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The isolation, purification and analysis of the melanin pigment extracted from *Armillaria mellea* rhizomorphs

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ABSTRACT

The aim of present study was isolation and characteriation of raw and purified melanin from *Armillaria mellea* rhizomorphs. Native melanin was isolated from the rhizomorphs of *A. mellea* by alkaline extraction. Obtained pigment was purifed by acid hydrolysis and washed by organic solvents. Chemical tests, FT-IR and Raman spectroscopy analysis were conducted to determine the melanin nature of the isolated pigment. UV-Vis, transmittance and colour properties were evaluated. Antioxidant activity was determined using ABTS and antibacterial activity by a well diffusion method. The results of the study demonstrated that melanins isolated from *A. mellea* rhizomorphs had antioxidant, light barrier and antibacterial properties. A purified form of melanin offered better light properties and higher antioxidant activity than the raw form. Both melanins showed antimicrobial activity, raw melanin form had broader activity compared to the pure form. This study revealed that *A. mellea* rhizomorphs may be considered as a promising source of natural melanin. Isolated pigments presented all the physical and chemical properties common to natural and synthetic melanins. Raw and purified melanins showed differences in chemical composition, antioxidant activity and light barrier properties. Results of this study suggest that, melanins from *A. mellea* could be applied in the food, cosmetics and pharmaceutical industries.

***Keywords*:** melanin, *Armillaria mellea*, rhizomorphs, antioxidant, antibacterial, light barrier

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1. INTRODUCTION

The genus *Armillaria* comprises of soil borne fungi that play an important role in the decomposition of wood. Fungi belonging to the genus *Armillaria* cause root disease of deciduous and coniferous trees and shrubs in forests, plantations, orchards and gardens in boreal, temperate and tropical habitats worldwide [1-8]. The root disease-causing capabilities of different *Armillaria* spp. on a given host species can be thought of as points along a continuum with strict saprotrophism at one extreme and relatively virulent parasitism at the other. Saprotrophic and parasitic *Armillaria* spp. are frequently sympatric [6,9]. It is well known that fruiting bodies of basidiomycetes of *Armillaria* genus are not able to emit visible light. Luminescence occurs only in mycelium, growing on the natural substrate (wood) or artificial substrates (nutrient medium) [10]. Most *Armillaria* species have the ability to spread in the soil by rhizomorphs to reach new food bases or potential hosts. Rhizomorphs (also called mycelial cords) are root-like fungal structures with diameters between 0.5 and 3 mm, that consist of an outer melanised cortex and an inner core, called medulla. Due to the melanin content, the cortex protects the rhizomorphs from environmental stress including attacks by antagonistic fungi and bacteria. The medulla is the main active structure of the rhizomorphs responsible for transport of water, nutrients, and oxygen. Rhizomorphs normally are growing out from a woody food base and can produce extensive networks in the soil [4,5,11,12].

Armillaria mellea (Vahl) P. Kumm is a basidiomycete fungus in the genus *Armillaria* in the Physalacriaceae family which grows on the stumps and roots of a wide variety of woody and herbaceous plants. It is also called honey mushroom and is a widespread fungus in Asia, Europe, North America and Africa [11-17]. In moderate climatic zones, the fruiting bodies are usually formed from July to November, with the most abundantly in October [17]. *A. mellea* is a fungus symbiotic with the Chinese medicinal herb "Tianma" (*Gastrodia elata* Blume). The fruiting bodies of *A. mellea* have been used in traditional Chinese medicine for the treatment of hypertension, headache, insomnia, dizziness and vertigo. Recently, the cultured mycelium of *A. mellea* became a health food in Taiwan and China and its tablets are used to treat geriatric patients with palsy, headache, insomnia, dizziness, and neurasthenia [2,13,16,18]. Although the fungus is considered a weak parasite or a saprophyte on forest trees, it can cause serious damage to a wide range of tree species, including *Abies* spp., *Quercus* spp., *Fagus sylvatica*, *Castanea sativa*, *Populus* spp. and *Ostrya carpinifolia*. In addition, *A. mellea* was commonly found on cultivated woody plants, causing damage, especially on apple, pear and in vineyards. *A. mellea* is known to occur in central and southern Europe, but is only common in the southern and western parts of this area. In Europe and North America, *A. mellea* occurs mainly in broadleaved forests, though less commonly in coniferous forests. The fungus is considered thermophilic, and in central Europe is restricted to low altitudes. In central and western Europe, *A. mellea* was not reported above 1000 m, whilst in the Mediterranean and Balkans regions, it has been found in altitudes up to 1750 m [7].

The known chemical compounds from *A. mellea* fruiting bodies and mycelial cultures have been reported include: carbohydrates (glucose, xylose, trehalose, D-mannitol, D-erythritol, glucans), peptides (prosomatostatin), sphingolipids (armillaramide), sterols (ergosterol, ergosterol peroxide), fatty acids (oleic, linoleic, palmitic and stearic acids), sesquiterpenoids (proto-illudan derivatives such as armillarin, melleolid, melledonal A, B and

C), phenolics (*p*-hydroxybenzoic and cinnamic acids), indole compounds (serotonin, tryptamine, tryptophan), ascorbic acid, enzymes and polysaccharides (exopolysaccharides and intracellular polysaccharides). Several studies describe the biological activities of *A. mellea* including immuno-modulating, anti-cancer, antioxidant, anti-inflammatory, antibacterial and fibrinolytic [2,13,16-22].

Melanins have been isolated from a variety of phylogenic sources: animals [24], plants [25], bacteria [26,57] and fungi [27,28,56]. Melanins are commonly represented as black and brown pigments, high molecular weight heterogenous polymers derived from the oxidation of monophenols and the subsequent polymeryzation of intermediate o-diphenols and their resulting quinones [29,30]. Melanins are types of pigments, possessing broad biological activities including; antioxidant, radioprotective, thermoregulative, chemoprotective, antitumor, antiviral, antimicrobial, immunostimulating and anti-inflammatory properties [24-30]. Based on these features, natural melanin has the potential to be of great value and application in the fields of pharmacology, cosmetics, functional foods and material modifications [30-33]. However, knowledge relating to the physiochemical properties and biological activities of melanins from *A. mellea* rhizomorphs including antioxidant, antimicrobial, light barrier properties is highly limited. In recent years there has been a revival of interest in the development of natural colorants as food additives, and also in the cosmetic and pharmaceutical industries. This has been encouraged by strong consumer demand, as synthetic colorants are frequently perceived as undesirable or harmful [27,28]. Owing to the high toxicity of synthetic compounds, the search for new natural colorants with antiradical, light barrier as well as antimicrobial properties still remains a challenge for modern science.

