



Purification, characterization, and biological activities of melanin pigment isolated from Indian squid *Uroteuthis duvaucelii*

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Abstract

Melanin is a natural pigment present in plants, animals, humans, and pigment-producing bacteria. Due to their biocompatibility and versatile biological activities, it was heavily used in cosmetic and pharmaceutical industries and also has market demand. The objective of this study is to develop a strategic method to convert the ink biomass obtained from the Indian squid *Uroteuthis duvaucelii* into a valuable and sensible healthcare product such as melanin. Initially, the squid was collected and processed and the ink was isolated and the powdered form was purified by 150- μm sieve with the respective procedure. Then, they were characterized by UV-Vis spectroscopy, field electron scanning electron microscopy, Fourier transform infrared spectroscopy, and gas chromatography-mass spectrometry. The presence of melanin was confirmed by FE-SEM and the elements present were analyzed using energy-dispersive spectroscopy. In addition, their biological activities such as antioxidant and antibacterial properties were evaluated. DPPH free radical scavenging activity revealed that the powdered ink sample has significant antioxidant potential with increasing concentrations. Also, it showed potent antibacterial properties against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Pseudomonas aeruginosa*. From the present study, we conclude that the melanin was extracted from the Indian squid, and it showed significant antioxidant and antibacterial activities. Furthermore, in the near future, this *Uroteuthis duvaucelii* biomass has to be studied further to formulate value-added products in healthcare and cosmetics by replacing synthetic compounds and formulations.

Keywords *Uroteuthis duvaucelii* · Indian squid · Melanin · Antioxidant activity · Antibacterial activity

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Introduction

The ocean covers more than 70% of the earth's surface which represents the largest habitat present on earth and a profuse resort of organisms including chemical as well as biological diversity (Lotze 2021; Lindequist 2016). A considerable amount of drug has been derived from marine sources even though most of the drugs derived now are from terrestrial sources. Since 2008, more than 30,000 marine-origin compounds have been discovered (Hu 2015). Moreover, every year, around 1000 different compounds are newly explored. Marine resources are characterized based on their diversity, structure, novelty, and complexity (Hu et al. 2015; Kiuru et al. 2014). Marine pharmacology is classified based on the source of the candidate drug as the genetically engineered (GE) marine organisms, pharmaceuticals, and nutraceuticals manufactured from marine sources and chemicals produced or present from marine organisms have pharmaceutical applications (Malve 2016). Marine drugs exhibit various biological actions such as anti-bacterial, anti-inflammatory, neuroprotective, antiparasitic, antiviral agents, anticancer, antimicrobial, and antimalarial activity (Chen et al. 2022; Jo et al. 2021; Murti et al. 2010; and Li et al. 2019).

The class Cephalopoda is one of the seven classes under phylum mollusks which exists in the marine environment for around 500 million years (Meza-Buendia et al. 2022). Cephalopods are further classified into two major orders: nautiloidea and coleoidea. All orders of coleoidea have inking behavior, whereas the order nautiloidea does not possess this behavior. They have an ink sac that releases the ink in response to predators acting as an escape or defense mechanism (Jaitly et al. 2022). Cephalopod ink is secreted from one of the two glands responsible for the ejection of the ink which contains the melanin pigment (Derby 2014). Melanin is the most abundant natural pigment and is responsible for skin color in humans (Brenner and Hearing 2008; Naik and Farrukh 2022). Tyrosine is an amino acid that is responsible for melanin synthesis. Melanin is a complex biopolymer characterized into two forms as eumelanin (brown to black) and pheomelanin (pink to red). The difference in pigment color depends on the molecular precursor. Eumelanin and pheomelanin are derived from tyrosine and cysteine respectively, whereas eumelanin is a polymer, composed of 5,6-dihydroxy indole-2-carboxylic acid (DHICA) and 5,6-dihydroxy indole (DHI) (Palumbo 2003; Prota 2000). Unlike eumelanin, pheomelanin has the composition of benzothiazole, monomers, and benzothiazine (Derby 2014; Morgan et al. 2013; Tanaka et al. 2018). It has been reported that melanin produces other important chemicals including dopamine, tyrosine, and dihydroxyphenylalanine (DOPA) and enzymes such as dopamine-rearranging enzymes, tyrosinases, and peroxidases (Derby 2014). Cephalopod ink also consists of catecholamines (Derby et al. 2007), peptidoglycans (Zong et al. 2013), amino acids (Derby et al. 2007), and metals.

