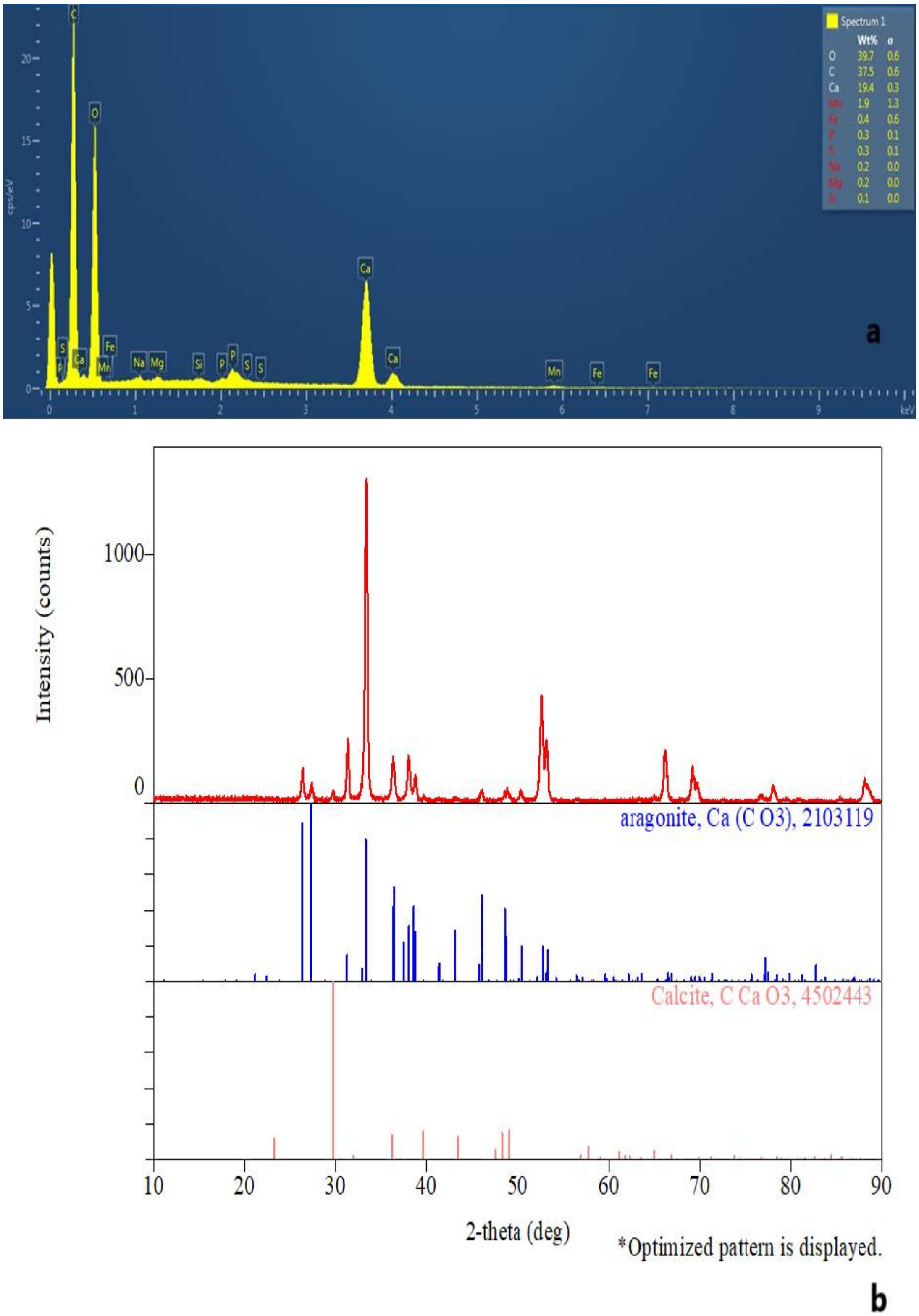

Figure 2: a) The SEM-EDS of cross sectioned surface b) XRD pattern of shells in *Radix auricularia.*

