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Research Article

CHARACTERIZATION OF SKIN PIGMENTS FROM MARINE CEPHALOPOD *UROTEUTHIS SIBOGAE BY* SPECTRAL ANALYSIS AND ITS PHARMACOLOGIC VALIDATION

[1]Rajashri Sharma C, [1]Shanmugapriya R, *[2]Nalini R and [1]Saravanan R

[1]Post Graduate and Research Department of Zoology, Dr Ambedkar Government Arts College, Vyasarpadi, Chennai-600039, Tamil Nadu, India
Department of Applied Sciences, Chemistry Division, University of Technology and Applied Sciences, Muscat, Sultanate of Oman

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ABSTRACT

Physical characterization of the ethanolic extracts of skin pigments isolated from marine cephalopod, *Uroteuthis sibogae* was performed by spectral studies. FT-IR analysis to determine the presence of different functional groups present in the extracts to confirm presence of pigment-based compounds. UV-VIS spectrophotometer analyzed the peaks between 200 to 600 nm to determine molecular identity of the ommochrome pigments. ^{1}H and ^{13}C NMR revealed the chemical shifts, protons and carbon resonances of the kynurenine based synthesized compounds xanthommatin and dihydroxanthommatin. 2D-NMR COSY spectrum provided structural elucidation of the various compounds present in the pigment extract. This study of marine cephalopod skin derived based pigment compounds provides pharmacophore leads from molluscan species being a source of target for degenerative disease management.

Keywords: *Uroteuthis sibogae,* FT-IR, UV-Visible spectrophotometer, ^{1}H, ^{13}C NMR.

INTRODUCTION

Among the invertebrate animal kingdom, phylum Mollusca has emerged as a prominent source of natural products, contributing substantially to the discovery of biologically active compounds with therapeutic potential effective against cancer and degenerative diseases (Chen *et al*., 2015). Biologically active compounds comprising pigments responsible for coloration in cephalopod skin are primarily ommochromes, a group of polycyclic aromatic compounds biosynthesized through the oxidative metabolism of an amino acid, tryptophan (Figon *et al*., 2020). These pigments are characterized by an extended framework formed around polycyclic, asymmetric aromatic cores that incorporate heteroatoms such as nitrogen and oxygen (Chatterjee *et al*., 2018; Williams *et al*., 2019). Ommochrome pigments that originate from the oxidative metabolic pathway are several intermediate and end products, that include kynurenine, kynurenic acid, 3-hydroxykynurenine, xanthommatin and ommatin D (Williams *et al*., 2016). Based on their core chemical framework, ommochromes are broadly categorized into two structural groups. The first group comprises ommatins, which are built around a phenoxazone ring, a ketone derivative of phenoxazine containing nitrogen and oxygen heteroatoms. The second group includes ommins or ommidins, characterized by a phenothiazine ring system incorporating nitrogen and sulphur atoms. Variations within the ommatin subgroup arise from substitutions on the aromatic ring, resulting in compounds such as dihydroxanthommatin (the reduced form with R = H), ommatin D (R = SO_3H), and decarboxylated xanthommatin formed through removal of a carboxyl group (Aubourg *et al*., 2016).

The ommochromes that are derived from the kynurenine pathway differ in their molecular framework, pigmentation properties and biological functions (Daniels and Reed, 2012). An unstable intermediate, uncyclized xanthommatin, is thought to serve as a crucial metabolic link between the kynurenine pathway and ommatin synthesis within specialized pigment-producing organelles known as

*Corresponding Author: Nalini R, Department of Applied Sciences, Chemistry Division, University of Technology and Applied Sciences, Muscat, Sultanate of Oman, India. Email: rsaravanan0268@gmail.com

ommochromasomes. Owing to its distinctive chemical architecture, uncyclized xanthommatin acts as a central branching point that drives the biosynthetic divergence and structural variability of both ommatins and ommins across a wide range of invertebrates, that comprise insects and cephalopods (Figon *et al*., 2020). The ommochromes compounds responsible for the pigmentation in the cephalopods are synthesized in the skin, produce colour in the biological system, preventing peroxidation in cellular liposomes (Bolognese and Liberatore, 1988). They act as primary antioxidant through their chelating activity by scavenging radicals such as singlet oxygen and superoxide anions. The skin of the Indian squid, *Uroteuthis sibogae* are considered as a waste discard from the animal, that possess significant bioactive compounds and evaluated as a source of useful nutraceuticals.