Sepia officinalis is a cuttlefish that belongs to the Cephalopoda family and is heavily reported for its biological activities and valuable products (Endress et al. 2022). *Uroteuthis duvaucelii* is an Indian squid belonging to the same family that is less explored and very little research was reported. This interested us to explore the functional and biological properties of *Uroteuthis duvaucelii*. So, the present study was mainly focused on the extraction of the ink from the squid (*Uroteuthis duvaucelii*), followed by its characterization by UV-Vis spectrophotometer, Fourier transform infrared (FTIR), field electron scanning electron microscopy (FE-SEM), and gas chromatography-mass spectrometry (GC-MS). Furthermore, it was tested for its antibacterial potential against *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Klebsiella pneumoniae* using zone of inhibition studies.

Materials and methods

Sample collection and preparation

The *Uroteuthis duvaucelii* (Indian squid) were purchased from the local market in Chennai, Tamil Nadu, India. Then they were washed clearly and safely transported to the lab in a chilled condition. The ink gland of the squid was dissected and the ink was collected in a sterile condition, and finally stored at -20°C until further use.

Extraction and purification of *Uroteuthisduvaucelii* ink

The extraction procedure was carried out as mentioned by Neifar et al. (2013) with slight modifications. To the 20 mL of pre-prepared ink, 15 mL of distilled water was added to remove the non-melanin layer from the melanin pigment. Then, the mixture was spun at $20,000 \times g$ for 10 min at 4°C , and the supernatant was discarded. To the pellet, 10 mL of hexane was added and spun at $10,000 \times g$ for 30 min at 4°C . Then, the pellet was collected and dried at $55\text{--}60^{\circ}\text{C}$ for 48 h. Finally, the dried sample was ground and the fine powder was collected through a $150\text{-}\mu$ sieve and stored in photosensitive dark tubes.

Characterization of *Uroteuthis duvaucelii*ink

UV-Vis spectroscopy analysis

The ink sample was mixed in hexane at 1:5 w/v and the absorbance was observed from 200 to 800 nm in UV-Vis spectrophotometer Shimadzu UV-1280. Synthetic melanin was used as the standard to compare the sample (Mboniyiriyuze et al. 2015).

FE-SEM-EDS analysis

The fine powder of the powdered ink was then further analyzed to study morphology through field emission scanning electron microscopy (FE-SEM), and also, the qualitative analysis was carried out through energy-dispersive spectroscopy (EDS), Thermo Fisher FEI-Quanta 250 FEG (Gigli et al. 2022).

FTIR analysis

The dried ink powder was mixed with KBr and subjected to Fourier transform infrared spectroscopy (FTIR), Thermo Nicolet iS50 with inbuilt ATR, to predict the functional groups present in the ink (Affandi et al. 2019).

GC-MS analysis

The gas chromatography-mass spectroscopy (GC-MS) analysis was performed to determine the volatile compounds present in the squid ink sample in Shimadzu-QP2010 Plus (Balakrishnan et al. 2018). For mobile phase and stationary phase conditions: hexane

with 1:10 split ratio mode was used as the mobile phase, and silica-coated column with 30 m length, 0.25 mm dia, and 0.25- μ m thickness was used as the stationary phase. For GC conditions, injector and column oven temperatures were set at 280 °C and 45 °C, and the flow rate was set at 1 mL/min with a cut-off time of 30 min. For MS conditions, ion source temperature and interface temperature were set at 250 and 300 °C temperature respectively. The voltage in the detector was set at 1.15 kV and the mass range was set as 50–1000 m/z. Then, NIST library was used to determine the detected compounds (Girija et al. 2014).

Free radical scavenging assay

The antioxidant potential of the squid ink was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay as described previously (Aarland et al. 2019). The various concentrations of the sample ranged from 200 to 1000 μ g/mL, and the ascorbic acid (standard) was added to 1 mL of 0.1 mM DPPH solution. After incubated at the dark condition for 1 h, the absorbance was measured at 517 nm and the radical scavenging activity was calculated as follows

$$\% \text{ DPPH scavenging} = \frac{\text{Control absorbance} - \text{Sample absorbance} \times 100}{\text{Control absorbance}}$$

Antibacterial activity

The antimicrobial activity of the squid ink sample was performed for two strains of gram-positive, namely *Staphylococcus aureus* and *Klebsiella pneumoniae*, and gram-negative strains, namely *Escherichia coli* and *Pseudomonas aeruginosa*. The samples were loaded in the sterile paper discs with various concentrations ranging from 25 to 100 μ g, and the zone of inhibition was determined using ciprofloxacin and erythromycin as standards for gram -ve and gram +ve organisms respectively (Sundaramoorthy et al. 2014).

