Isolation, characterisation and biological activity of melanin from *Exidia nigricans*

Łukasz Łopusiewicz

Center of Bioimmobilisation and Innovative Packaging Materials,
Faculty of Food Sciences and Fisheries, West Pomeranian University of Technology Szczecin,
35 Janickiego Str., Szczecin 71-270, Poland

E-mail address: lukasz.lopusiewicz@zut.edu.pl

ABSTRACT

The aim of present study was isolation and characteriation of raw and purified melanin from *Exidia nigricans*. Native melanin was isolated from the fresh *E. nigricans* fruiting bodies 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 *E. nigricans* 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 inhibited the growth of *E. facealis* and *P. aeruginosa*. This study revealed that *E. nigricans* 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. Melanin may play pivotal role in physiology of *E. nigricans* protecting it against UV radiation and dessication.

*Keywords***:** melanin, pigment, *Exidia nigricans*, antioxidant, light barrier, antimicrobial

1. INTRODUCTION

The genus *Exidia* comprises of saprotrophic basidiomycetes from family *Auriculariaceae* (an order of the *Agaricomycetes*) growing as pioneer fungi on dead or recently fallen wood, and produce gelatinous (jelly) fruiting bodies (basidiocarps) [1,2]. *Exidia* taxa are distributed worldwide and have been reported from the northern hemisphere (including North America, South America, Europe and New Zeland) [3-9] and are involved in wood biodegradation (production of wood degrading enzymes) [10-12]. Some studies describe the biological activities of *Exidia* species including antioxidant and antimicrobial activity [13].

Exidia nigricans (With.) P. Roberts (syn. *Exidia plana*), commonly known as Witches's butter, occurs as a wood-rotting species on dead branches of a wide range of broadleaf trees [14]. When mature, forms dark, blackish jelly fruit bodies that are button shaped and form clusters. The dark colour of fruiting bodies is caused by melanin. When the fruit bodies are dried they can shrink to form a flattened black crust.

Melanins have been isolated from a variety of phylogenic sources: animals [15], plants [16], bacteria [17,18] and fungi [19-25]. 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 [26]. Melanins are types of pigments, possessing broad biological activities including: antioxidant, radioprotective, thermoregulative, chemoprotective, antitumor, antiviral, antimicrobial, immunostimulating and anti-inflammatory properties [15-26]. Based on these features, natural melanin has the potential to be of great value and application in the fields of pharmacology, cosmetics, and functional foods [21].

However, knowledge relating to pharmacological and the biological activities of melanins from *E. nigricans* 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, pharmaceutical and medicinal industries. This has been encouraged by strong consumer demand, as synthetic colorants are frequently perceived as undesirable or harmful [27]. 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 this study was to isolate and characterise properties of raw and purified melanins isolated form *E nigricans*. This represents a first report on the isolation and biological activities of melanins from *E. nigricans*.

2. MATERIALS AND METHODS

2. 1. Test fungus

The tests were made up of fresh mature fruiting bodies of E. nigricans within this study. The fruiting bodies were collected in September 2017 in a mixed forest near Szczecin, Poland (53°20' N, 14°49' E) and for taxonomic identification according to microscopic and macroscopic features of fungal specimen the identification keys were used [28-30].

2. 2. Chemicals

NaOH, HCl, $AgNO_3$, $FeCl_3$, H_2O_2, acetone, ethanol, ethyl acetate, chloroform, dimethyl sulfoxide (DMSO) and methanol (Chempur, Poland) were used to extract, purify and offer up a characterisation of the active substances from the *E. nigricans* fruiting bodies. Gallic acid, ABTS and KBr (Sigma Aldrich) were also used in this study.

To verify the antimicrobial properties of any melanin, Mueller-Hinton broth and Mueller-Hinton agar media (Merck, Germany) were used. All media were prepared according to the Merck protocol.

2. 3. Extraction and purification

The isolation and purification of melanin was performed as described by Harki et al. [19] with minor modifications. 100 g of material (fresh fruiting bodies) were homogenised in 500 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 EN-RM (*Exidia nigricans* 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 (EN-PM – *Exidia nigricans* pure melanin) was dried, ground to a fine powder in a mortar and stored at −20 °C until testing.

2. 4. Chemical tests

Different diagnostic tests, as described by Selvakumar et al. [23], were conducted on the EN-RM and EN-PM isolated melanins in comparision with L-DOPA melanin used as a melanin standard. The testing organic solvents included acetone, chloroform, ethanol, ethyl acetate, DMSO and methanol.

2. 5. 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 of melanins were also calculated [16]. Transmittance values were measured between 200 and 800 nm at 0.01; 0.05; 0.1; 0.5 and 1 mg/mL for EN-RM and EN-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. 6. IR spectroscopy

The IR spectra of melanins solid samples were obtained at room temperature by attenuated total reflection with a Fourier transform infrared spectrometer (Spectrum 100 Perkin Elmer). The samples (10 mg) were evenly mixed with 100 mg 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) [16].

2. 7. 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.

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 according to Cuevas-Juárez et al. [16]. 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_2. The mixture was allowed to stand in darkness for 30 min at 40 ºC, 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 antioxidant activity (ABTS assay)

An ABTS assay was performed according to Cuevas-Juárez et al. [16]. Radical 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS·+) 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 (EN-RM, EN-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·+ 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 % A A = $[(A_c-A_m)/A_c] \times 100$; where A_c and A_m are absorbances for the control and melanin sample, respectively.

2. 10. 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. 11. The antibacterial activity of isolated melanins

Test microorganisms, including Bacillus cereus ATCC14579, Enterococcus faecalis ATCC29212, Escherichia coli DSMZ1576, Pseudomonas aeruginosa ATCC2753 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

The results of the study demonstrated that raw and purified black pigments from *E. nigricans* had antioxidant, antibacterial and light barrier properties. The EN-RM and EN-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. The results are summarized in Table 1, which also shows the properties of the L-DOPA melanin sample used for calibration.