The aim of present study was to isolate, characterize and investigate the antioxidant, antimicrobial and light barrier properties of raw and purified melanins form *A. mellea* rhizomorphs.

2. MATERIALS AND METHODS

2. 1. Test fungus

The tests were made up of rhizomorphs of *A. mellea* within this study. The rhizomorphs were collected in October 2017 in a mixed forest near Szczecin, Poland (53° 20' N, 14° 49' E).

2. 2. Extraction and purification

The isolation and purification of melanin was performed as described by Łopusiewicz [27,28]. The rhizomorphs were washed with distilled water to remove any impurities, powdered in blender and used for melanin extraction. To summarise, 5 g of the rhizomorphs powder were homogenised in 50 ml of 1 M NaOH, extracted in orbital shaker (150 rpm, 50 °C, 24 h) and centrifuged at 6000 rpm for 10 min to remove fungal tissue. Alkaline AM-RM (*Armillaria mellea* raw melanin) mixture was first adjusted to pH 2.0 with 1 M HCl to precipitate melanin, followed by centrifugation at 6000 rpm for 10 min and a pellet was collected. Then, the pellet was hydrolyzed in 6 M HCl (90 °C, 2 h), centrifuged (6000 rpm, 10 min) and washed by distilled water five times to remove acid. The pellet was washed with chloroform, ethyl acetate and ethanol three times to wash away lipids and other residues. Finally, the purified melanin (AM-PM – *Armillaria mellea* pure melanin) was dried, ground to a fine powder in a mortar and stored at −20 °C until testing.

2. 3. Chemical tests

Different diagnostic tests, as described by Selvakumar et al. [34], were conducted on the AM-RM and AM-PM isolated melanins in comparison with L-DOPA melanin used as a melanin standard. The testing organic solvents included ethanol, methanol, chloroform, ethyl acetate, acetone and DMSO.

2. 4. Ultraviolet-visible absorption and transmittance spectra

Melanin solutions were prepared at concentration 0.1 mg/mL and UV-Vis absorption spectra were measured between 200 and 800 nm. The absorbance ratio (A300/A600) values and plots of optical densities against wavelengths of melanins were also calculated [25,27,28]. Transmittance values were measured between 200 and 800 nm at 0.01; 0.05; 0.1; 0.5 and 1 mg/mL for AM-RM and AM-PM; for L-DOPA melanin 0.01; 0.05; 0.1; 1 mg/mL concentrations were measured. All spectrophotometric assays were conducted in a Thermo Scientific Evolution 220 spectrophotometer.

2. 5. IR spectroscopy

The IR spectra of melanins solid samples were obtained at room temperature by attenuated total reflection with a Fourier transform infrared spectrometer (Perkin Elmer). The samples were evenly mixed with KBr, and pressed into tablets, then scanned at a range between 650 cm^{-1} and 4000 cm^{-1} (64 scans and 1 cm^{-1} resolution) [27,28]. Obtained spectra have been normalized, baseline corrected and analysed using SPECTRUM software.

2. 6. Raman spectroscopy

Melanin samples were analysed using a Raman station (RamanStation 400F, Perkin Elemer) with point-and-shot capability using an excitation laser source at 785 nm, 100 micron spot size, 25% laser power, 4 shots, 8 second exposition time. Obtained spectra have been normalized, baseline corrected and analysed using SPECTRUM software.

2. 7. The antioxidant activity (ABTS assay)

An ABTS assay was performed according to Łopusiewicz [27,28]. Radical 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid ($ABTS^{\cdot+}$) was produced by mixing 7 mM ABTS with 2.45 mM potassium persulfate (5 mL of ABTS + 5 mL of potassium persulphate 4.9 mM). The mixture was incubated in darkness at room temperature for 16 h, diluted with 7 mM phosphate buffer (pH 7.4) to reach an absorbance of between 1.0 and 1.2 at 734 nm. For the ABTS assay, 50 µL of melanin (AM-RM, AM-PM, L-DOPA melanin; 0.0625; 0.125; 0.25, 0.5; 1 mg/mL), or dissolvent as control, were mixed with 1.95 mL of $ABTS^{\cdot+}$ solution, incubated in darkness for 10 min at 37 °C, and then the absorbance was measured at 734 nm and antioxidant activity (%AA) was calculated as $\%AA = [(A_c-A_m)/A_c]\times100$; where A_c and A_m are absorbances for the control and melanin sample, respectively.

2. 8. Determination of the total phenolics content (TPC) of melanins

The total phenolics content (TPC) of the melanins were determined by the Folin-Ciocalteu reaction. Melanin dissolved in DMSO (20 µl) was mixed with 1.58 ml of deionized water and 100 µl of the Folin-Ciocalteu reagent, stirred gently for 5 min and added with 300

µl of saturated solution of Na_2CO_3. The mixture was allowed to stand in darkness for 30 min at 40 °C, the absorbance was then measured at 765 nm. A calibration curve of gallic acid in water-methanol (1:1, v/v) (0, 50, 100, 200, 400 and 500 µg/mL) was prepared and TPC was calculated as miligrams of gallic acid equivalents (GAE)/gram of melanin (mg GAE/g). The results were presented as an average of three samples with standard deviation.

2. 9. The visual colour of melanins

The visual colour of melanin solution (0.1 mg/mL) values were measured by a Konica Minolta CR-5 colorimeter with the Hunter LAB colour system. The colour values were expressed as L* (brightness/darkness), a* (redness/greenness) and b* (yellowness/blueness) as an averages of five measurements.