MATERIALS AND METHODS

Collection of samples

The cephalopod species, *Uroteuthis sibogae* were collected from Kasimedu fish landing centre during the month of March 2024. The whole animal specimens were selected based on same size and physical appearance. About two kg of the squid specimen were conveyed to the laboratory in insulated boxes with dry ice to maintain freshness of the animal. Using running tap water, the sand, mucus and debris were rinsed out of the body parts thoroughly. The arms along with their suckers, ink gland, pen and the tentacles was precisely removed and discarded. The skin layer excised and washed with distilled water. The skin tissues were further processed for preparation of ethanolic extract.

Identification and authentication of the species

Whole animal specimen transported to the laboratory, were identified using FAO identification keys (Jereb and Roper, 1984). International databases and authorised taxonomic references were used to identify the specimens morphologically and anatomically (FAO, 1984). The cephalopods were authenticated by Zoological Survey of India, Marine Biological Regional Centre, Santhome, Chennai. Authentication ID: F.No 4-49/2024-2025/Tech/426 dated 13/05/2024.

Preparation of ethanolic extract and lyophilization

The outer skin layer enclosing the mantle was removed using sterile forceps, washed with distilled water and cut into small pieces. Using 10 ml of ethanol, 10g of the tissue were homogenized by using mortar and pestle following aseptic techniques. To the homogenate 100 ml ethanol was added and incubated for 72 hours at room temperature before further processing. The extracted skin homogenate was then lyophilized using phosphate buffered saline (PBS) in the ratio of 1:10 using a lyophilizer.

Isolation of skin pigments

The isolation of the chromatophoric granules and the extraction of the pigments (ommochromes) were carried out by means of two processes. Solubilization of the ommochromes in acidified ethanol (1% v/v HCl), followed by successive steps of centrifugation. Pigment extraction was done using acidified ethanol. The pigment extraction process from the lyophilized skin extract (w/v) in acidified ethanol (99:1; Ethanol: HCl), The mixture was kept under constant stirring for 10 min in a vortex and sonicated in an ultrasonic bath for 10 min at room temperature 25ºC followed by centrifugation at 10,000 × *g* for 15 min. The pigmented supernatant was collected. The extraction was repeated three times (Van den Branden and Decleir, 1976).

Concentration of the skin pigment extract

The supernatant obtained was collected and concentrated under vacuum in a rotary evaporator (Buchi) at 30ºC and further evaporated to atmospheric conditions. A rotating evaporator with reduced pressure concentrated the solvents and extract was filtered through Whatman No.1 filter paper. The filtrate was poured in previously weighed petridishes and left for further evaporation to dryness. A dark brownish red sticky viscous residue was produced. The dried extract was used for further experimental analysis

Spectral studies of skin pigments from *Uroteuthis sibogae* to analyze the bioactive compounds

Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectra of the skin pigment sample were obtained using a Spectrum 2, IR spectrophotometer, Perkin Elmer, USA to examine the presence of functional groups. The skin pigment residue was characterized by mixing with IR-grade potassium bromate (KBr) and compressed into a disk to create transparent and uniform pellet. The pellet was then positioned in the light beam and scanned over a range of 400-4000cm^{-1} and recorded in diffuse reflectance.

Ultraviolet - visible (UV- Vis) spectroscopy

The UV-Vis-NIR absorption spectrum of pigment extracted from squid skin, obtained using the Perkin Elmer UV WinLab Data Processor and Viewer Version 1.1.00 (integration time: 100 ms), demonstrates characteristic chromophore absorption patterns across the 200-600 nm wavelength range.

Nuclear magnetic resonance (NMR) spectroscopy

Structural elucidation of skin pigment was determined by NMR measurements performed on a Bruker Avance III 500 MHz instrument with an operating frequency of 500 MHz for ^{1}H and 125 MHz for ^{13}C and equipped with a 5 mm triple resonance broadband probe. Experiments were performed in deuterated methanol (MeOD) solution at ambient temperature (298^{0}K). The chemical shifts are reported in ppm, relative to the residual solvent signals at 3.33 ppm for MeOD and 4.9 for moisture. ^{1}H and ^{13}C NMR

spectrum of skin pigment are reported as a chemical shift (δ ppm).

Two-dimensional (2D) correlated homonuclear spectroscopy (COSY)

The skin pigments were analysed additionally by COSY spectra. Two-dimensional ^{1}H-^{1}H COSY experiments were recorded at 500 MHz using methanol- deuterated (MeOD) as solvent at 294-295 K. Phase-sensitive COSY spectra were acquired using a standard cosygpppqf pulse sequence with adequate digital resolution in both F1 and F2 dimensions. Expanded COSY plots (COSY-1 to COSY-4) were generated to facilitate detailed analysis of individual chemical-shift regions.