Table 1. Diagnostic tests for melanins

| No	Test	Result		
		EN-RM	**EN-PM**	**L-DOPA melanin**
1.	Solubility in water	Insoluble		
2.	Solubility in organic solvents (acetone, chloroform, ethanol, ethyl acetate, methanol, DMSO)	Soluble only in DMSO		
3.	Solubility in 1 M NaOH	Soluble		
4.	Precipitation in acidic conditions	Readily precipitation		
5.	Reaction with oxidizing agents (H_2O_2)	Decolorized		
6.	Reaction with ammoniacal $AgNO_3$ solution	Gray coloured silver precipitate on tube side		
7.	Reaction for polyphenols ($FeCl_3$ test)	Brown precipitate		
8.	Colour	Black		
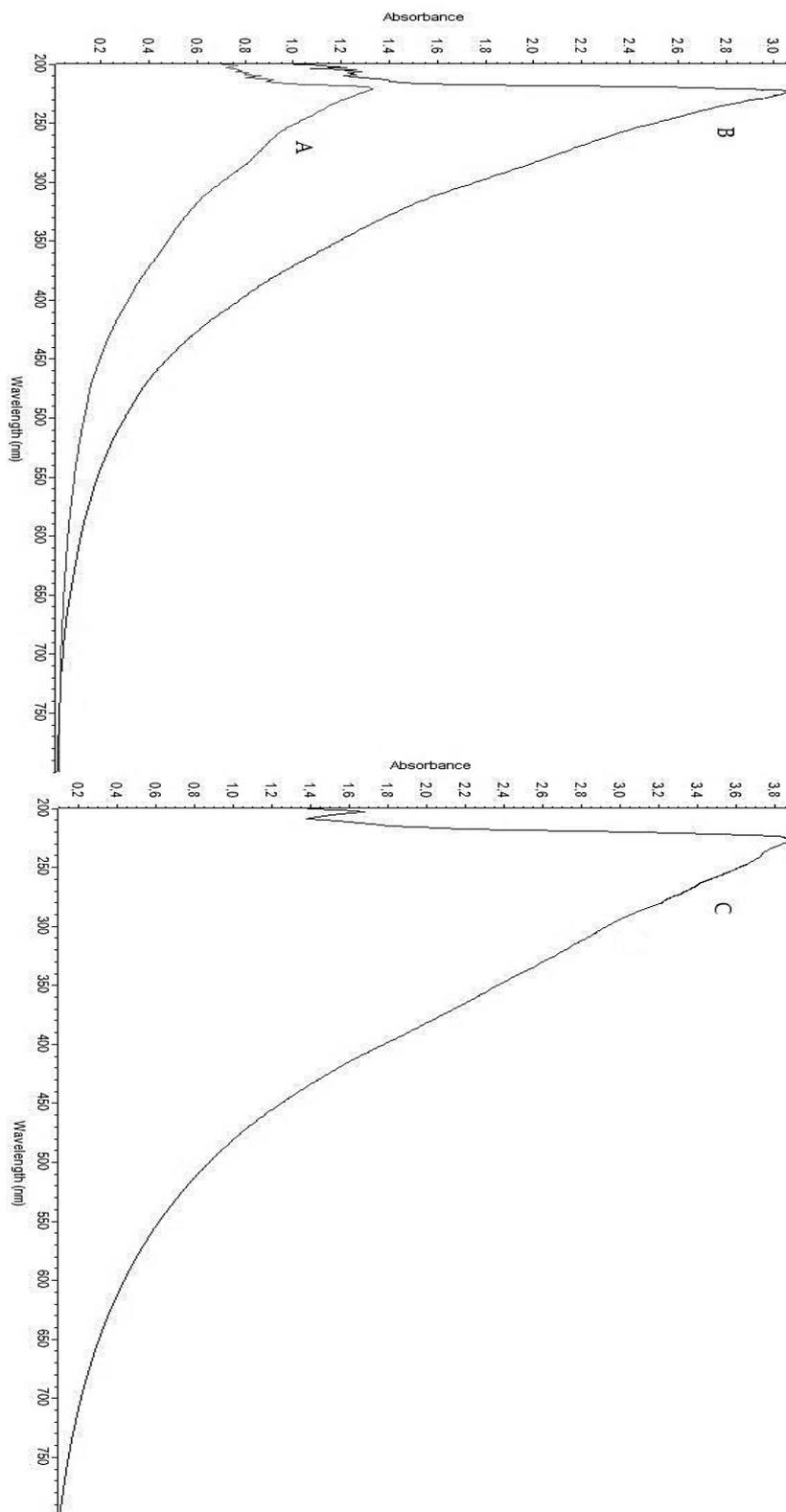
Figure 1. The absorbance of EN-RM (A), EN-PM (B) and L-DOPA melanin (C)

Figure 1 shows EN-RM (A) and EN-PM (B) have maximum absorption peaks at 221 nm and 224 nm, respectively, and exhibited an exponential decrease in the visible region. This behaviour in EN-RM and EN-PM were similar to the melanin synthetized from L-DOPA (C), which is used as a melanin standard.
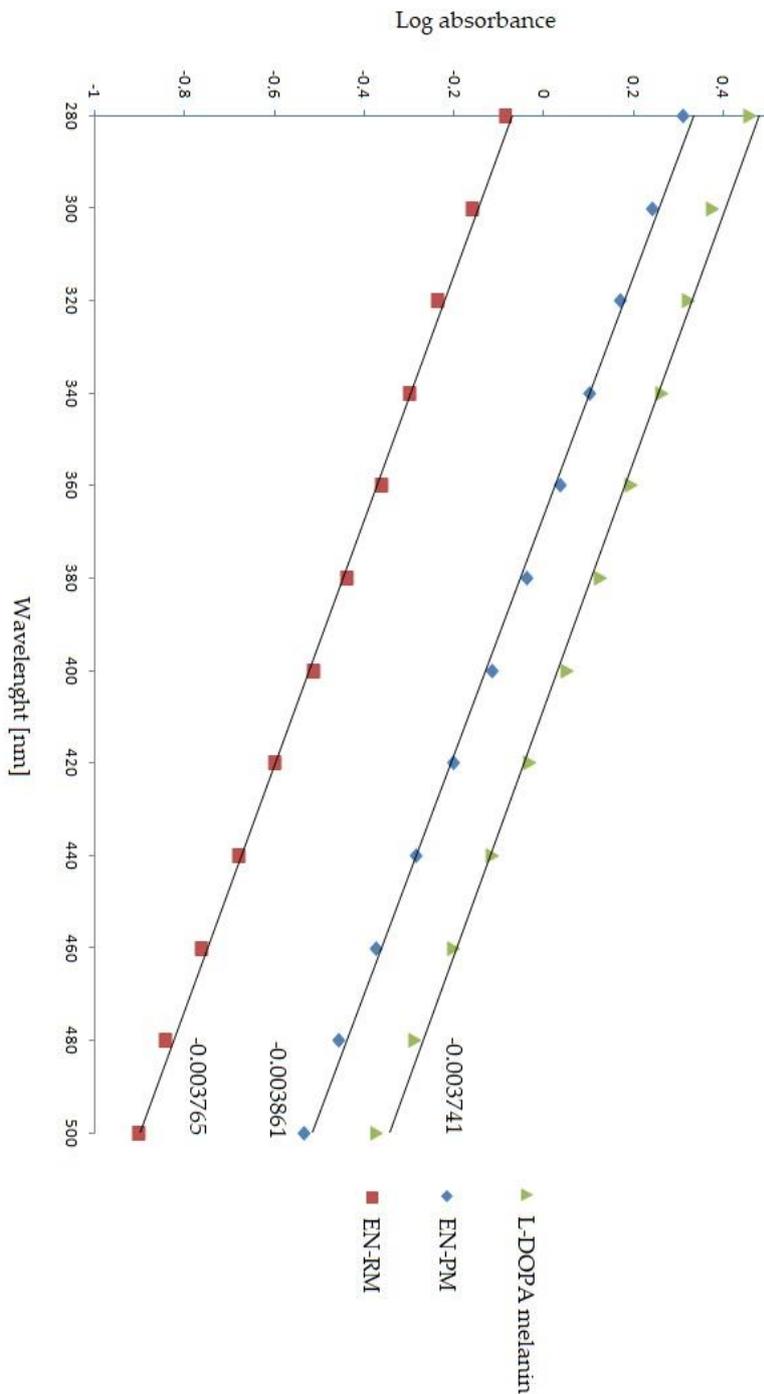