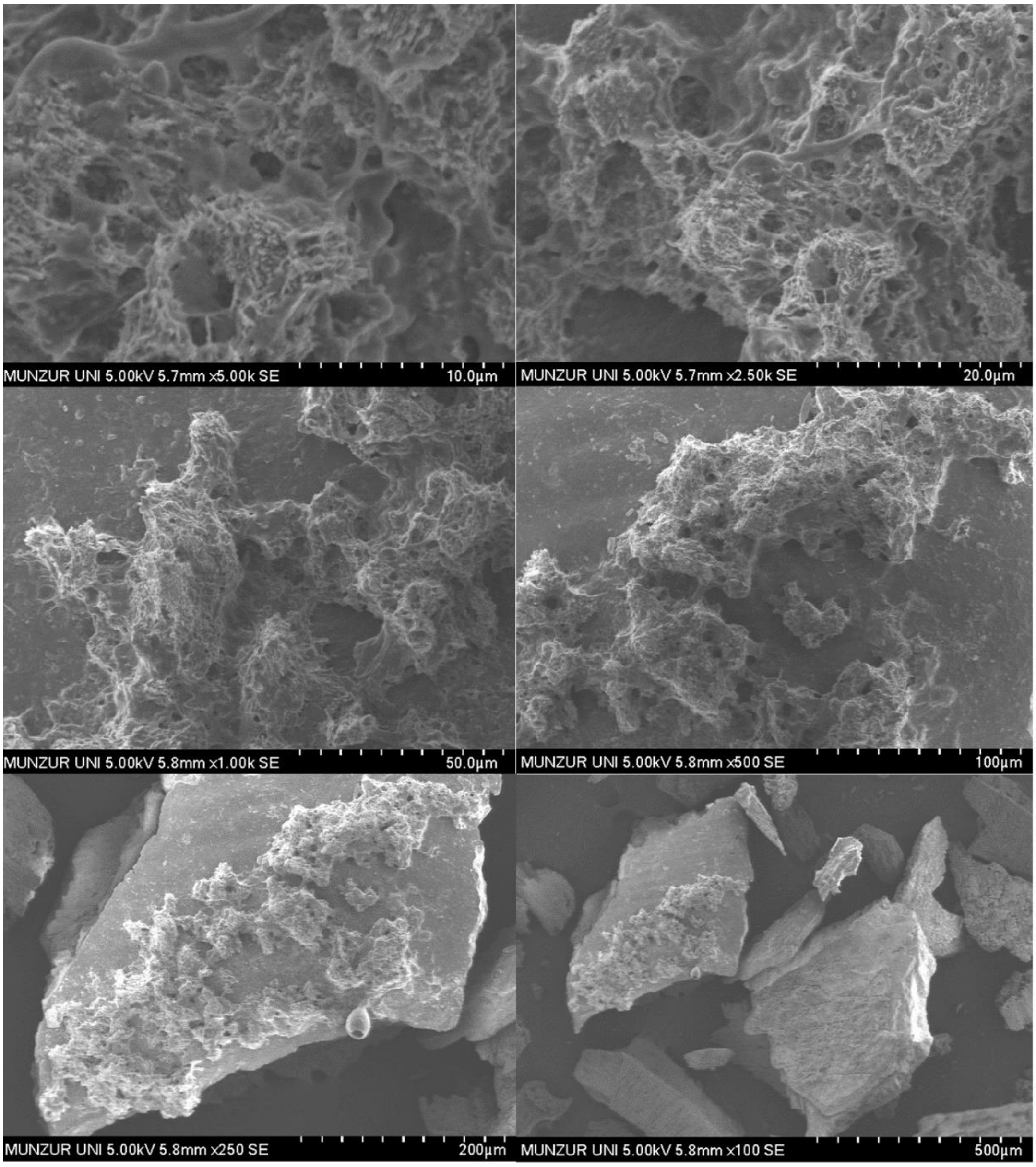

Figure 3: Structural aspect of the organic matrix of *Radix auricularia* shells (SEM) at different magnifications. Organic matrix fibers forming parallel layers of different widths and densities observed after surface treatment by EDTA (surface decalcification).