Results and discussion

Purification of *Uroteuthis duvaucelii* ink

Uroteuthis duvaucelii was carefully collected as shown in Fig. 1. It is a widely consumed marine organism due to its high omega-3 fatty acid content and nutritional values (Chakraborty

Fig. 1 Pictorial representation of Indian squid (*Uroteuthis duvaucelii*)



et al. 2021). It was consumed for its tasty meat, and some of the value-added products such as squid gels and surimi were also prepared from *Uroteuthis duvaucelii* (Lin et al. 2020).

The ink from the *Uroteuthis duvaucelii* was thoroughly purified using n-hexane followed by spinning and collection of samples. Then, the samples were dried and made into powder using mortar and pestle. Furthermore, the fine powder was obtained by filtering in the 250- μ sieve, and finally, the ink powder sample was purified and stored for further characterization and study of their biological activities. The squid ink extract, the dried form of ink, and the fine powder of the purified *Uroteuthis duvaucelii* ink are shown in Fig. 2.

Analytical characterization of *Uroteuthisduvaucelii* ink

UV-Vis absorbance spectra

The UV-visible absorbance spectra showed a maximum wavelength at 220 nm as shown in Fig. 3, for both synthetic melanin and squid melanin, indicating that the extracted *Uroteuthis duvaucelii* ink has the melanin content. According to Mbonyiryivuze et al. (2015), since the absorption was found to be the highest at the ranges between 200 and 250 nm, there was negligible absorption in the visible region. A sharp peak was noticed at 220 nm, and hence, the maximum UV absorption was found to be between 200 and 300 nm; this can be attributed to $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ of aromatic, amino, and carboxylic moieties (Newberry et al. 2017). The blue energy in UV transition contributes to the nonbonding transition from the “n” orbital to an antibonding orbital π^* ($n \rightarrow \pi^*$), which was found to occur primarily in carbonyl bonds (C=O), where these bonds are present in melanin abundantly. In 200–1000 nm region, there is a quasi-constant along with a larger absorbance, which serves as a major source of the color black of the squid melanin. Strong absorption transitions involving the orbital energy of the antibonding π^* and the bonding π ($\pi \rightarrow \pi^*$) are responsible for the broad and extensive spectrum absorption. The aromatic-unsaturated C bonds later experience this (Magarelli et al. 2010). The significant absorption of sepia melanin in the red region of visible squid melanin is mostly owing to the presence of numerous carbonyl groups in its indolic groups. This is the source of squid melanin’s black color (Magarelli et al. 2010).



Fig. 2 The extraction of squid ink using n-hexane (a), dried form of squid ink (b), and fine powder sieved in 150- μ mesh (c)

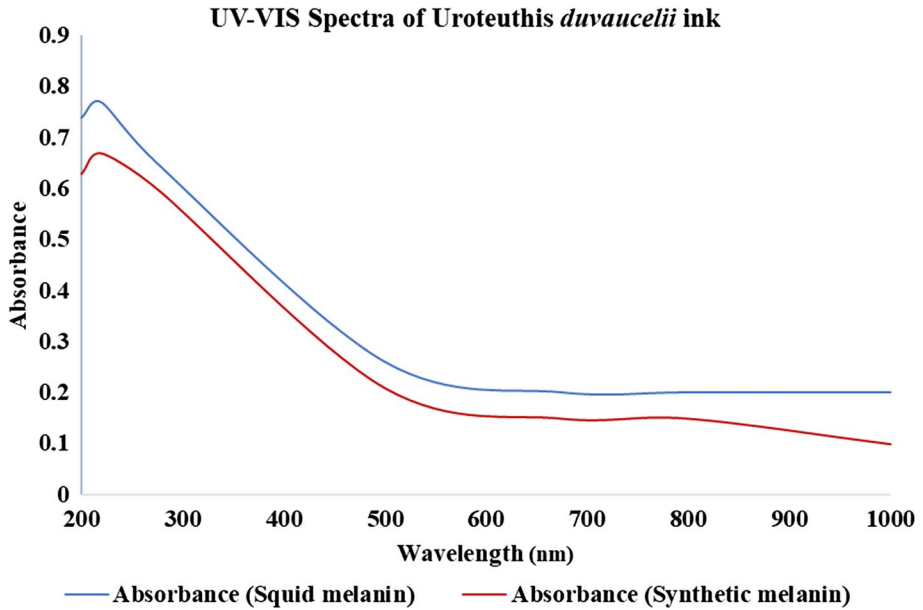


Fig. 3 The peak absorbance at 220 nm with reference to synthetic melanin (red) against squid melanin (blue)