Figure 2. A plots of log of optical denisty of EN-RM, EN-PM and L-DOPA melanin against wavelength

Figure 2 shows the log of optical density of a melanin solutions when plotted against the wavelength of EN-RM, EN-PM and L-DOPA melanin. EN-RM, EN-PM and L-DOPA melanin had straight lines with negative slopes of -0.003765, -0.003861 and -0.003741, respectively.
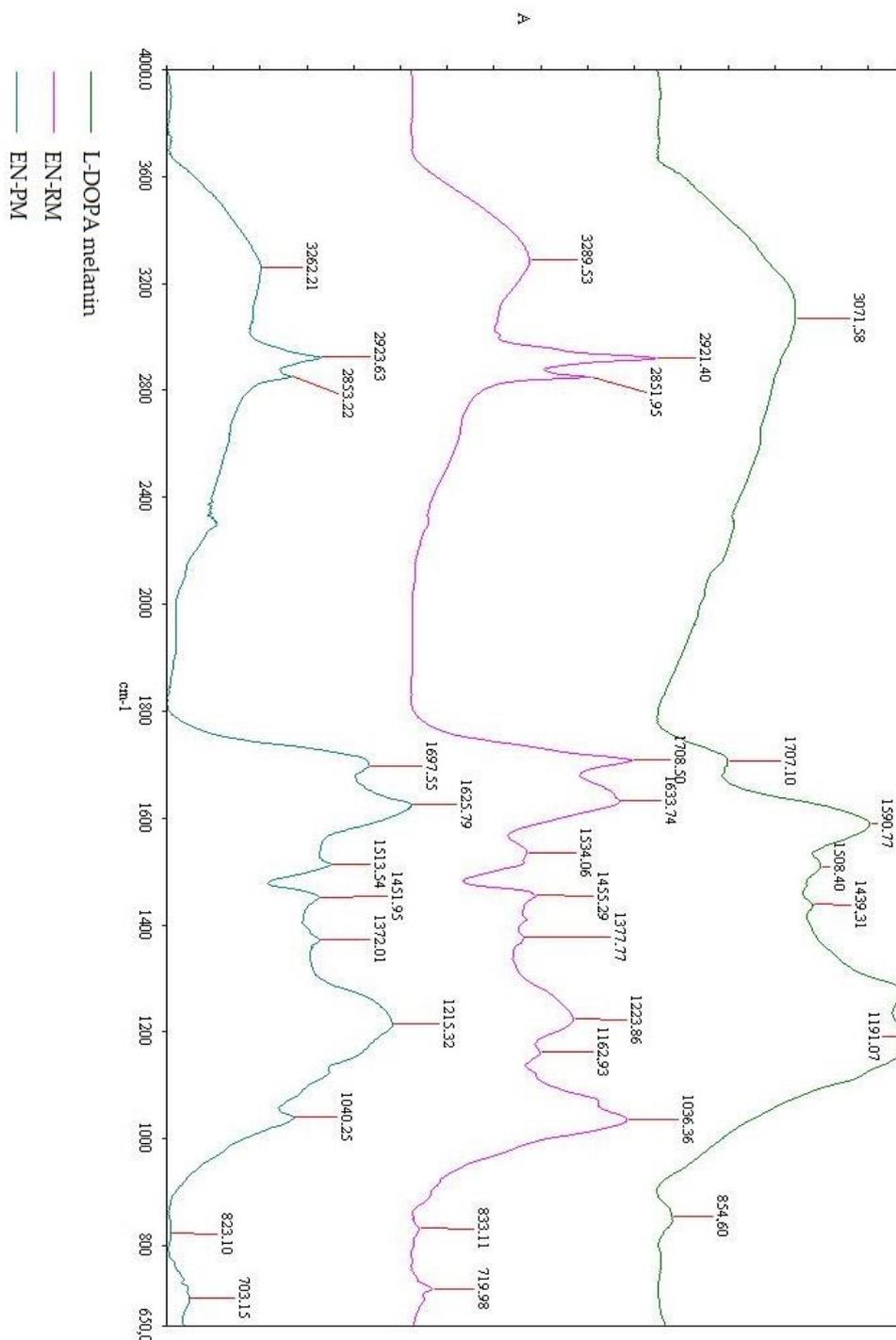