2. 10. The antibacterial activity of isolated melanins

Test microorganisms, including *Bacillus athrophaeus* ATCC49337, *Bacillus cereus* ATCC14579, *Bacillus subtilis* ATCC6633, *Enterococcus faecalis* ATCC29212, *Escherichia coli* DSMZ1576, *Micrococcus luteus* ATCC4698, *Pseudomonas aeruginosa* ATCC2753, *Pseudomonas putida* ATCC12633 and *Staphylococcus aureus* DSMZ346 were separately cultivated in Mueller-Hinton broth. The antibacterial activity was tested through a well diffusion method. 50 mL of Mueller-Hinton broth was inoculated by a single bacterial strain and incubated at 37 °C for 24 h. Mueller-Hinton agar was autoclaved and on reaching approx. 45 °C, 200 µL of bacterial suspension was added to 20 mL of the medium, vigorously vortexed and poured on 90 mm Petri dishes. Wells were cut out by sterile tips (9 mm diameter) in triplicate on each plate and 100 µL of melanin solutions at 0.1 mg/mL in DMSO were placed in the wells. DMSO served as a control. Plates were incubated at 37 °C for 24 h. The inhibition zones were measured after incubation. The results were presented as an average of three samples with standard deviation.

3. RESULTS

Table 1. The results are summarized, which also shows the properties of the L-DOPA melanin sample used for calibration.

No	Test	Result		
		AM-RM	AM-PM	L-DOPA melanin
1.	Solubility in water	Insoluble	Insoluble	Insoluble
2.	Solubility in organic solvents (acetone, chloroform, ethanol, ethyl acetate, methanol)	Insoluble	Insoluble	Insoluble

3.	Solubility in 1 M NaOH	Soluble	Soluble	Soluble
4.	Precipitation in acidic conditions	Precipitation	Precipitation	Precipitation
5.	Reaction with oxidizing agents (H_2O_2)	Decolorized	Decolorized	Decolorized
6.	Reaction with ammoniacal $AgNO_3$ solution	Positive*	Positive*	Positive*
7.	Reaction for polyphenols ($FeCl_3$ test)	Brown precipitate	Brown precipitate	Brown precipitate
8.	Colour	Black	Black	Black

*Positive – gray-coloured silver precipitate on tube side

The results of the study demonstrated that raw and purified black pigments from *A. mellea* had antioxidant, antibacterial and light barrier properties. The AM-RM and AM-PM pigments presented all of the physical and chemical properties common to natural melanins and the experimental data within this work were found to be comparable to those reported in literature (Table 1). Figure 1 shows the log of optical density of a melanins solutions when plotted against the wavelength of AM-RM, AM-PM and L-DOPA melanin. AM-RM, AM-PM and L-DOPA melanin had straight lines with negative slopes of -0.004695, -0.003644 and -0.003741, respectively.

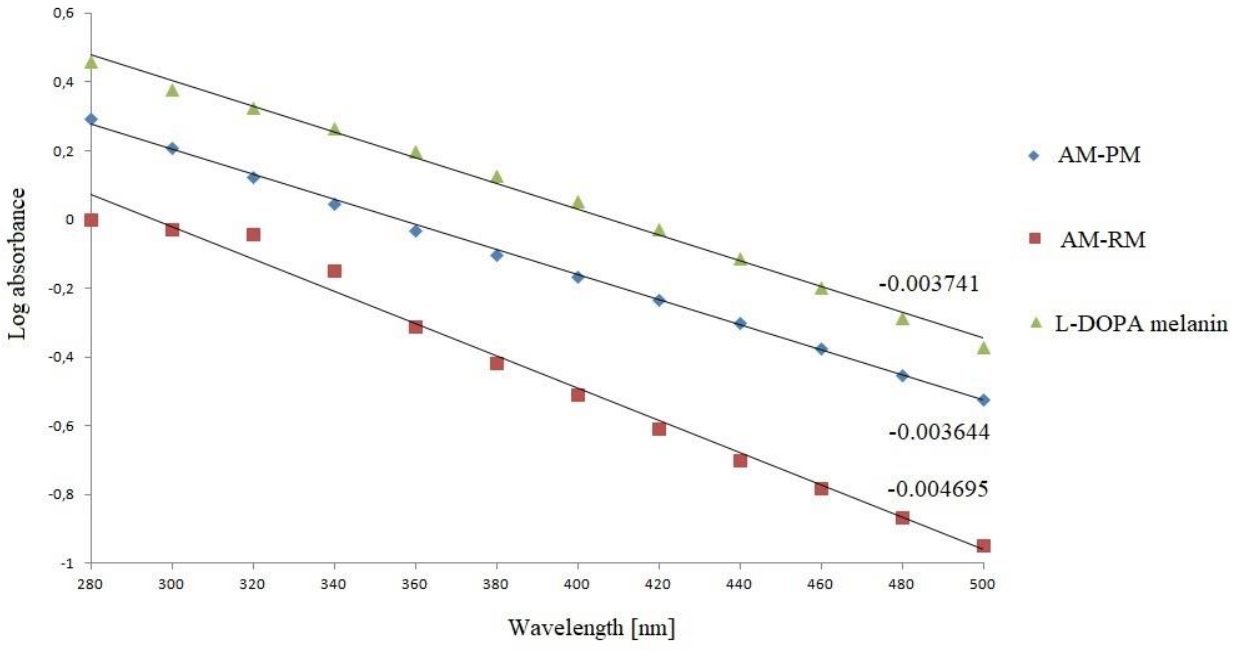

Figure 1. A plots of log of optical denisity of AM-PM, AM-RM and L-DOPA melanin against wavelength