FE-SEM-EDS

The field emission scanning electron microscope/energy-dispersive spectroscopy (FE-SEM/EDS) is generally employed to visualize the shape, size, and topology of the particles (Nasrollahzadeh et al. 2021). The morphology of the purified was visualized in the FE-SEM/EDS, and the 2- μm and 1- μm images are shown in Fig. 4. The FE-SEM clearly depicts the presence of melanin in the purified squid ink as showcased by Gigli et al. (2022). The microscopic visualization indicates that the melanin was spherical in shape, and the particle size

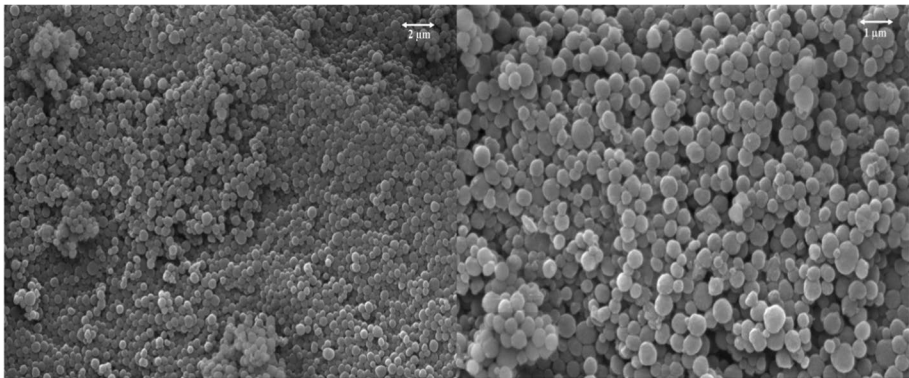


Fig. 4 Field emission scanning electron microscopy (FE-SEM) showing the purified and morphology of squid ink melanin at 2- μm (left) and 1- μm (right) range

ranges from 90 to 100 nm. Also, the carbon (C), oxygen (O), potassium (K), chloride (Cl), sulfur (S), magnesium (Mg), sodium (Na), calcium (Ca), and copper (Cu) were mapped using EDS and visualized at 2.5 μm as shown in Fig. 5. In addition, these elements were also quantified in weight% by EDS as shown in Fig. 6 and Table 1.

FTIR analysis

The Fourier transform infrared (FTIR) is generally employed to determine and evaluate the biochemical content and the structural changes of biomolecules (Rodriguez-Saona and Allendorf 2011; Wang and Wang 2021). The FTIR spectra of the squid ink are shown in Fig. 7, and the FTIR peak shows the presence of functional groups such as 3500–3370 cm^{-1} which represents the N-H group, 1535–1640 cm^{-1} represents diketones, and 1270–1150 cm^{-1} represents the ester carbonyl group.

GCMS analysis

The gas chromatography coupled mass spectrometry analysis is usually utilized to determine the volatile chemical compositions present in the biological sample (Balakrishnan et al. 2018; Purushothaman et al. 2018; Purushothaman et al. 2020). The squid ink was subjected to GCMS analysis to determine the chemical composition present in it.