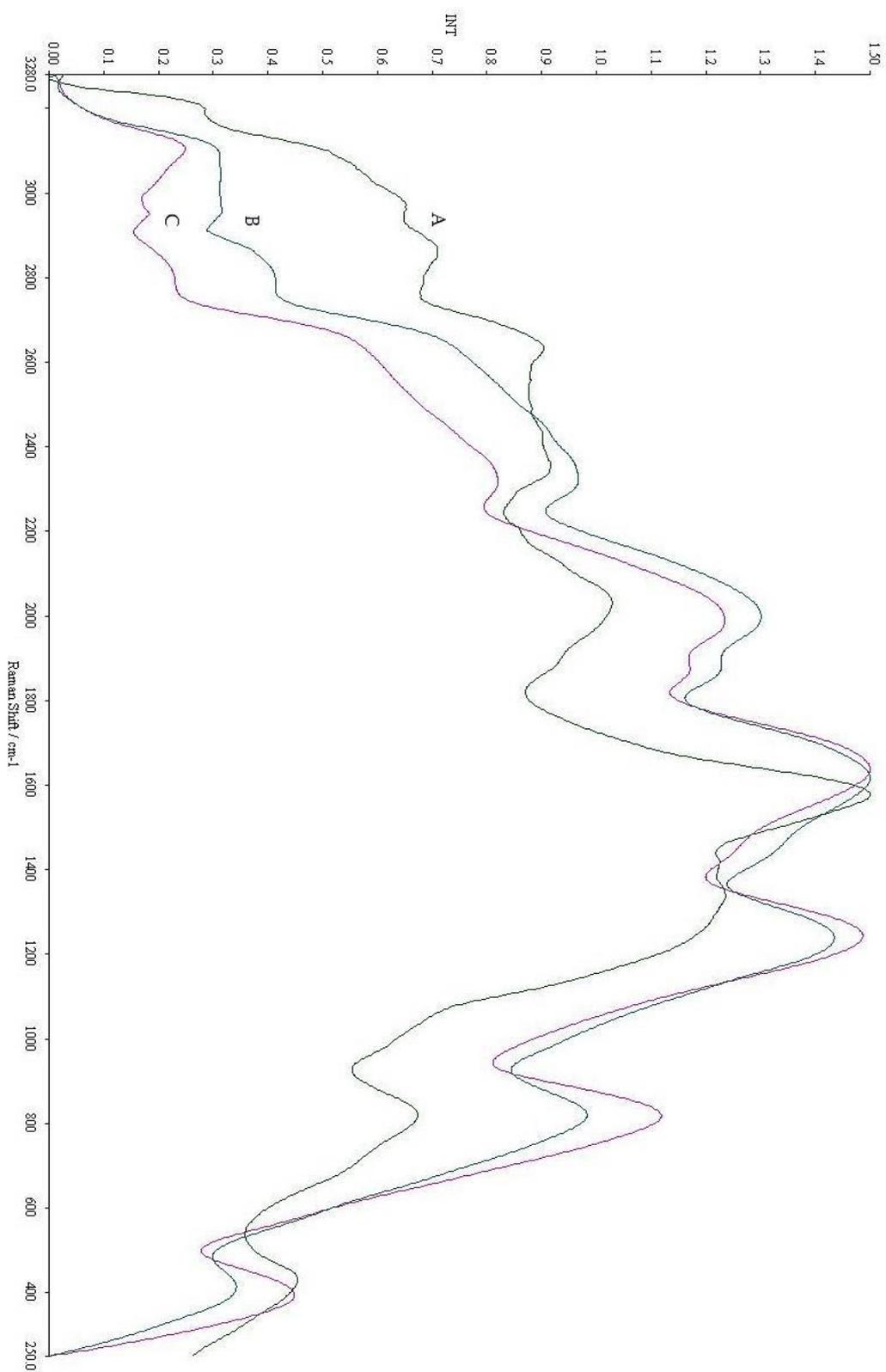**Figure 3.** FT-IR spectra of EN-RM, EN-PM and L-DOPA melanin

Figure 4. Raman specta of L-DOPA melanin (A), EN-PM (B) and EN-RM (C)

Figure 3 shows the FT-IR spectra of EN-RM, EN-PM and L-DOPA melanin. Extra display broad absorption bands at 3600-3000 cm^{-1} were noted, attributed to stretching vibrations of hydrogen –bound groups such as -CH, -NH and/or -OH. The peaks at 2921.40 cm^{-1}; 2851.95 cm^{-1} (EN-RM) and 2923.63 cm^{-1}; 2853.22 cm^{-1} (EN-PM) might result form the oscillations of aliphatic groups –CH$_2$ and –CH$_3$, respectively, more intensive in raw form of melanin. The peaks in the regions of 1708.50 cm^{-1} and 1697.55 cm^{-1} may result from the oscillations of the C=O groups within acids, esters and ketones. The peaks at 1633.74 cm^{-1} and 1625.79 cm^{-1} corresponded to the vibration of aromatic C=C strechtching bonds and COO$^-$ groups. The –NH bending vibration peaks at 1534.06 cm^{-1} and 1513.54 cm^{-1} indicate that *E. nigricans* melanin had indole structure. Phenolic –COH stretchings at 1223.86 cm^{-1} and 1251.52 cm^{-1} relate to phenolic compunds. The peaks centered at 1036.36 cm^{-1} and 1040.25 cm^{-1} are the indication of –CH in-plane vibrations of aliphatic structures. Weak peaks at 833.11 cm^{-1} and 823.10 cm^{-1} are probably due to aromatic –CH groups. Also, weak bands in the regions of 719.98 cm^{-1} and 703.15 cm^{-1} are ascribed to alkene substitiutions in melanin molecules, and are absent in spectrum of synthetic melanin.

However, spectrum of EN-PM (after acid hydrolysis and purification) showed some bands that were reduced in comparision to the EN-RM spectrum. Purification process reduced the amount of –CH$_2$ and –CH$_3$ groups (2923.63 cm^{-1}; 2853.22 cm^{-1}), C=O groups resulted from acids, esters and ketones, bonded to native melanin molecules. The most noticeable peak intensivity chage was noted in the region of 1036.36 cm^{-1} attributed to –CH in-plane vibrations of aliphatic structures. Stronger bands attributed to phenolics were noted in pure form of E. *nigricans* melanin.

Figure 4 shows the Raman spectra of synthetic melanin (A), EN-PM (B) and EN-RM (C). EN-RM and EN-PM Raman spectra were similar to spectrum of L-DOPA melanin. The Raman spectrum of EN-RM is dominated by two intense and broad peaks at about 1640 cm^{-1} and 1240 cm^{-1}, while at EN-PM spectrum peaks at 1620 cm^{-1} and 1230 cm^{-1} were observed. A peak at 2000 cm^{-1} from both melanins is noticeable. Peaks 385 cm^{-1}; 2010 cm^{-1} and 395 cm^{-1}; 1998 cm^{-1}, for EN-RM and EN-PM, respectively, are present.

The light barrier properties of EN-RM (A), EN-PM (B) and L-DOPA melanin (C) are shown in Figure 5. It was noted that in all analysed concentrations, the EN-RM transmittance values were higher than those of the corresponding EN-PM, which suggests that in purified form, melanin had better light barrier properties, even when the transmittance values of EN-PM were smaller than the synthetic melanin.