Discussion

As the major component, antioxidant potential, protective effects, and anti-inflammatory properties of Eicosane has been reported in both *in vitro* models of glutamate-induced cell damage and *in vivo* models of NMDA-induced retinal ganglion cell (RGC) injury in mouse glaucoma by Wu and Pang (2024). The anti-inflammatory, antimicrobial and antifungal properties of Eicosane have been reported by Karanja *et al.* (2012), Nandhini (2015) and Beema Shafreen *et al*. (2022). Trivedi *et al.* (2019) detected that the level of eicosane was notably elevated in the sebum of Parkinson's patients compared with healthy individuals. Additionally, Eicosane acts as a ligand for the taste receptor BminGR59b, which is found in *Bactrocera minax*. This interaction is pivotal in the insect's taste system, facilitating precise host plant selection for oviposition (Zhang *et al*., 2023). Apart from these, Eicosane has been used in cosmetics, lubricants, plasticizers (Larranaga *et al.,* 2016).

9-Octadecen-1-ol, (Z)-/ Oleyl alcohol, one of the main components of mucus detected in the study via GC-FID-MS is an unsaturated fatty alcohol and a non-polar derivative of oleic acid (Mahmoud *et al.*, 2022). It has been used in cosmetics and skincare products, lubricants, permeation, and penetration enhancers to facilitate topical drug delivery (Kováčik *et al*., 2023). The purified compound in *R. auricularia* mucus could have applications in pharmaceuticals and health sectors.

Among the major bioactive constituents detected in mucus, 1,2-Benzenedicarboxylic acid has been reported potential antimicrobial and anti-gonorrhoeal activities and suggested as a pharmaceutical candidate for treating venereal diseases by Vijayakumar *et al.* (2018).

Aragonite crystals possess unique crystalline properties, enabling shells to offer structural flexibility as an alternative biological material for construction purposes (Willinger *et al.,* 2015; Parveen *et al.,* 2020; Kutluyer Kocabaş and Kocabaş, 2023). The shells of gastropods have utility in various applications such as soil amelioration, wastewater treatment, biodiesel production, bone materials, fillers, alternatives to mortars, and biosorbents for dye and heavy metal removal, effectively repurposing them from waste into valuable resources because of their features and structures of shells (Chakraborty *et al.,* 2020; Kutluyer Kocabaş and Kocabaş, 2023). EDS spectra indicated that shells were composed of atomic elements O (39.7%), C (37.5%), Ca (19.4%), Mn (1.9%), Fe (0.4%), P (0.3%), S (0.3%), Na (0.2%), Mg (0.2%) and Si (0.1%). The presence of C, O, and Ca is expected, as these elements are commonly found in most carbonate-based biominerals (Jacob *et al.,* 2008; Kutluyer Kocabaş and Kocabaş, 2023). The existence of orthorhombic aragonite phase in the XRD pattern was revealed with the intense peaks at (111) and (012) planes.

Conclusion

In summary, EDS analysis revealed Ca, C and O as the main components of shell and aragonite crystals were confirmed by XRD. As a byproduct, shells offer versatile potential for reuse across various applications. The characterization of the active components from *R. auricularia* showed the presence of Eicosane, 9-Octadecen-1-ol, (Z)-, *N,N*-Dımetylpalmıtamıde, citroflex a and 1,2-Benzenedicarboxylic acid. Due to contain valuable bioactive compounds, it is promising for the use of *R. auricularia* mucus in cosmetic and health and pharma sectors and, different industrial sectors (e.g. food, manufacturing) with multiple techniques or analytical methods in future. Moreover, the mucus obtainment from a freshwater snail is easy and sustainable as it does not require killing the organism.

Conflicts of interest

The authors declare that they have no conflict of interest.

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