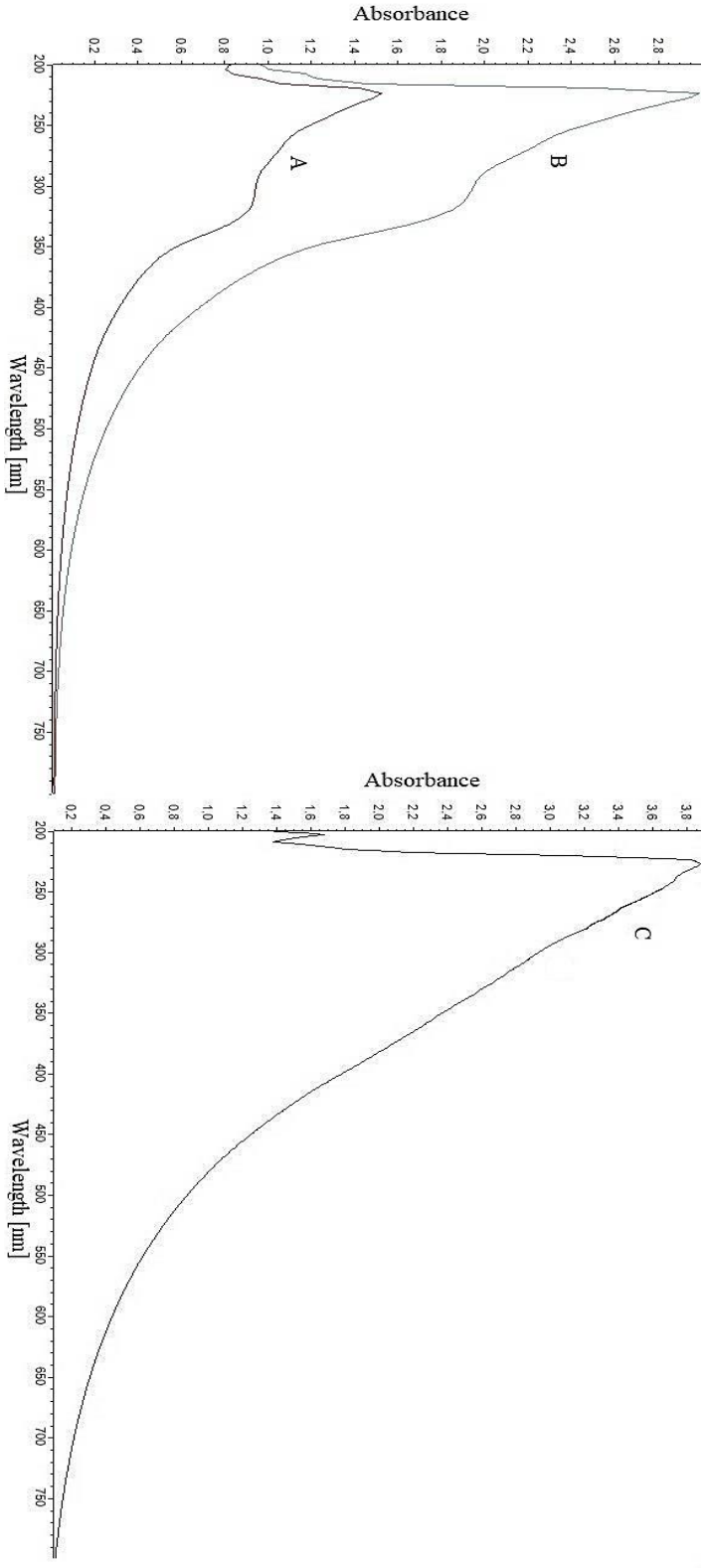

Figure 2. The absorbance of AM-RM (A), AM-PM (B) and L-DOPA melanin (C)