RESULTS AND DISCUSSION

The FTIR spectra of ethanolic extract of skin pigment chemical constituents of squid, *Uroteuthis sibogae* show frequency assignment (3466-750 cm^{-1}) depicted (Table 1; Figure1). The main signals were observed at 3446-3013 cm^{-1} (N-H; aromatic C-H stretching). A sharp intensity of 2925 $cm^{-1}$and 2851 cm^{-1}corresponds to asymmetric and symmetric stretching bands of CH_2 (C-H stretching vibrations). 1557-1465 cm^{-1} (N-H and C-H bending vibrations), 1223-1029 cm^{-1} (C-O and C-O-C asymmetric stretching vibrations). The wave numbers corresponding to 1731-1640 cm^{-1} for carbomethoxy C=O (1731 cm^{-1}) and quinonic C=O (1640 cm^{-1}) indicated that squid pigments contained ommochromes compounds (Bolognese and Scherillo, 1974; Chan-Higuera, 2019).

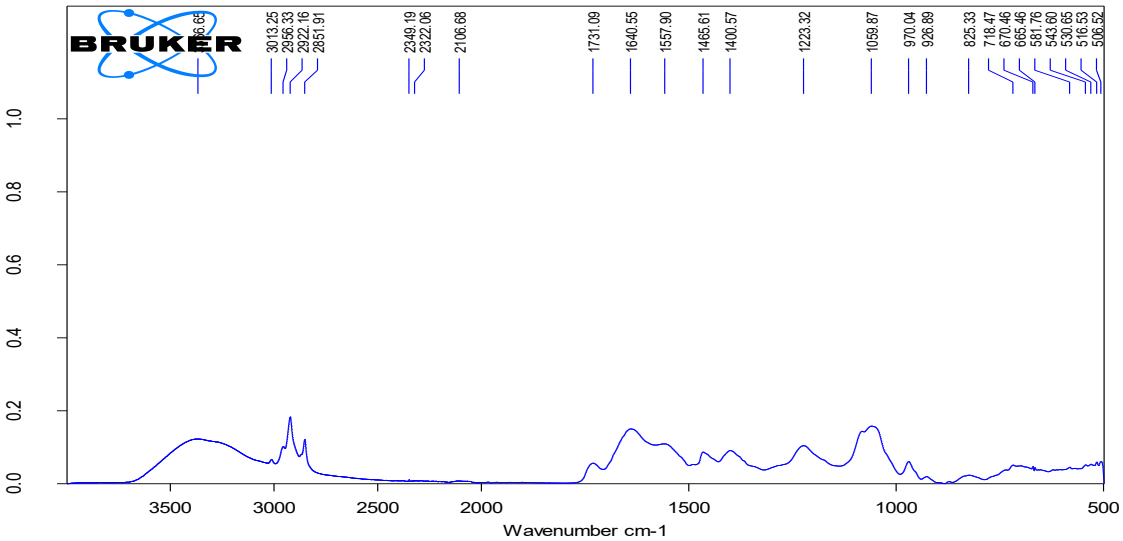


Figure 1. FTIR spectral analysis of *Uroteuthis sibogae* skin pigment extract depicting various peaks and wave number**.**

Table 1. FTIR assignment of functional groups from the skin pigment extract of *Uroteuthis sibogae*.

S.No	Peak (cm^{-1})	Group Interference
1	3466	— O-H or N-H stretching of primary amine
2	3013	Aromatic C-H stretching
3	2925	C-H asymmetric and symmetric stretching (Aliphatic -CH_2 or -CH_3 groups)
4	2851	C -H Stretching vibration
5	1731	aromatic ketone groups (C=O)
6	1640	C=N / C=C stretching
7	1557	NH bending and aromatic C=C stretching
8	1465	C-N / C-H bending
9	1223	C-O stretching
10	1029	C-H in-plane bending / C-O-C asymmetric stretching
11	970 -750	C-H in-plane bending / C-O-C asymmetric stretching

The UV- visible spectra shows broad absorption in the UV- region (Figure 2). The peak at ~220-240 nm attributed to aromatic systems or conjugated double bonds and type of $\pi \rightarrow \pi^*$, indicating the presence of phenolic / aromatic amine structures. Peak at 250 nm (shoulder peak) attributed to non-bonding electrons on oxygen or nitrogen (-OH, -NH_2, -C=O) and type of $n \rightarrow \pi^*$ indicating groups such as carbonyls, amines, or hydroxyls within a conjugated system.