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

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

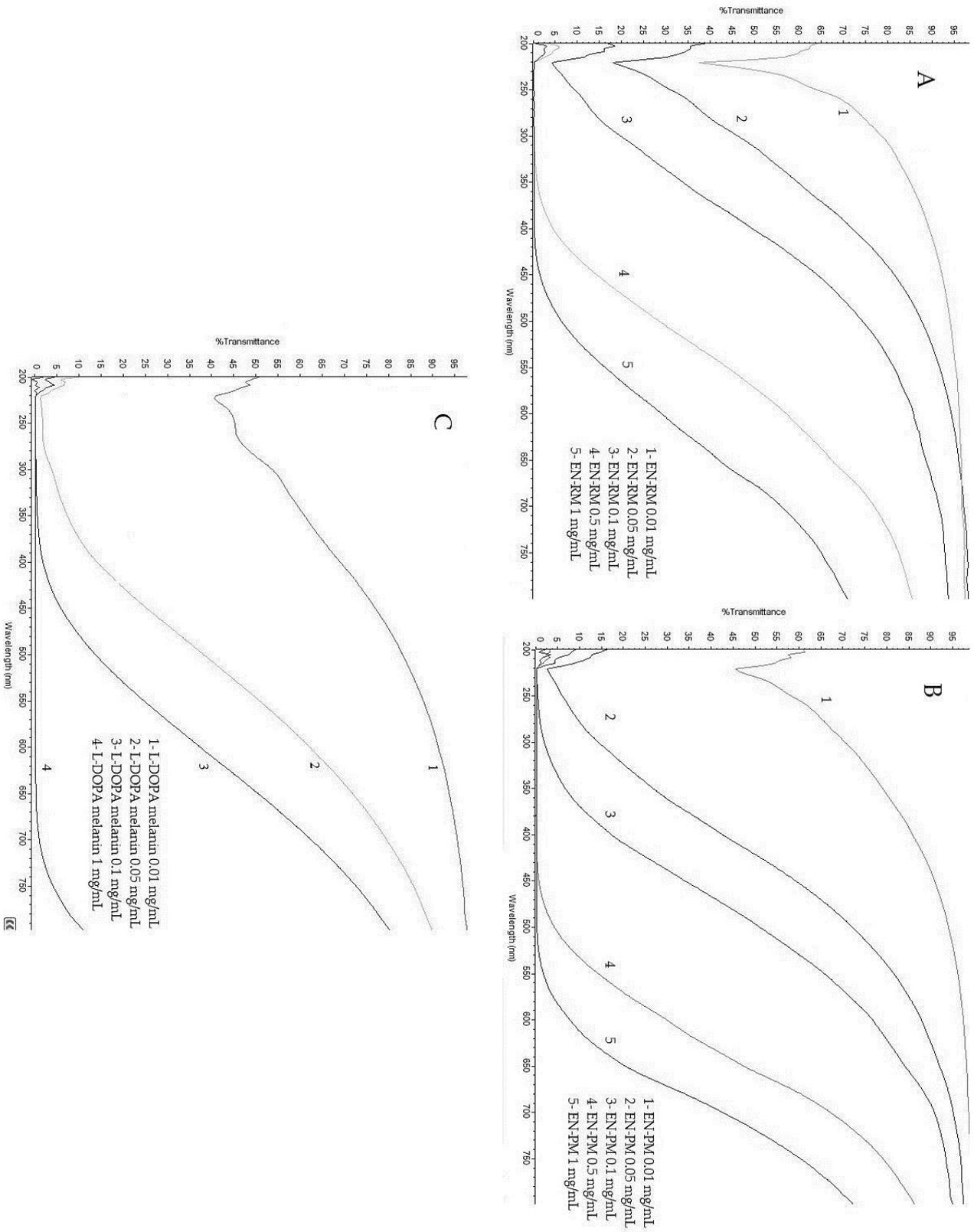|	L*	a*	b*
EN-RM	94.14±0.01	0.49+±0.00	13.58±0.01
EN-PM	84.72±0.00	4.03±0.02	32.85±0.00
L-DOPA melanin	74.87±0.00	10.56±0.00	47.74±0.02

Figure 5. Transmittance values of EN-RM (A), EN-PM (B) and L-DOPA melanin (C)

The TPC values for melanins were 0.10±0.03; 0.23±0.09; 0.29±0.05 mg GAE/g for EN-RM, EN-PM and L-DOPA melanin, recpectively.

In general, the % AA values of EN-PM were higher than those of EN-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 EN-RM, EN-PM and L-DOPA
melanin (mean ±SD, n=3)

EN-RM	%AA	EN-PM	%AA	L-DOPA melanin	%AA
0.0625 mg/mL	0.57±0.05	0.0625 mg/mL	0.71±0.10	0.0625 mg/mL	20.31±0.26
0.125 mg/mL	0.68±0.08	0.125 mg/mL	2.68±0.08	0.125 mg/mL	31.51±1.04
0.25 mg/mL	6.67±0.25	0.25 mg/mL	9.53±0.44	0.25 mg/mL	50.75±0.18
0.5 mg/mL	19.27±0.23	0.5 mg/mL	21.03±0.19	0.5 mg/mL	95.91±0.33
1 mg/mL	59.20±0.13	1 mg/mL	66.75±1.23	1 mg/mL	97.16±0.05

The results of an antibacterial activity assessment of EN-RM and EN-PM are illustrated in Table 4. The zones of growth inhibition of *E. faecalis* and *P. aeruginosa* were 12.3±0.1 mm and 14.5±0.2 mm for EN-RM, respectively, while EN-PM, were 11.1±0.1 mm and 12.2±0.3 mm. No inhibition on *B. cereus*, *E. coli* and *S. aureus* was observed.

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

	BC	EC	EF	PA	SA
EN-RM	-	-	12.3±0.1	14.5±0.2	-
EN-PM	-	-	11.1±0.1	12.2±0.3	-
L-DOPA melanin	-	-	11.4±0.2	13.1±0.1	-

"-" – no inhibition zone, "BC" - *B. cereus* ATCC14579, "EC" - *E. coli* DSMZ1576, "EF" - *E. faecalis* ATCC29212, "PA" - *P. aeruginosa* ATCC2753, "SA" - *S. aureus* DSMZ346

4. DISCUSSION

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

Chemical tests, FT-IR and Raman spectroscopy analysis conducted on isolated pigments in comparison to the synthetic L-DOPA melanin clearly demonstrated that they are

melanins. Purified melanin (EN-PM) was obtained by acid hydrolysis, repeated precipitation and purification through the use of organic solvents [19]. 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 [22].

There was no absorption peak between 260-280 nm in the EN-PM UV-Vis spectrum, indicating, that melanin do not contain proteins and nucleic acids [24]. EN-RM UV-Vis spectrum shows weak absorbtion peak in 260-280 nm region, suggesting that EN-RM molecules may contain some amounts of linked proteins, peptides or nucleic acids. The UV-Vis absorption spectra of the impure (RM) and purified (PM) melanin were similar to those reported in other literature [16-24,27].

A decrease in absorption with increasing wavelength is almost linear in the case of melanins. Hence, the slopes of linear plots are often used to identify melanin. The log of optical density of a melanin solution when plotted against the wavelength produces a linear curve with negative slopes. Such characteristic straight lines with negative slopes have been obtained in some terrestian and marine fungi [19-22,31]. EN-RM and EN-PM had straight lines with negative slopes of -0.003765 and -0.003861, respectively, in comparision to L-DOPA melanin (-0.003741), indicating that the isolated dark pigments are melanins.