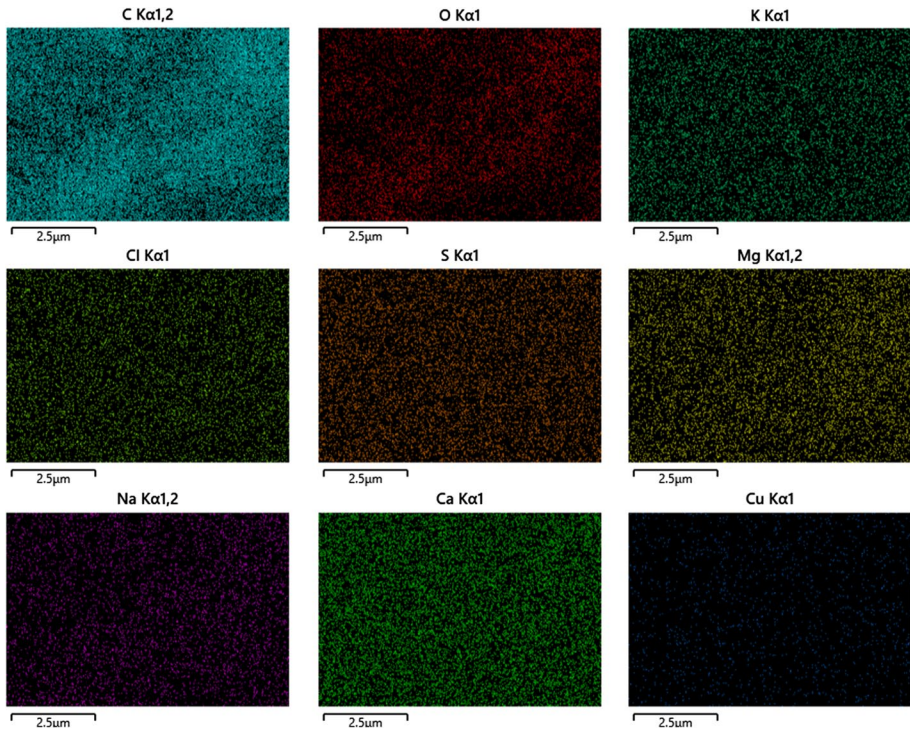


Fig. 5 Energy-dispersive spectroscopy (EDS) mapping representing the elements present in squid ink melanin

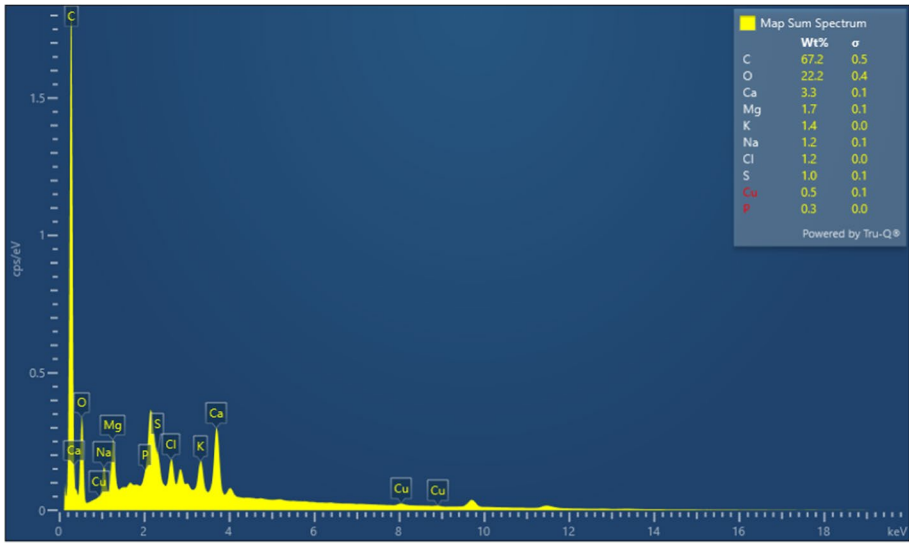
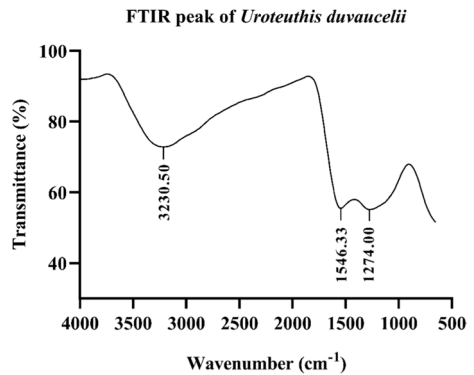


Fig. 6 Energy-dispersive spectroscopy (EDS) analysis showing the presence of carbon (C), oxygen (O), calcium (Ca), magnesium (Mg), potassium (K), sodium (Na), chloride (Cl), and sulfur (S)

Table 1 Elemental analysis based on weight%

Elements	Weight%	Σ
C	67.2	0.5
O	22.2	0.4
Ca	3.3	0.1
Mg	1.7	0.1
K	1.4	0.0
Na	1.2	0.1
Cl	1.2	0.0
S	1.0	0.1
Cu	0.5	0.1
P	0.3	0.0