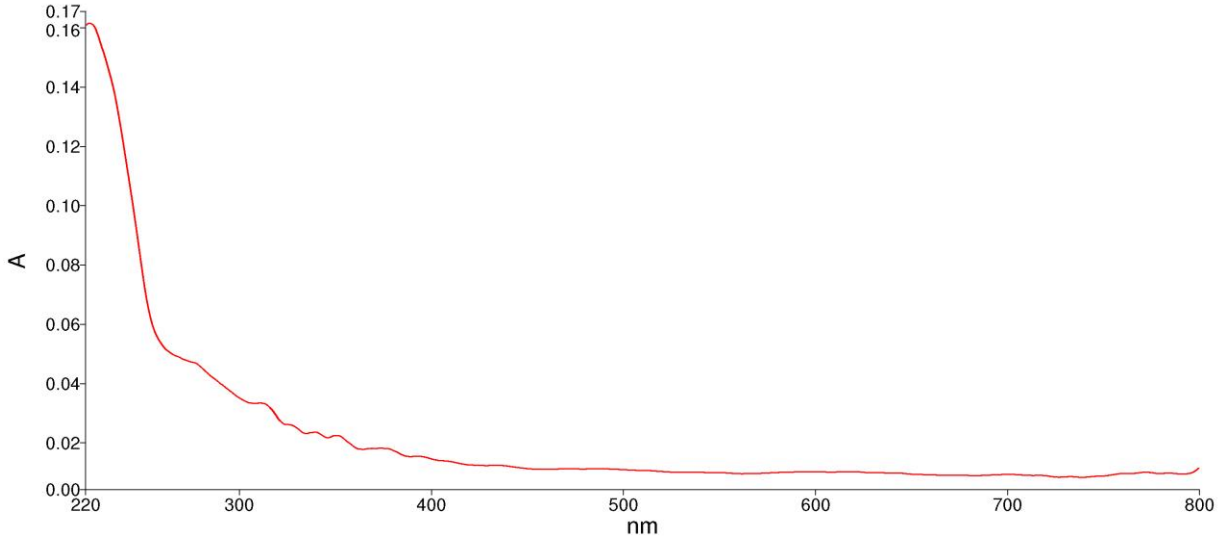


Figure 2. UV-Visible spectrum of skin pigment extract from *Uroteuthis sibogae.*

The typical peaks of ommochromes are commonly characterized by their absorbance spectra with strong absorption at ~220-240 nm (A ≈ 0.16-0.17) and an intense band. Further, two peaks, one at 250-350 nm and another wide band extended between 400-500 nm in the visible region with limited conjugation and n → π* transitions with weak intensity indicating a colored compound in nature. The wavy peaks of different ommochromes occur in the visible region in the 400-500 nm range. This indicates simple aromatic rings, alkenes and non-conjugated systems with basic chromophores**.** In the UV-visible region, characteristic peaks at 240, 300 and 430 nm were identified by their absorbance spectra. The spectra matched the ommatin group of ommochromes (Francikowski *et al*., 2019). The wide and extended bands are attributed to a combination of different ommochrome compounds. The main absorbance reported for xanthommatin and dihydroxanthommatin are observed in the UV and near UV range 430-520 nm (Liu *et al*., 2012; Figon and Casas, 2018).

The ^{1}H NMR (proton NMR) spectrum displays signals that reveal the number of different types of protons, and the integration of these signals indicates the relative proportions of these protons (Figure 3). The ^{13}C (carbon NMR) contribute signals related to aromatic and heteroaromatic carbon resonances, side-chain and substituent carbons (Figure 4). NMR spectra of the skin pigments of *Uroteuthis sibogae* highlight the characteristic spectroscopic signatures of phenoxazinone-based pigments, which are dominated by strongly deshielded aromatic and quinonoid resonances.

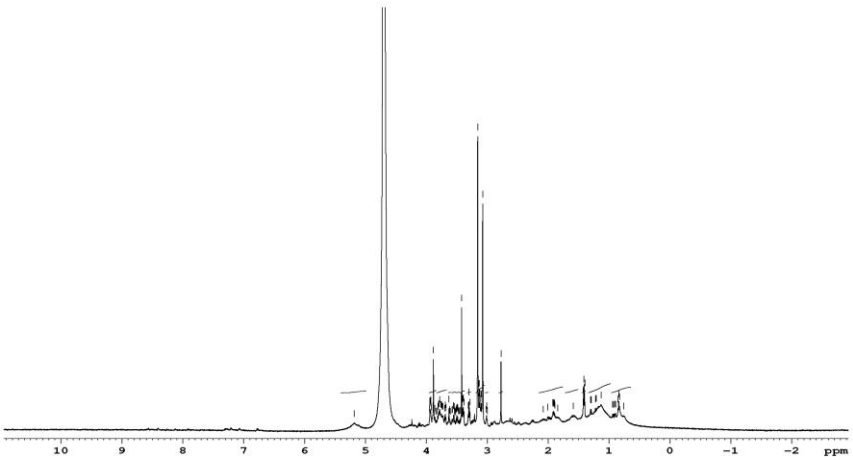
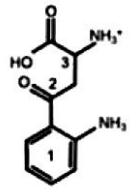
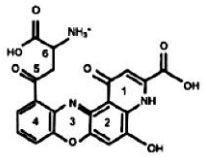
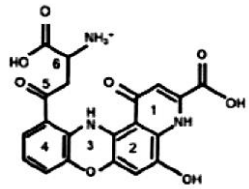