The A300/A600 ratios offer information about the oxidation state and the range size of melanin molecules [16]. 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) [16]. EN-PM showed a higher value (15.67) than its corresponding raw EN-RM (12.06) but lower than L-DOPA melanin (16.00). This data supports the fact than EN-PM are a more complex mixture of melanin molecules than that of EN-RM, with a variability in size and degree of oxidation. These data are consistent with the results of Cuevaz-Juárez et al. [16] and also with observations made by Hung et al. [32] 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.

Infrared spectroscopy has been used in the chemical structure study of many melanins. It has been suggested that identical melanin structures do not exist in nature and their chemical characterization is a complicated task. Their composition depends not only on their different monomeric units, but also on environmental conditions during polymerization. Infrared spectrometric techniques offer information on the main functional groups in the melanin structure. A detailed comparative analysis of the infrared spectra of the melanins studied may supply valuable information on the effect of each treatment step used to purify the melanin and the distinct functional groups prevailing in the various samples [16-24].

The Raman spectrum of EN-RM is dominated by two intense and broad peaks at about 1640 cm^{-1} and 1240 cm^{-1}, while EN-PM spectrum peaks at 1620 cm^{-1} and 1230 cm^{-1} were noted. The peaks can be interrelated as originating from the in-plane stretching of the aromatic rings and the linear stretching of the C-C bonds within the rings, along with some contributions from the C-H vibrations in the methyl and methylene groups [33].

A peak at 2000 cm^{-1} from both melanins is similar to those obtained by Galvan et al. [34] from eumelanin and may be caused by the stretching of three of the six C-C bonds within the melanin aromatic rings. It was noted, that on both melanin Raman spectra peaks (385 cm^{-1} and 395 cm^{-1}, for EN-RM and EN-PM, respectively) are present, which are thought to correspond to peaks obtained from pheomelanin and eumelanin and are caused by an out-of-plane deformation of phenyl rings. Peaks 2010 cm^{-1} and 1998 cm^{-1}, for EN-RM and EN-PM, respectively, these are also similar to peaks seen in pheomelanin and are probably due to overtone or combination bands [34].

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 [16,26]. The high antioxidant activity of the melanins was expected due to the protection against UV-radiation and free radical scavenging being their main functions [35-38]. 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 [39]. The high antioxidant activity of melanin isolated from various sources has been reported by other authors [24-40].

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 the environmental conditions, as well as at the developmental stage. The presence of melanins in fungi adapted to different environments and the fact that the same melanin is synthetized by a fungus, indecently of environmental conditions, suggests that this molecule plays different crucial biological roles in fungal physiology [35-37]. 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 [19-21,35-37.40,41]. In several plant pathogens melanin a plays pivotal role in generating osmotic pressure within the appresorium, when hyphae penetrate cell walls [35]. Melanin may play also important role in *E. nigricans* physiology, supporting wood and bark inhabiting.

In fungi the amount of melanin produced is associated with the level of resistance to radiation [35]. Fungi living on rocks, exposed surfaces or in extreme environments are often heavily pigmented and able to resist elevated temperatures and UV radiation [41,42]. *E. nigricans* grows on wood surface and bark, which are often exposed on direct sun radiation. The *Auriculariales* are wood-decomposers inhabiting various hosts, from the tropics to the subartic zone. Some of them are able to survive under extreme climatic conditions, in particular, in arid regions or dry habitats. Two main strategies to persist the drought occur in the order. One of them is represented by genera *Auricularia* and *Exidia*: their gelatinous basidiocarps are able to dry out and revive again during the raining season [1].

Exidia species are known to outlast longer periods of drought than other wood-decaying species (e.g. gilled fungi) because they store comparably large amounts of water.

If arid conditions and evaporation of water from the fruit bodies continue, they can survive in a desiccated condition. Their tissues absorb water rapidly, and growth and spore discharge can continue a few minutes after rewetting [43]. The fungi ability to protect fertile structures from rapidly drying out, to develop spore producing organs inside and under the surface of substrates and to tolerate desiccation together with a rapid uptake of water and rapid development of spore producing structures made these species preferable inhabitants of dead wood [43]. Therefore melanin may play a putative role in mechanism of *E. nigricans* adaptation to drought survival as it is known in other fungi [35,44,45]. Fernandez and Koide [44] suggest that porostiy of cell wall is decreased by melanin and reduced porosity may slow the rate of water loss from the cell, at least initially, upon reduction in soil osmotic potential or water content. Also, sudden rewetting may result in very high turgor press, creating enough strain on the cell wall to cause cell lysis. Melanization may strenghten the cell wall, allowing the cell to withstand elevated turgor pressure resulting from rewetting. Moreover, water stress can lead to the production of free radical, which are harmfull to cell function, and one of the most important melanin functions is scavenging and nautralizing oxidant, which may be of particular advantage during dessication and rewetting. Melanin from *E. nigricans* shown antioxidant activity. There are some reports, that melanin-defficient fungi mutants are more sensible and less resistant for high temperature, dessication, UV-radiation, freezing, digestion by hydrolytic enzymes and have reduced tensile strenght [46].

Rajagopal et al. [31] suggest that apart from protecting fungal hyphae form desiccation, microbial attack, UV radation and osmotic shock melanin may also protect fungi from host defense reactions to overcome host barriers and to survive in harsh environments such as bark and leaf. Considering the mutltifarious role-played by melanin, it would certainly be advantageous for a wood-rotting fungus living on the surface of wood to synthetize this pigment. Finding of this study amply support this that *E. nigricans* the dark colored fungus had melanin. Thus, it is obvious, that *E. nigricans* isolated from bark have evolved panoply of adaptations, which include adaptations for overcoming host barriers, sucessfull competition with other microorganisms and surviving harsh enviornmental conditions.

Antimicrobial assessment results are partially consistent with results found by other authors. Both melanins showed antibacterial activity againt *P. aeruginosa* and *E. faecalis*. No antibacterial activity towards *B. cereus*, *E. coli* and *S. aureus* was observed. Helan Soundra Rani et al. [25] noted the antimicrobial activity of melanin isolated from halophilic black yeast *Hortaea werneckii*. Laxmi et al. [47] 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. [48] 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 [49]. From the other hand there are some reports that melanins have antibiofilm activity agains pathogenic bacteria including *P. aeruginosa* and could interfere with bacterial quorum-sensing system, regulate its associate functions and prevent bacterial pathogenesis [48,50,51].

References

[1] Malysheva V., Spirin V. Taxonomy and phylogeny of the *Auriculariales* (*Agaricomycetes*, Basidiomycota) with steroid basidiocarps. *Fungal Biol*. 2017, 121, 689-715, DOI:10.1016/j.funbio.2017.05.001.