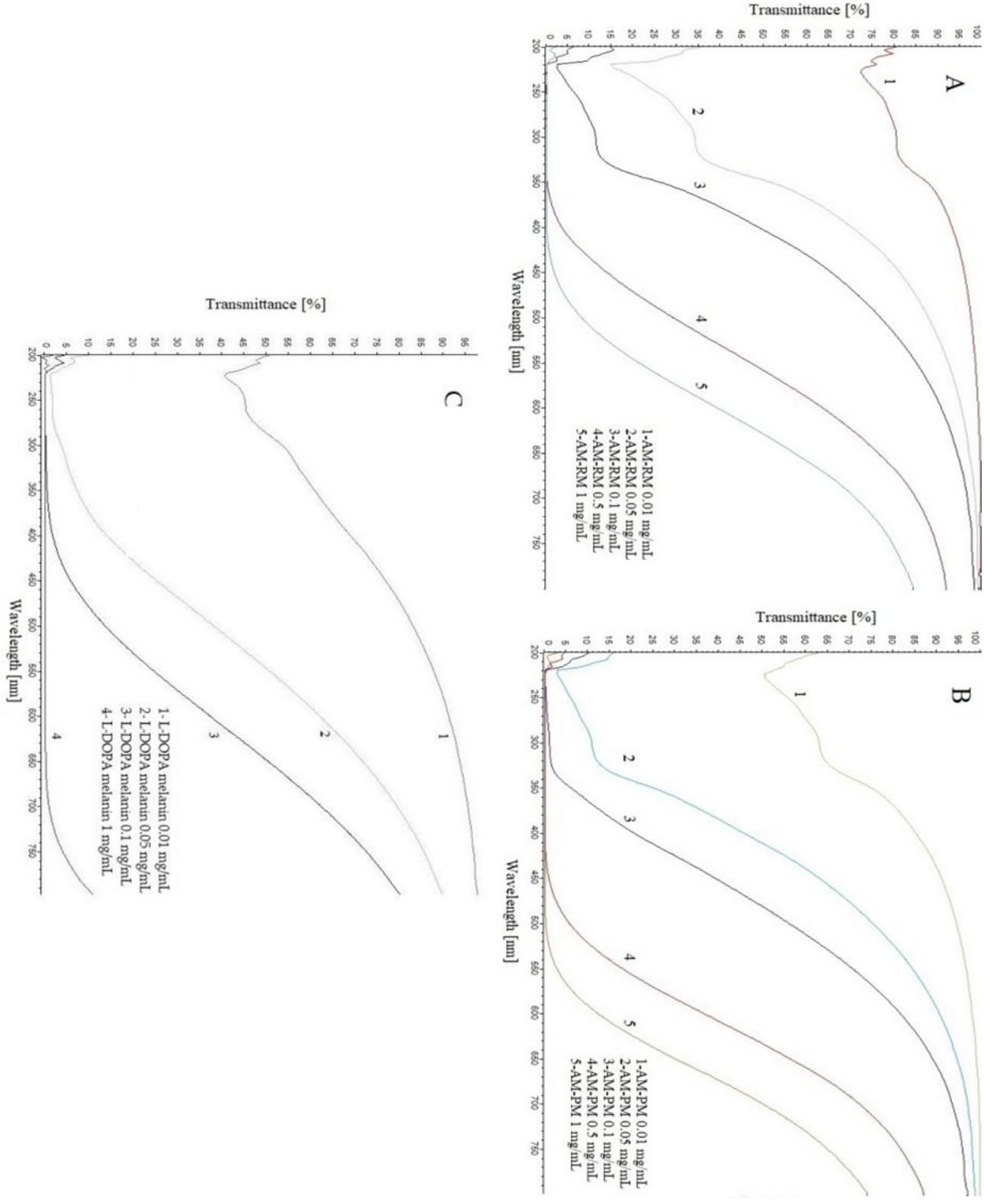

Figure 3. Transmittance values of AM-RM (A), AM-PM (B) and L-DOPA melanin (C)

Figure 2 shows AM-RM (A) and AM-PM (B) have maximum absorption peaks at 224 nm and 227 nm, respectively, and exhibited an exponential decrease in the visible region. This behaviour in AM-RM and AM-PM were similar to the melanin synthesized from L-DOPA (C), which is used as a melanin standard.

The light barrier properties of AM-RM (A), AM-PM (B) and L-DOPA melanin (C) are shown in Figure 3. It was noted that in all analysed concentrations, the AM-RM transmittance values were higher than those of the corresponding AM-PM, which suggests that in purified form, melanin had better light barrier properties, even when the transmittance values of AM-PM were smaller than the synthetic melanin.

The colour values of AM-RM, AM-PM and synthetic melanin are shown in Table 2. Results from the colorimeter indicated that AM-PM presented lower L* value, and higher a* and b* values than AM-RM in Hunter Lab colour system.

Table 2. The visual colour values of AM-RM, AM-PM and L-DOPA melanin (mean ± SD, n = 5)

	L*	a*	b*
AM-RM	94.99±0.00	0.58±0.00	11.39±0.01
AM-PM	89.37±0.01	3.30±0.00	29.90±0.00
L-DOPA melanin	74.87±0.02	10.56±0.01	47.74±0.01

The TPC values for melanins were 0.12±0.02; 0.25±0.01; 0.29±0.05 mg GAE/g for AM-RM, AM-PM and L-DOPA melanin, respectively. In general, the %AA values of AM-PM were higher than those of AM-RM, and the %AA of both melanins were lower than the corresponding concentrations of L-DOPA melanin, as shown in Table 3.

Table 3. The antioxidant activity (%AA values, [%]) of AM-RM, AM-PM and L-DOPA melanin at various concentrations [mg/ml] (mean ± SD, n = 3)

AM-RM [mg/mL]	%AA [%]	AM-PM [mg/mL]	%AA [%]	L-DOPA melanin [mg/mL]	%AA [%]
0.0625	9.67.1±0.14	0.0625	16.09±0.45	0.0625	20.31±0.26
0.125	15.05±0.34	0.125	28.02±0.19	0.125	31.51±1.04
0.25	27.22±0.89	0.25	44.88±0.22	0.25	50.75±0.18
0.5	39.78±0.33	0.5	61.33±0.56	0.5	95.91±0.33
1	68.12±0.51	1	89.17±0.12	1	97.16±0.05

Figure 4 shows the IR-spectra of AM-RM, AM-PM and L-DOPA melanin. Extra display broad absorption bands at 3600-3000 cm^{-1} were noted, attributed to stretching vibrations of C-H, N-H and/or O-H groups. The C-H could be due to the presence of aromatic rings, with strong bands at 1629 cm^{-1} and 1628 cm^{-1}, for AM-RM and AM-PM, respectively, which corresponds to the vibration of aromatic C=C, and more intense in AM-PM. Two peaks at 2915 cm^{-1} to 2847 cm^{-1} in both melanins may result from the oscillation of aliphatic CH_2 and CH_3 groups. The bands at 1212 cm^{-1} and 1236 cm^{-1} due to C-N and C-O, would support the presence of phenols and aromatic amines. It is difficult to state whether there is an amide group, as the C=O group that it complements might be joined in the band corresponding to the aromatic C=C. Noticeable intense peak at 1024 cm^{-1} in AM-RM spectrum could be due to the presence of glucose residues, suggesting polysaccharides components linked with AM-RM. There are differences between the AM-RM and AM-PM spectra which may be a result of the purification process.