Figure 3. ^{1}H NMR study to determine molecular identity and skin pigment compounds of *Uroteuthis sibogae* by proton signals.

^{1}H NMR (500 MHz, D_2O) of the sample in the spectrum is dominated by well-resolved aliphatic resonances in the range δ 2.7-3.1 ppm, attributable to the β-CH_2 protons, along with a characteristic signal at δ ~4.0 - 4.2 ppm corresponding to the α-CH proton of kynurenine. A simple aromatic multiplet observed around δ 7.1-7.6 ppm is consistent with the phenyl ring of kynurenine. Xanthommatin exhibit a dense and predominantly downfield aromatic or heteroaromatic proton pattern (δ 6.5-8.5 ppm), with broad signals arising from extensive conjugation and hydrogen bonding. Aliphatic protons in aliphatic region are below 3 ppm. Further exchangeable protons associated with -NH and -OH groups are observed. Dihydroxanthommatin, the reduced form of xanthommatin introduce δ 3-4 ppm signals, and retain a strong conjugated aromatic fingerprint with broad conjugated signals (Kumar *et al.*, 2018).

Table 2. Identified ommochrome derivatives by ^{1}H NMR from skin pigment of *Uroteuthis sibogae*.

Ommochrome derivatives identified	**Chemical shift (δ ppm) Protons**	**Structure**
Kynurenine	2.7 - 4.2	
Xanthommatin	6.5-8.5	
Dihydroxanthommatin	3-4	

^{13}C NMR spectra, exhibit deshielded aromatic and heteroaromatic carbon resonances, side-chain and substituent carbons contribute signals in the mid-field and aliphatic regions. Xanthommatin exhibits distinct signals in the δ ~175-185 ppm region, corresponding to quinone and phenoxazinone carbonyl carbons, along with numerous aromatic and heteroaromatic carbons resonating above δ 140 ppm. Dihydroxanthomatin shows a similar pattern, retaining downfield aromatic and heteroatom-substituted carbon resonances despite partial reduction of the quinonoid system. Multiple downfield signals (>150ppm) are due to the presence of quinone/ phenoxazinone C=O and C=N and strongly conjugated aromatic carbons. Key regions are δ160-185ppm (carbonyl/conjugated heterocycles) and 130-155ppm (aromatic/ heteroaromatic carbons). The compound displays significant signals with very few aliphatic carbons in the region between δ10-60 ppm which can be attributed to methylene and methine carbons in the amino acid-derived side chain and associated substituents. In addition, resonances in the δ126-131 ppm region are consistent with the aromatic carbons of a conjugated ommochrome core. The observed resonances therefore support a highly functionalized, partially conjugated pigment framework. The NMR data support kynurenine as the major detectable component, along with the slight detection limit for xanthommatin and dihydroxanthommatin (Table 2). These compounds are highly conjugated heteroaromatic systems (phenoxazinone derivatives). The downfield signals arise from protons within highly conjugated heterocyclic frameworks and are regarded as diagnostic for ommochrome pigments (Esparza-Espinoza *et al*.,2022). Aliphatic proton resonances in the upfield region (δ < 3.5

ppm). Additional signals in the oxygenated aromatic and carbonyl region of the spectrum, reflect the presence of phenolic, quinonoid, and carboxyl functionalities. The overall pattern is therefore compatible with an ommochrome pigment mixture containing xanthommatin and hydroxylated derivatives (Chan-Higuera *et al*., 2019a)**.** The spectrum reveals a single dominant proton spin system extending continuously across the 0.8-5.5 ppm chemical-shift range.