Fig. 7 Fourier transform infrared peak of *Uroteuthis duvaucelii* melanin



Nearly 328 individual compounds were identified with specific peak area % and unique retention time (RT) as shown in Fig. 8. The top 5 higher composition compounds were tabulated in Table 2. The compounds, namely butanedioic acid-dimethyl ester; decanal; 2,6-octadien-1-ol, 3,7-dimethyl-, (Z); guanosine; and 3-O-methyl-d-glucose, were identified as the top 5 hit compounds with area % of 1.76, 0.55, 5.17, 8.22, and 7.3 and retention time of 8.569, 12.039, 13.082, 17.591, and 21.47 respectively.

Antioxidant activity of squid ink

The oxidative stress results from the overproduction of intracellular reactive oxygen species (ROS) levels, which in turn involved in various pathogenesis including cancer and cardiovascular diseases (Mekala et al. 2021, 2022a, b). The DPPH free radical scavenging potential of the compound/drug/extract is used to measure their antioxidant activity (Ramalingam et al. 2017). The DPPH free radical scavenging activity of the purified squid ink was determined, and the % free radical scavenging activity is tabulated in Table 3. Table 3 represents the DPPH free radical scavenging activity of squid ink with various concentrations ranging from 200 to 1000 $\mu\text{g/mL}$ in comparison with the ascorbic acid (standard). The data clearly depicts that the DPPH free radical scavenging potential is increasing with increasing concentrations of the squid ink, which shows potential activity but is lesser than the ascorbic acid. The maximum DPPH scavenging activity was observed as $76.25 \pm 0.5\%$ at 1000 $\mu\text{g/mL}$ concentration, while the standard showed $81.25 \pm 0.5\%$ at the same concentration.

Antibacterial activity of squid ink

Human pathogenic microbes involved in the progression of different pathogenesis in humans including inflammation and severe infections (Sundaramoorthy et al. 2014). Herein, we have determined the antibacterial potential of the squid ink against human

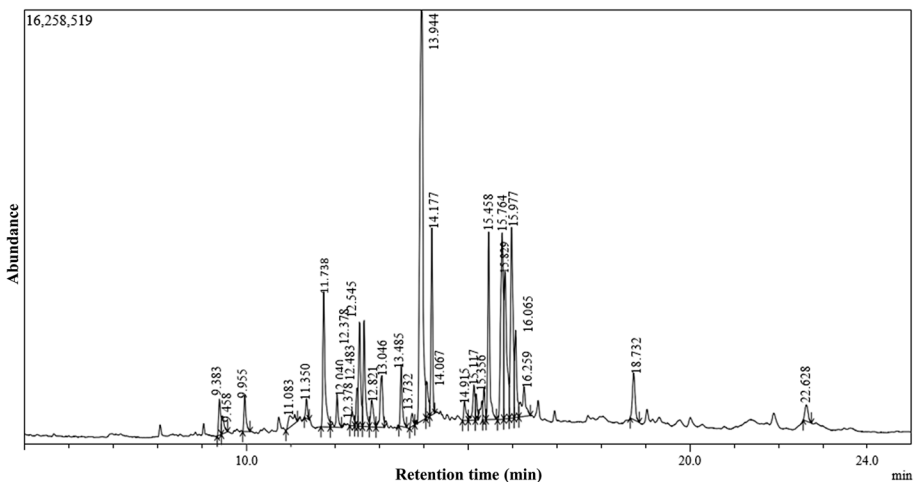


Fig. 8 Chromatogram of squid ink up to 24 RT using gas chromatography-mass spectroscopy