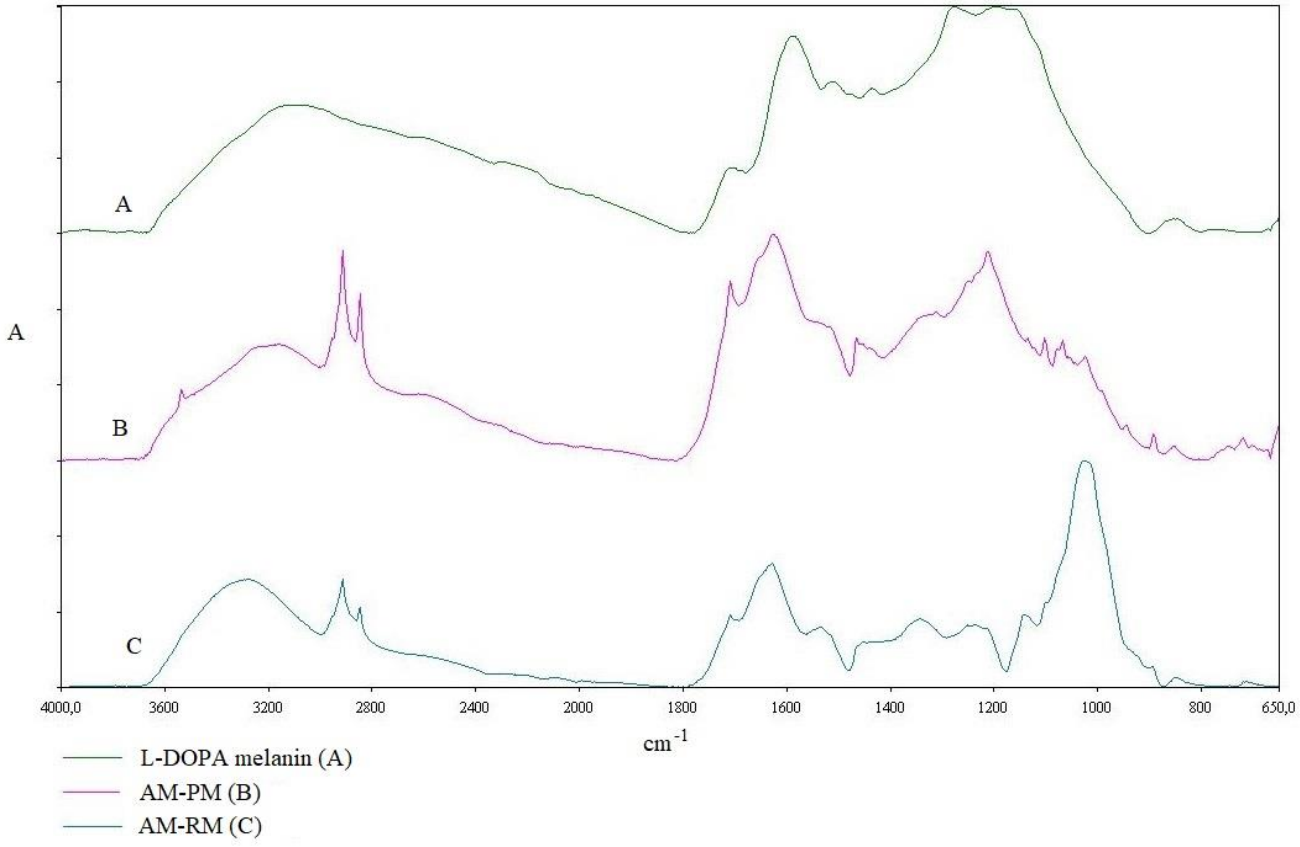

Figure 4. FT-IR spectra of AM-RM, AM-PM and L-DOPA melanin

Figure 5 shows the Raman spectra of synthetic melanin (A), AM-PM (B) and AM-RM (C). AM-RM and AM-PM Raman spectra were similar to spectrum of L-DOPA melanin. The Raman spectrum of AM-RM is dominated by two intense and broad peaks at about 1618 cm^{-1} and 1239 cm^{-1}, while at AM-PM spectrum peaks at 1622 cm^{-1} and 1241 cm^{-1} were observed. A peak at 1970 cm^{-1} from both melanins is noticeable. Peaks 391 $cm^{-1}$and 398 $cm^{-1}$for AM-RM and AM-PM, respectively, are present.

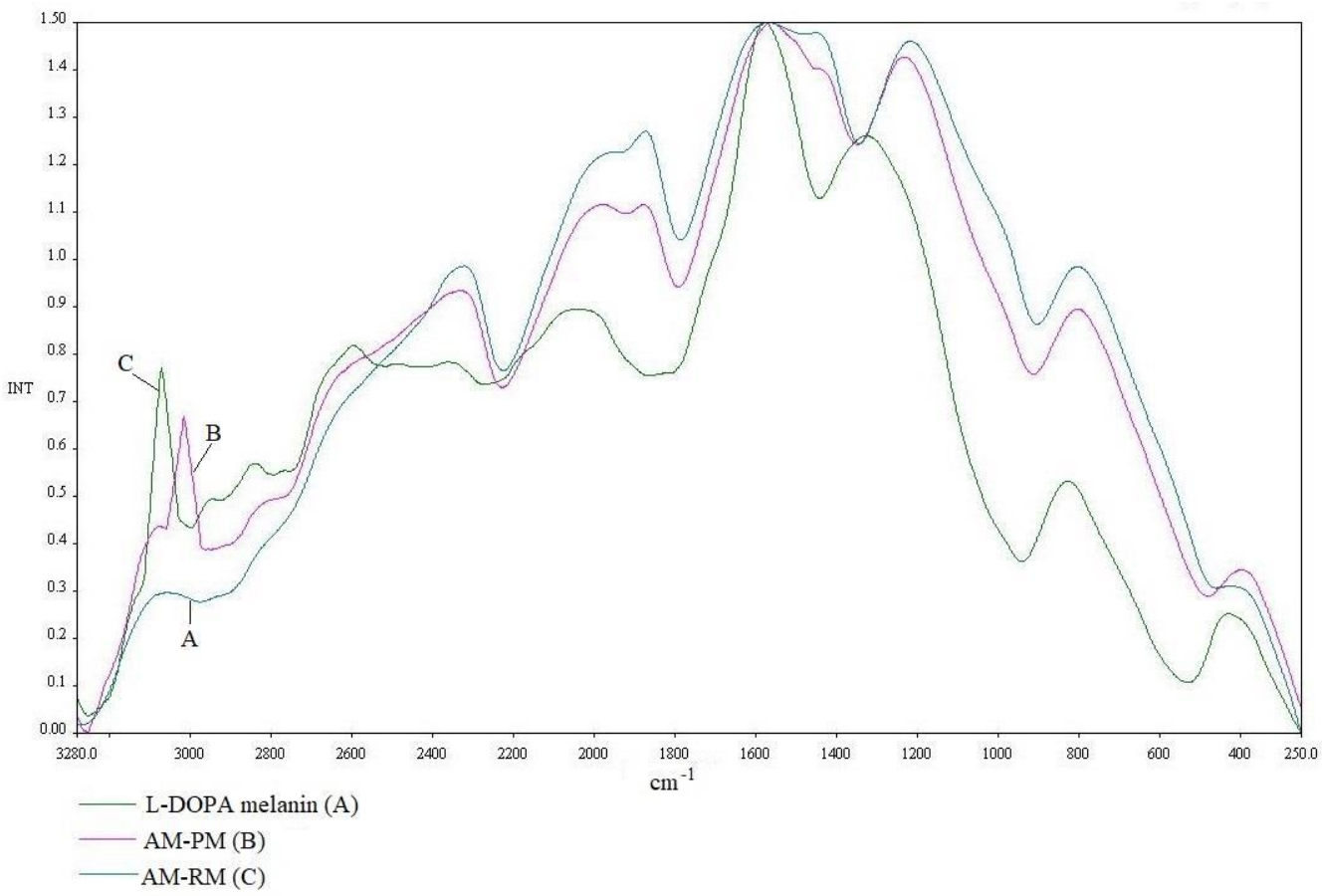

Figure 5. Raman spectra of L-DOPA melanin (A), AM-PM (B), AM-RM (C)