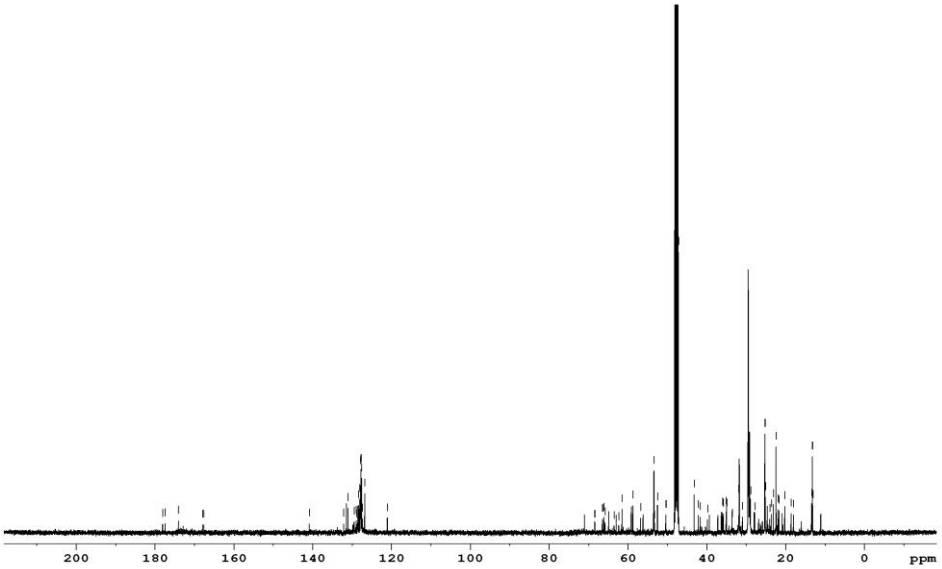


Figure 4. ^{13}C NMR study to determine molecular identity and skin pigment compounds of *Uroteuthis sibogae* by carbon resonances.

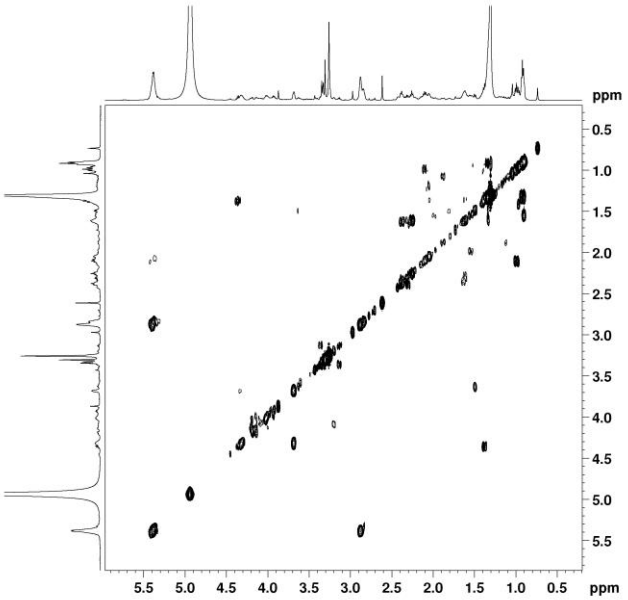


Figure 5a. Proton connectivity of the identified ommochrome pigments in the skin of marine cephalopod, *Uroteuthis sibogae*.

Numerous symmetric off-diagonal cross-peaks are observed, confirming scalar ($^2J/^3J$) coupling between adjacent protons. COSY correlations are detected in the aromatic region (≈6.5-8.5 ppm), indicating aromatic proton networks within the detectable range (Figure 5a). The aromatic COSY "box" and the dominance of aliphatic-heteroatom- adjacent correlations suggest that the principal component of the sample is detectable aromatic and structurally dominated by aliphatic and polar functional groups. The spectrum expansion highlights strong and well-defined cross-peaks among resonances in the 0.8-2.4 ppm region. These correlations correspond to methylene and methine protons forming part of a saturated carbon backbone. Continuous COSY correlations between signals near ~1.6-2.1 ppm are characteristic of CH_2 groups adjacent to carbonyl functionalities, where deshielding shifts the resonances toward ~2 ppm (Figure 5b). The presence of multiple, mutually coupled aliphatic protons with no isolated singlets indicates a flexible, contiguous aliphatic chain, rather than discrete alkyl substituents or terminal methyl groups. This region establishes the foundation of an amino-acid-like side chain.