[2] Boddy L., Rayner A.D.M. Ecological roles of *Basidiomycetes* forming decay communities in attached oak branches. *New Phytol*. 1983, 93, 77-88, DOI:10.1111/j.1469-8137.1983.tb02694.x.

[3] Pippola E., Kotiranta H. The genus Tremella (Basidiomycota, Tremellales) in Finland. *Ann. Bot. Fennici* 2008, 45, 401-434, DOI:10.5735/085.045.0601.

[4] McNabb R.F.R. New Zealand *Tremellales − I. N. Z. J. Bot*. 1964, 2, 403-414, DOI:10.1080/0028825X.1964.10428761.

[5] Toma F.M., Ismael H.M., Faqi Abdulla N.Q. Survey and identification of mushrooms in Erbil Governorate. *Res. J. Enviorn. Earth Sci*. 2013, 5, 262-266.

[6] Vasco-Palacios A.M., Franco-Molano A.E. Diversity of Colombian macrofungi (Ascomycota − Basidiomycota). Mycotaxon 2013, 121, 1-58, DOI:10.5248/121.499.

[7] Redhead S. Macrofungi of British Columbia: Requirements for Inventory. *Res. Br., B.C. Min. For., and Wildl. Br., B.C. Min. Environ., Lands and Parks, Victoria, B.C. Work. Pap*. 1997, 28.

[8] Salamaga A., Ślusarczyk D. Diversity of macromycetes in the Białaczów nature reserve (Central Poland). *Acta Mycol*. 2014, 49, 99-107, DOI:10.5586/am.2014.012.

[9] Alvarenga R.L.M., Xavier-Santos S. A checklist of Jelly Fungi (*Agaricomycotina*: Basidiomycota) recorded in Brazil. *Mycotaxon* 2015, 130, 1-14.

[10] Liers Ch., Pecyna M.J., Kellner H., Worrich A., Zorn H., Steffen K.T., Hofrichter M., Ullrich R. Substrate oxidation by dye-decolorizing peroxidases (DyPs) from wood- and litter-degrading *Agaricomycetes* compared to other fungal and plant heme-peroxidases. *Appl. Microbiol. Biotechnol*. 2013, 97, 5839-5849, DOI:10.1007/s00253-012-4521-2.

[11] Peláez F., Martínez M.J., Martínez A.T. Screening of 68 species of basidiomycetes for enzymes involved in lignin degradation. *Mycol. Res*. 1995, 99, 37-42, DOI:10.1016/S0953-7562(09)80313-4.

[12] de Koker T.H., Zhao J., Allsop S.F., Janse B.J.H. Isolation and enzymic characterisation of South African white-rot fungi. *Mycol. Res*. 2000, 104, 820-824, DOI:10.1017/S0953756299002373.

[13] Atiphasaworn P., Monggoot S., Gentekaki E., Brooks S., Pripdeevech P. Antibacterial and Antioxidant Constituents of Extracts of Endophytic Fungi Isolated from *Ocimum basilicum* var. *thyrsiflora* Leaves. *Curr. Microbiol*. 2017, 74, 1185-1193, DOI:10.1007/s00284-017-1303-1.

[14] Roberts P. *Exidia nigricans*: a new and legitimate name for *Exidia plana*. *Mycotaxon* 2009, 109, 219-220, DOI:10.5248/109.219.

[15] Sun X., Wu B., Zhou L., Liu Z., Dong Y., Yang A. Isolation and characterization of melanin pigment from yesso scallop *Patinopecten yessoensis*. *J. Ocean. Univ. China*. 2017, 16, 279-284, DOI:10.1007/s11802-017-3162-6.

[16] Cuevas-Juárez E., Yuriar-Arredondo K.Y., Pío-León J.F., Montes-Avila J., López-Angulo G., Díaz-Camacho S.P., Delgado-Vargas F. Antioxidant and α-glucosidase inhibitory properties of soluble melanins from the fruits of *Vitex mollis* Kunth, *Randia echinocarpa* Sessé et Mociño and *Crescentia alata* Kunth. *J. Funct. Foods* 2014, 9, 78-88, DOI:10.1016/j.jff.2014.04.016.

[17] Kurian N.K., Nair H.P., Bhat S.G. Melanin producing *Pseudomonas stutzeri* BTCZ10 from marine sediment at 96 m depth (Sagar Sampada cruise #305). *Int. J. Curr. Biotechnol.* 2014, 2, 6-11.

[18] El-Naggar E-N., El-Ewasy S.M. Bioproduction, characterization, anticancer and antioxidant activities of extracellular melanin pigment produced by newly isolated microbial cell factories *Streptomyces glaucescens* NEAE-H. *Scientific Reports* 2017, 7, 1-19, DOI:10.1038/srep42129.

[19] Harki E., Talou T., Dargent R. Purification, characterisation and analysis of melanin extracted from *Tuber melanosporum* Vitt. *Food Chem.* 1997, 58, 69-73, DOI:10.1016/S0308-8146(96)00215-4.

[20] Suryanarayanan T., Ravishankar J., Venkatesan G., Murali T. Characterization of the melanin pigment of a cosmopolitan fungal endophyte. *Mycol. Res.* 2004, 108, 974-978, DOI:10.1017/S0953756204000619.

[21] Zhang M., Xiao G., Thring R.W., Chen W., Zhou H., Yang H. Production and Characterization of melanin by submerged culture of culinary and medicinal fungi *Auricularia auricula*. *Appl. Biochem. Biotechnol*. 2015, 176, 253-266, DOI:10.1007/s12010-015-1571-9.

[22] Zhan F., He Y., Zu Y., Li T., Zhao Z. Characterisation of melanin isolated form a dark septate endpophyte (DSE), *Exophiala pisciphila*. *World J. Microbiol. Biotechnol*. 2011, 27, 2483-2489, DOI:10.1007/s11274-011-0712-8.

[23] Selvakumar P., Rajasekar S., Periasamy K., Raaman N. Isolation and characterization of melanin pigment from *Pleurotus cystidiosus* (teleomorph of *Antromycopsis macrocarpa*). *World J. Microbiol. Biotechnol.* 2008, 24, 2125-2131, DOI:10.1007/s11274-008-9718-2.

[24] Ye M., Wang Y., Qian M., Chen X., Hu X. Preparation and properties of the melanin from *Lachnum singerianum*. *IJBAS-IJENS* 2011, 11, 51-58.

[25] Helan Soundra Rani M., Ramesh T., Subramanian J., Kalaiselvam M. Production and Characterization of Melanin Pigment from Halophilic Black Yeast *Hortaea werneckii*. *IJPRR* 2013, 2, 9-17.