Table 4. The antibacterial activity of EN-RM, EN-PM and L-DOPA melanin (zones of growth inhibition, mm), (mean ± SD, n = 3)

	BA	**BC**	**BS**	**EC**	**EF**	**ML**	**PA**	**PP**	**SA**
AM-RM	12.1 ±0.1	11.5 ±0.3	13.8 ±0.2	10.3 ±0.1	14.4 ±0.2	11.1 ±0.5	14.5 ±0.2	11.3 ±0.3	10.4 ±0.2
AM-PM	-	10.7 ±0.2	12.4 ±0.4	-	12.2 ±0.1	-	12.2 ±0.3	-	-
L-DOPA melanin	-	-	-	-	11.4 ±0.2	-	13.1 ±0.1	-	-

"-" – no inhibition zone, "BA" – *B. athrophaeus* ATCC49337, "BC" - *B. cereus* ATCC14579, "BS" – *B. subtilis* ATCC6633, "EC" - *E. coli* DSMZ1576, "EF" - *E. faecalis* ATCC29212, "ML" – *M. luteus* ATCC4698, "PA" - *P. aeruginosa* ATCC2753, "PP"- *P. putida* ATCC12633, "SA" - *S. aureus* DSMZ346

The results of an antibacterial activity assessment of AM-RM and AM-PM are presented in Table 4. It was noted that AM-RM melanin was active agains all selected strains. AM-PM was active against *B. cereus*, *B. subtilis*, *E. faecalis* and *P. aeruginosa*, and showed no activity against *B. atrophaeus*, *E. coli*, *M. luteus*, *P. putida* and *S. aureus*.

4. DISCUSSION & CONCLUSIONS

Through the work of this study, it is clear that the melanins isolated from *A. mellea* rhizomorphs possess promising antioxidant, light barrier and antibacterial properties.

Chemical tests and FT-IR conducted on isolated pigments in comparison to the synthetic L-DOPA melanin clearly demonstrated that they are melanins. Purified melanin (AM-PM) was obtained by acid hydrolysis, repeated precipitation and purification through the use of organic solvents. The structure of melanin polymers is poorly understood and an accurate definition of melanin is still required. However, the following criteria indicate melanin is: black/brown in colour, insoluble in water and most other organic solvents, resistant to degradation by hot or cold acids, bleached by oxidizing agents and solubilised by alkali solutions [27,28,35].

There was no absorption peak between 260-280 nm in the UV-Vis spectra, indicating, that melanins do not contain proteins and nucleic acids [27,28]. The UV-Vis absorption spectra of the impure (RM) and purified (PM) melanins were similar to those reported in other literature. Also light barrier properties are in accordance with previous studies, showed that purified melanins form *E. nigricans* and *S. citrinum* offered better light properties than the raw form [27,28].

In general, melanins are dark because they do not re-radiate the absorbed visible or invisible light, but transform the energy into rotational and vibrational activity within the molecule and then dissipate it as heat. This phenomenon protects melanised tissues against light-induced damage [23-30]. In previous study it has been also observed that purified melanins showed lower L* and higher a* and b* values [25,27,28]. The high antioxidant activity of the melanins was expected due to the protection against UV-radiation and free radical scavenging being their main functions [23-30]. The ability of melanin to scavenge reactive oxygen species (ROS), such as singlet oxygen, hydroxyl radical and superoxide anion, has been firmly established in model systems, suggesting that melanin could protect pigmented cells against oxidative stress that may accompany the formation of ROS in cells. Even though critical damage to oxidatively stressed cells may result from the reaction of crucial cellular constituents with ROS, an efficient antioxidant may protect the cells by scavenging other oxidizing radicals such as the peroxyl radical, and by interacting with molecular oxygen [36]. The high antioxidant activity of melanin isolated from various sources has been reported by other authors [23-28]. Antioxidant activity of *A. mellea* melanins is comparable to those analyzed in previous studies, also polyphenolic content, which suggest that presence of polyphenolics in melanin molecules is linked with antioxidant activity [27,28].

The A300/A600 ratios offer information about the oxidation state and the range size of melanin molecules [27,28]. Melanin oxidation induces lower absorbance values at 600 nm (A600), and the A300/A600 absorbance ratio was proposed as a measure of oxidation extent, with high values corresponding to greater oxidized melanin molecules. Also, it was argued

that during the melanin oxidation, phenolics are converted to semiquinones or quinones, which produce more oxidized (higher A300/A600 absorbance ratios) and smaller melanins (molecular weight < 1000 Da) [27,28]. AM-RM showed a higher value (22.45) than its corresponding pure AM-PM (11.56) and L-DOPA melanin (16.00). This data supports the fact than AM-RM are a more complex mixture of melanin molecules than that of AM-PM, with a variability in size and degree of oxidation. These data are consistent with previous study showed that raw form of *S. citrinum* melanins are more complex that the pure form [28]. In contrast, for *E. nigricans* melanins, opposite observations have been made, showed that pure form of melanin had higher A300/A600 value being more complex than the raw form [27]. These data are also consistent with the results of Cuevaz-Juárez et al. [37] and also with observations made by Hung et al. [38] who noted that oxidized and reduced melanins obtained from black tea have variances in their absorption spectra. Reduced forms of melanin have phenolic form prevalence, which when oxidized, forms show preponderance for quinone forms.