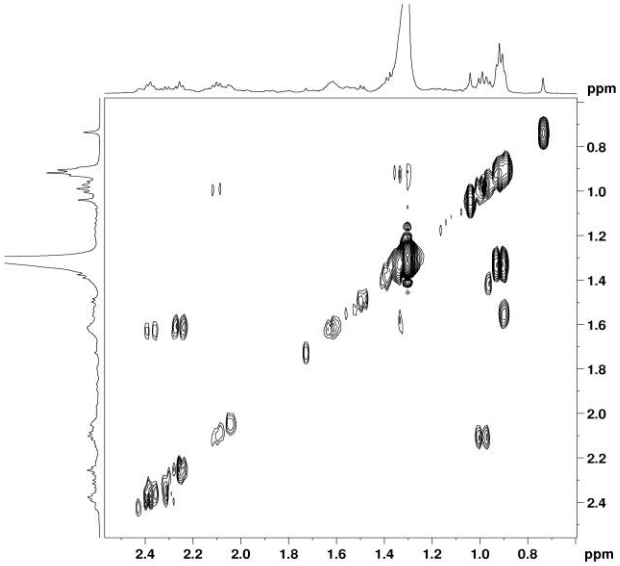


Figure 5b. Aliphatic backbone region of the identified ommochrome pigments in the skin of marine cephalopod, *Uroteuthis sibogae.*

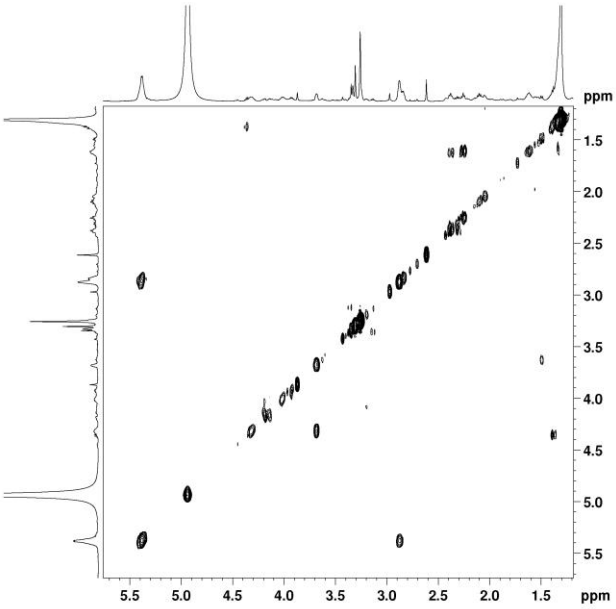


Figure 5c. Backbone-heteroatom connectivity of the identified ommochrome pigments in the skin of marine cephalopod, *Uroteuthis sibogae.*

The spectrum bridges the purely aliphatic region and higher-ppm resonances associated with heteroatom substitution. Clear cross-peaks are observed between resonances at approximately ~2.0-2.3 ppm and ~3.7-4.2 ppm, indicating vicinal coupling between a methylene group and a deshielded methine proton (Figure 5c). Such a pattern is diagnostic of an α-amino acid framework, where the α-CH proton (attached to -NH_2 and -COO^-/-COOH) couples strongly to the adjacent β-CH_2 group. The uninterrupted connectivity from COSY-2 into COSY-3 confirms that the aliphatic backbone is directly linked to a polar, heteroatom-substituted center.

The expansion focuses on the 3.0-5.5 ppm region and reveals cross-peaks among resonances characteristic of protons bound to carbons adjacent to nitrogen and/or oxygen atoms. Signals in the ~3.8-4.4 ppm range exhibit COSY correlations consistent with the α-CH of an amino acid, while additional weaker or broadened features likely reflect proximity to exchangeable NH_2 protons, which are partially averaged in MeOD (Figure 5d). The persistence of COSY correlations in this region indicates that these protons are scalar-coupled and part of the same spin system, rather than isolated heteroatom-bound singlets. This behavior is fully consistent with a zwitter ionic or highly polar amino-acid-derived structure.

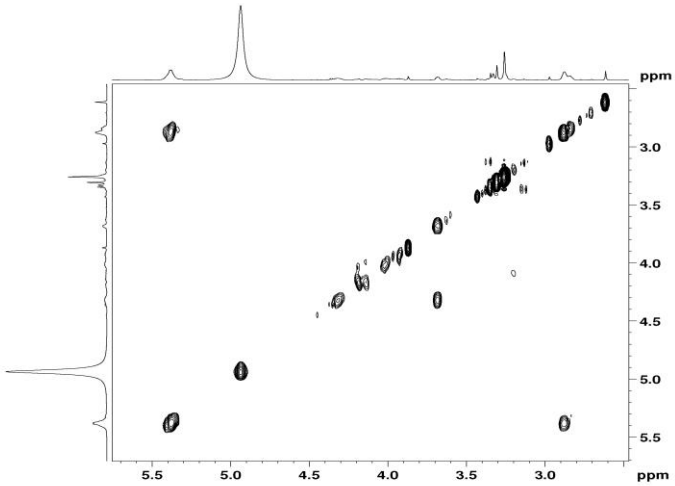


Figure 5d. Heteroatom-adjacent proton region of the identified ommochrome pigments in the skin of marine cephalopod, *Uroteuthis sibogae.*