[26] Solano F. Melanins: skin pigments and much more – types, structural models, biological functions, and formation routes. *New J. Sci*. 2014, 1-28, DOI:10.1155/2014/498276.

[27] Yao Z., Qi J., Wang L. Isolation, Fractionation and Characterization of Melanin-like Pigments from Chestnut (*Castanea mollissima*) Shells. *J. Food Sci*. 2012, 77, 671-676, DOI:10.1111/j.1750-3841.2012.02714.x.

[28] Breitenbach J., Kränzlin F. *Pilze der Schweiz*, Band 2; Publisher: Verlag Mykologia, Switzerland, 1986; pp. 62-63, ISBN 3-85604-020-X.

[29] Hagara L. *Ottova Encyklopedie Hub*, 1st ed.; Publisher: Ottovo Nakladelství, Czech Republic, 2014; pp. 156, ISBN 978-80-7451-407-4.

[30] Linton A. *A Field Guide to Mushrooms of Britain and Europe*; Publisher: New Holland Publishers, United Kingdom, 2016; pp. 142, ISBN 9781921517730.

[31] Rajagopal K., Kathiravan G., Karthikeyan S. Extraction and characterization of melanin from *Phomopsis*: A phellophytic fungi isolated from *Azadirachta indica* A. Juss. *Afr. J. Microbiol. Res*. 2011, 5, 762-766, DOI:10.5897/AJMR10.514.

[32] Hung Y-Ch., Sava V., Makan S., Chen Tz-HJ., Hong M-Y., Huang G.S. Antioxidant activity of melanins derived from tea: comparision beteen different oxidative states. *Food Chem*. 2002, 78, 233-240, DOI:10.1016/S0308-8146(01)00403-4.

[33] Huang Z., Lui H., Chen M.X.K., Alajlan A., McLean D.I., Zeng H. Raman spectroscopy of in vivo cutaneous melanin. *J. Biomed. Opt*. 2004, 9, 1198-1205, DOI:10.1117/1.1805553.

[34] Galván I., Jorge A., Ito K., Tabuchi K., Solano F., Wakamatsu K. Raman spectroscopy as non-invasive technique for quantification of melanins in feathers and hairs. *Pigment Cell Melanoma Res*. 2013, 26, 1-7, DOI:10.1111/pcmr.12140.

[35] Toledo A.V., Franco M.E.E., Lopez S.M.Y., Troncozo M.I., Saparrat M.C.N., Balatti P.A. Melanins in fungi: Types, localization and putative biological roles. *Physiol. Mol. Plant Pathol*. 2017, 99, 2-6, DOI:10.1016/j.pmpp.2017.04.004.

[36] Fogarty R.V., Tobin J.M. Fungal melanins and their interactions with metals. *Enzyme Microb. Technol*. 1996, 19, 311-317, DOI:10.1016/0141-0229(96)00002-6.

[37] Eisenman H.C., Casadevall A. Synthesis and assembly of fungal melanin. *Appl. Microbiol. Biotechnol*. 2012, 93, 931-940, DOI:10.1007/s00253-011-3777-2.

[38] d'Ischia, M., Wakamatsu K., Napolitano A., Briganti S., Garcia-Borron J-C., Kovacs D., Meredith P., Pezzella A., Picardo M., Sarna T., Simon J.D., Ito S. Melanins and melanogenesis: methods, standards, protocols. *Pigment Cell Melanoma Res*. 2013, 26, 616-633, DOI:10.1111/pcmr.12121.

[39] Różanowska M., Sarna T., Land E.J., Truscott T.G. Free radical scavenging properties of melanin: interaction of eu- and pheo-melanin models with reducing and oxidizing radicals. *Free Radic. Biol. Med*. 1999, 26, 518-525, DOI:10.1016/S0891-5849(98)00234-2.

[40] Al-Laaeiby A., Kershaw M.J., Penn T.J., Thornton Ch.R. Targeted Disruption of Melanin Biosynthesis Genes in the Human Pathogenic Fungus *Lomentospora prolificans* and Its Consequences for Pathogen Survival. *Int. J. Mol. Sci*. 2016, 17, 444, DOI:10.3390/ijms17040444.

[41] Onofri S., Selbmann L., de Hoog G.S., Grube M., Barreca D., Ruisi S., Zucconi L. Evolution and adaptation of fungi at boundaries of life. *Adv. Space Res*. 2007, 40, 1657-1664, DOI:10.1016/j.asr.2007.06.004.

[42] Gunde-Cimerman N., Sonjak S., Zalar P., Frisvad J.C., Diderichsen B., Plemenitaš A. Extremophilic fungi in artic ice: a relationship between adaptation to low temperature and water activity. *Phys. Chem. Earth* 2003, 28, 1273-1278, DOI:10.1016/j.pce.2003.08.056.

[43] Unterseher M., Tal O. Influence of small scale conditions on the diversity of wood decay fungi in a temperate, mixed deciduous forest canopy. *Mycol. Res*. 2006, 110, 169-178, DOI:10.1016/j.mycres.2005.08.002.

[44] Fernandez Ch.W., Koide R.T. The function of melanin in the ectomycorrhizal fungus *Cenococcum geophilum* under water stress. *Fungal Ecol*. 2013, 6, 479-486, DOI:10.1016/j.funeco.2013.08.004.

[45] Vasantahakumar A., De Araujo A., Mazurek J., Schilling M., Mitchell R. Pyomelanin production in *Penicillium chrysogenum* is stimulated by L-tyrosine. *Microbiology* 2015, 161, 1211-1218, DOI:10.1099/mic.0.000030.

[46] Rehnstrom A.L., Free S.J. The isolation and characterization of melanin-deficient mutants of *Monilinia fructicola*. *Physiol. Mol. Plant. Pathol*. 1996, 49, 321-330, DOI:10.1006/pmpp.1996.0057.

[47] Laxmi M., Kurian N.K., Smitha S., Bhat S.G. Melanin and bacteriocin from marine bacteria inhibit biofilms of foodborne pathogens. *Indian J. Biotechnol*. 2016, 15, 392-399.

[48] Xu C., Li J., Yang L., Shi F., Yang L., Ye M. Antibacterial activity and a membrane damage mechanism of *Lachnum* YM30 melanin against *Vibrio parahaemolyticus* and *Staphylococcus aureus*. *Food Control* 2017, 73, 1445-1451, DOI:10.1016/j.foodcont.2016.10.048.

[49] Correa N., Covarrubias C., Rodas P.I., Hermosilla G., Olate V.R., Valdés C., Meyer W., Magne F., Tapia C.V. Differential Antifungal Activity of Human and Cryptococcal Melanins with Structural Discrepancies. *Front. Microbiol*. 2017, 8, 1-13, DOI:10.3389/fmicb.2017.01292.