It was noted that AM-RM melanin was active against all selected strains. AM-PM was active against *B. cereus*, *B. subtilis*, *E. faecalis* and *P. aeruginosa*, and showed no activity against *B. atrophaeus*, *E. coli*, *M. luteus*, *P. putida* and *S. aureus*. This data are partially supported by previous study showed that melanins from *E. nigricans* and *S. citrinum* have been active against *P. aeruginosa* and *E. faecalis* [27,28]. Antimicrobial assessment results are also partially consistent with results found by other authors. Helan Soundra Rani et al. [39] noted the antimicrobial activity of melanin isolated from halophilic black yeast *Hortaea werneckii*. Laxmi et al. [40] observed that growth of *P. aeruginosa* was inhibited on the presence of melanin obtained from *Providencia rettgeri*, but in their study some *Bacillus* species were sensitive to melanin. Xu et al. [41] analysed the antimicrobial activity of melanin from *Lachnum* YM30 and noted that it was active against a wide spectrum of bacteria, including *S. aureus*. The authors suggest that melanin antibacterial activity might result from damage of the cell membrane and affect bacteria membrane function. A discrepancy in melanin antimicrobial activity may result in differences within the molecule structure and composition [42]. In fact, FT-IR spectra showed that AM-RM melanins are probably linked with high content of polysaccharides. Some fungal polyssacharides are known from their antimicrobial activity [43], and it is tempting to suggest that broad antimicrobial activity of AM-RM may result from the presence of polysaccharides linked with melanin molecules. From the other hand there are some reports that melanins have antibiofilm activity against pathogenic bacteria including *P. aeruginosa* and could interfere with bacterial quorum-sensing system, regulate its associate functions and prevent bacterial pathogenesis [44-46].

Members of the *Armillaria* genus are among the most damaging root pathogens of forest and orchard trees worldwide. Rhizomorphs, similar to roots in external appearance, are produced by these fungi on infected root systems. They grow through the soil, where they may contact roots of neighbouring trees [47]. *Armillaria* infects trees either by the rhizomorph penetration of healthy roots or through physical contact of a susceptible root with a diseased root. Rhizomorphs are important in the dissemination and survival of the pathogen, also playing pivotal role in their aeration [4]. During infection rhizomorphs penetrate the bark and form mycelial fans, which spread within the inner bark and cambium of the host root [48]. The black pigment found in rhizomorphs is a melanin type pigment, and has a protective role against unfavourable environmental conditions [4,47,49]. It is commonly observed that rhizomorph tips that are in contact the atmosphere become brown. Worral *et al*. suggest that

enzyme laccase participates in the formation of melanin in rhizomorphs [47]. This observation is accordance with fact that the formation of melanin occurs mainly in aerobic conditions [30]. Many fungi contain melanins in vegetative as well reproductive structures (e. g. spores). Melanins such as 1,8-dihydroxynaphtalene and L-3,4-dihydroxyphenylalanine (L-DOPA) types have been mostly associated to ascomycetous and basidiomycetous forms, respectively. Some fungi have the ability to synthetize a type of melanin according to environmental conditions, as well as at a developmental stage. The presence of melanins in fungi adapted to different environments and the fact that the same melanin is synthesized by a fungus, independent of environmental conditions, suggests that this molecule plays various crucial biological roles in fungal physiology [50-52]. Melanins enhance the tolerance of fungi to environmental stresses, improving their survival. Melanins protect fungal structures from UV radiation, temperature, desiccation, oxidizing agents and toxic compounds, such as antibiotics or heavy metals, due to their chelating ability [4,50-52]. In several plant pathogens melanin a plays pivotal role in generating osmotic pressure within the appresorium, when hyphae penetrate cell walls. They also may protect rhizomorphic structures from microbial lysis in the soil [47].

During their growth through soils, rhizomorphs can take up mineral nutrients at their growing tips [6]. The cortex of the rhizomorphs have the ability to absorb metal ions from natural soils [11,53]. The chemical structure of melanin presents many oxygen-containing groups, including carboxyl, phenolic and alcoholic hydroxyl, carbonyl, and methoxy groups, which have the ability to bind to a broad spectrum of substances. In literature, studies have confirmed that fungal melanin acts as a metal chelator, enhancing biomass-metal interaction, and consequently its biosorption capacity [54]. Rizzo *et al*. reported a binding of metals to the mycelial melanin of the *Armillaria* spp. finding that the melanized rhizomorph mycelia concentrated Al, Zn, Fe and Cu ions. Through their ability to bind metal ions and to produce extensive networks in the soil *Armillaria* rhizomorphs may contribute to the stabilization of heavy metals in contaminated soils. Fungal melanins contain various functional (chemical) groups, which provide binding sites for metal ions and microorganisms find them rather difficult to decompose [51-52]. Therefore, rhizomorphic melanin might still bind heavy metals as part of the soil organic matter after the death of the rhizomorphs. In fungi the amount of melanin produced is associated with the level of resistance to radiation. Fungi living on rocks, exposed surfaces or in extreme environments are often heavily pigmented and able to resist elevated temperatures and UV radiation [55]. *A. mellea* rhizomorphs often grow on wood surfaces and bark, which are often exposed on direct sun radiation. Thus, it is obvious, that fungi evolved panoply of adaptations in rhizomoprhs, which include adaptations for overcoming host barriers, successfully compete with other microorganisms and are able to survive harsh environmental conditions. Melanins are also responsible for dark lines formed in the inhabited wood. Fungal melanin deposition in spatial demarcation could be triggered by limited water availability. The anticipation of desiccation might lead to the development of an effective strategy to ensure the survival of the colonies. They produce high resistance, melanin-type pigment that surrounds the fungal community like a barrier, blocking water exchange within the wood substrate. This may appear as fine delimitation lines in sections. The formation of melanised mycelium in zone lines, in inter- and intraspecific antagonistic reactions, offer a perspective on substrate and environmental conditions that influence such formations in natural settings. Such lines have been also reported for *A. mellea*, which occupies wood moister than ambient [49].

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