COSY-1 through COSY-4 define a single, extended spin system characterized by a β-methylene group (~2.0 ppm) coupled to an α-methine proton (~3.8-4.2 ppm), which in turn is associated with nitrogen- and oxygen-substituted functional groups. The combined COSY-1 to COSY-4 spectra reveal a single, continuous aliphatic-heteroatom proton spin system spanning 0.8-5.5 ppm (Figure 5e). The observed β-CH_2 ↔ α-CH correlations and characteristic deshielded α-methine resonances are consistent with an amino-acid-derived structure, specifically a kynurenine-type framework (Englert *et al*., 1990). Multiple aromatic protons on a rigid fused ring system are shown. An amino-acid-derived side chain (α-CH and β-CH_2). Aromatic protons: ~7.4-8.4 ppm. Coupling between adjacent aromatic protons. Side-chain α-CH: ~3.8-4.0 ppm and Side-chain β-CH_2: ~3.5-3.6 ppm. These aromatic protons show strong COSY cross-peaks among themselves, forming a characteristic aromatic COSY box typical of phenoxazinone systems.

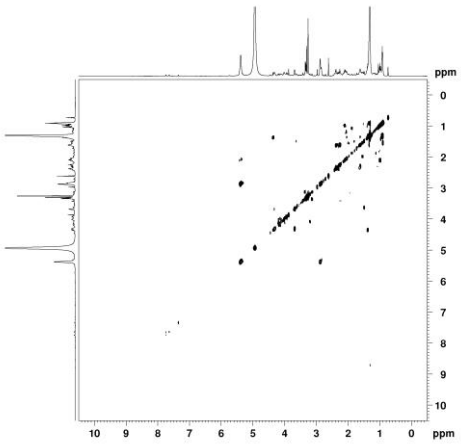


Figure 5e. Integrated interpretation of the chemical shift δ (ppm) and COSY spectrum of the identified ommochrome pigments in the skin of marine cephalopod, *Uroteuthis sibogae.*

This continuous connectivity pattern is diagnostic of a kynurenine-type amino-acid derivative, where the side chain comprises a carbonyl-linked methylene group attached to an α-amino acid center. The COSY data are fully consistent with reported ^{1}H-^{1}H COSY fingerprints of kynurenine (3-anthraniloyl-L-alanine) and related metabolites, in which the aliphatic amino-acid backbone dominates COSY correlations, while aromatic protons may appear outside the displayed windows or be broadened under polar solvent conditions. A higher proportion of kynurenine, which may act as an effective electron donor, and a lower ratio in the aromatic amino acid groups (δ 5.0-3.0 ppm) of xanthommatin and dihydroxanthommatin, were detected in the squid skin pigmented extracts in previous studies (Romero and Martínez, 2015) suggesting that ethanol-HCL solvent has more affinity for aromatic protons and amino group protons. Owing to these distinctive chemical features, phenoxazine based ommochromes and their derivatives have attracted increasing scientific and technological attention, with emerging applications across food science, pharmaceutical development, biomedical research, and materials engineering (Sadhu and Mitra, 2024). Reports of our earlier pharmacological studies indicate that ethanolic extract of skin pigments from *Uroteuthis sibogae* has predictive roles on negative bacterial and fungal species (Shanmugapriya *et al*., 2025a). Research studies from this laboratory establish *Uroteuthis sibogae skin* ethanolic extracts as a source of bioactive compounds with significant antioxidant properties. Concentration-dependent radical scavenging activities of DPPH, ABTS, β-carotene bleaching, reducing power and total antioxidant capacity was reported (Shanmugapriya *et al*., 2025b).

CONCLUSION

For the development of marine-derived bioactive compounds to be successful as innovative medicines in the treatment / prevention of chronic diseases, new technologies and researchers are essential. *Uroteuthis sibogae* squid skin can be an alternative source of bioactive constituents due to their biological activity based on the studies reported from this laboratory in this species of cephaolopod. These natural pigments can be promising pharmacological preparations for the prevention and treatment of pathologies associated with the development of oxidative stress. These skin wastes could be reused and converted into useful products based upon their chemical nature by proper processing which helps to revalue them.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest

ETHICS APPROVAL

Not applicable

FUNDING

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AI TOOL DECLARATION

The authors declare that no AI and related tools are used to write the scientific content of this manuscript.

DATA AVAILABILITY

Data will be available on request

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