Table 2 GCMS peak data of squid ink

Peak#	Retention time	Area %	Name
1	9.383	1.26	Dodecanoic acid
2	9.458	0.80	2(4 H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4
3	9.955	1.29	1,2-Benzenedicarboxylic acid, diethyl ester (C
4	11.083	1.13	LYXITOL, 1-O-NONYL-
5	11.350	0.60	5,7-OCTADIEN-2-ONE, 3-ACETYL-
6	11.738	5.23	Decanal
7	12.040	1.28	Tetradecanoic acid, ethyl ester (CAS) Ethyl m
8	12.378	0.64	(-)-Loliolide
9	12.483	1.26	2-Cyclohexen-1-one, 4-hydroxy-3,5,5-trimeth
10	12.545	3.73	NEOPHYTADIENE
11	12.647	3.74	2-Undecanone, 6,10-dimethyl-
12	12.821	1.31	Pentadecanoic acid
13	13.046	2.51	Phthalic acid, butyl undecyl ester
14	13.485	1.99	Pentadecanoic acid, 14-methyl-, methyl ester (
15	13.732	0.53	13-Methylpentadec-14-ene-1,13-diol
16	13.944	23.33	Guanosine
17	14.067	1.39	Dibutyl phthalate
18	14.177	5.11	Hexadecanoic acid, ethyl ester (CAS) Ethyl p.a.
19	14.915	0.80	Heptadecanoic acid
20	15.126	1.46	1-Hexadecanol
21	15.175	0.73	Sulfurous acid, hexyl nonyl ester
22	15.356	0.93	Cyclopentanol, 2,4,4-trimethyl-
23	15.458	6.26	Phytol
24	15.764	9.02	3-O-Methyl-d-glucose
25	15.829	7.02	Butanedioic acid, dimethyl ester
26	15.977	8.26	2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-
27	16.065	3.27	ETHYL LINOLEATE
28	16.259	2.04	Octadecanoic acid, ethyl ester (CAS) Ethyl ste
29	18.732	2.17	4,8,12,16-Tetramethylheptadecan-4-olide
30	22.628	0.91	Di-n-octyl phthalate

Table 3 DPPH activity of the squid ink

Concentration ($\mu\text{g}/\text{mL}$)	Ascorbic acid (standard)	Squid ink
200	36.5 ± 0.5	29.5 ± 0.75
400	42.75 ± 0.75	37.75 ± 0.5
600	54.25 ± 0.5	49.5 ± 0.5
800	69.5 ± 0.25	62.25 ± 0.25
1000	81.25 ± 0.5	76.25 ± 0.5

Table 4 Zone of inhibition potential of the squid ink

Organisms Name	Zone of inhibition (mm)				
	25 µg	50 µg	75 µg	100 µg	Ciprofloxacin/ erythromycin
<i>Escherichia coli</i>	8	11	15	18	21
<i>Pseudomonas aeruginosa</i>	6	9	12	15	17
<i>Staphylococcus aureus</i>	6	9	11	14	18
<i>Klebsiella pneumoniae</i>	4	9	13	16	19

pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Klebsiella pneumoniae* using the paper disc method. Ciprofloxacin was used as a standard for gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*, and erythromycin was used as a standard for gram-positive bacteria such as *Staphylococcus aureus* and *Klebsiella pneumoniae*. The various concentrations of the samples (25–100 µg) were loaded in the sterile disc and zone of inhibition in comparison with the standards ciprofloxacin/ erythromycin and tabulated in Table 4. The zone of inhibition was observed to increase with respect to the increase in the concentration of the ink. All concentrations showed potential antibacterial activity against all 4 tested pathogens but showed lesser activities than the standards.

Conclusion

In recent decades, marine organisms are attracting more interest due to their valuable products which offer versatile biological activities, thus having a huge market in the pharmaceutical and cosmetic industries. In the present study, we have focused on the isolation, purification, characterization, and biological activity evaluation of the melanin pigment from the Indian squid *Uroteuthis duvaucelii*. The squid ink was collected and processed, and the powdered ink sample was purified. Then, the chemical composition of melanin pigment in the ink samples was characterized by UV-Vis spectroscopy, FE-SEM, FTIR, and GCMS analysis. In addition, it also shows potent free radical scavenging activity confirmed by DPPH assay and significant antibacterial activity against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Pseudomonas aeruginosa* pathogenic microorganisms. Finally, we conclude that the melanin pigment from squid ink was successfully isolated and characterized, and their biological activities were also assessed. Thus, it has to be studied further to formulate more value-added products in healthcare and cosmetics by replacing synthetic compounds and formulations in the near future.

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Author contribution Sujatha Elangovan: conceptualization, methodology, investigation, validation, writing—original draft. Sivakumar Arumugam: supervision, writing—reviewing and editing. Both the authors proofread the manuscript.

Data availability Not applicable

Code availability Not applicable

Declarations

Ethical approval Not applicable

Consent to participate Not applicable

Consent for publication Not applicable

Competing interests The authors declare no competing interests.

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