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Original Article

# Bioactive properties of cuttlefish, *Sepia pharaonis*, ink extracted with different solvents

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**Abstract:** Cuttlefish ink, a multifunctional marine byproduct, has attracted increasing interest due to its bioactive properties and potential applications in the food, pharmaceutical, and other industries. This study investigated the antimicrobial activity, physicochemical characteristics, and biochemical composition of *Sepia pharaonis* ink extracted using various solvents. The well diffusion method revealed that isopropanol-extracted (IPA) ink exhibited the highest antibacterial activity (27±0.41 mm), while distilled water showed the lowest activity. Biochemical groups in ink and ink extract were identified by FTIR spectroscopy. The ink powder showed strong absorbance peaks followed by IPA, ethanol, and methanol, respectively. Different peaks of absorbance of ink powder and ink extracted with different solvents, indicating the presence of varying absorbance value peaks of phenolic, alcohol, and amine groups. Among the solvents, IPA exhibited absorbance peaks close to those of the ink powder. The FTIR transmittance was found to be significantly lower in the ink powder (76.2±0.04%) and in the ink extracted with IPA (83.81±0.03%). Proximate analysis indicated that ink powder contained higher levels of lipid (0.21%), ash (16.27±0.05%), and crude protein (45.99±0.72%) compared to ink extracts. The DPPH radical scavenging activity assay showed significantly higher activity in ink extracted with distilled water (66.21±0.12% at the concentrate test at 12.5 mg/mL) than with IPA (52.82±0.97% at the tested concentration of 1000 mg/mL). IPA demonstrated superior extraction efficiency, achieving an average yield of 101.31±0.51%. Regarding functional properties, the water absorption index (WAI) remained stable, while the water solubility index (WSI) increased significantly with longer mixing times, reaching a maximum value of 22.09±0.40 after 24 hours. These results demonstrate the potential of *S. pharaonis* ink, depending on the solvent type, to have a significant impact on the effectiveness and functionality of extraction. IPA serves as an effective solvent for ink extraction, exhibiting high absorption characteristics and a significantly larger antimicrobial zone, which indicates potent bioactivity. These findings suggest that IPA-extracted ink possesses promising potential as a natural antioxidant. Ink powder and ink extract have the potential to be implemented, and their commercial viability and safety can be evaluated in the future for biomedicine applications.

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## Introduction

The increasing volume of by-products generated by the food processing industry has raised global concerns regarding environmental sustainability. This issue has prompted the development of value-added strategies, particularly the extraction of bioactive compounds, including proteins, amino acids, vitamins, fatty acids, minerals, and polysaccharides, from bio-residues (Patra et al., 2022). Cephalopod ink, a common byproduct of squid and cuttlefish processing, has garnered attention due to its nutritional, antioxidant, and antimicrobial properties (Zaharah and Rabeta, 2018; Shazwani and Rabeta, 2020; Le et al., 2024). It has been proposed as a potential functional feed additive for both humans and aquaculture, supporting health promotion and disease prevention (Sasaki et al., 1997; Lian et al., 2005; Riyad et al., 2020; Salsabila et al., 2024). Beyond its nutritional value, cephalopod ink exhibits promising therapeutic potential due to its biofunctional compounds, making it suitable for applications in food preservation, pharmacology, and biomedical research (McConnell et al., 1994; Nair et al., 2011; Aulia et al., 2013; Vate and Benjakul, 2013; Hamdi et al., 2024).

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*Sepia pharaonis are in* the order Sepiida and are widely distributed in the Indo-Pacific. Cephalopods employ a range of defensive measures to evade predators, including jet propulsion, camouflage, venom release, inking, and benthic behavior (Hanlon and Messenger, 1996; Hanlon and Ament, 1999; Norman and Reid, 2000; Zohar and Mylonas, 2001). *Sepia pharaonis* exhibits complex reproductive behavior, depositing eggs on hard substrates, such as coral reefs (Okamoto et al., 2017). The species produces a distinctive brown ink, which is stored in specialized ink sacs and secreted as a defense mechanism. This ink consists primarily of melanin, proteins, polysaccharides, alkaloids, and amino acids, which contribute to its strong biological activity (Hanlon and Ament, 1999; Derby, 2014; Okamoto et al., 2017).

Their ink, formed during maturation in a viscous, initially colorless state, becomes pigmented due to the presence of melanin (Liu et al., 2011). The coloration differs across species: black in octopus, blue-black in squid, and brown in cuttlefish (Jeyasanta and Patterson, 2020). Cephalopod ink is especially rich in melanin and antioxidant compounds, which have been widely recognized as multifunctional marine bioactives (Nadarajah et al., 2017; Riyad et al., 2020). These compounds have been shown to exhibit antimicrobial, antiviral, and even anticancer activities (Ismail and Riad, 2018; Shaikh et al., 2018; Hamdi et al., 2024).

One of the properties of ink is its antibacterial nature; several studies have revealed strong inhibitory effects of squid ink against both Gram-positive and Gram-negative bacteria. For instance, Girija et al. (2014) and Shaikh et al. (2018) demonstrated that extracts from squid ink glands inhibited *Escherichia coli* and *Staphylococcus aureus* with inhibition zones up to 28 mm. Diaz and Thilaga (2016) reported that crude squid ink inhibited a wide range of microbial pathogens, including *Vibrio* spp. and *Aeromonas* spp. Such pathogens are common in aquaculture environments, where bacterial infections remain a major cause of mortality. Therefore, squid ink presents a promising natural alternative to antibiotics, which are increasingly restricted due to concerns over antibiotic resistance (Fadjar et al., 2016; Affandi et al., 2019; Islamy, 2019).

Moreover, the antioxidant features of cuttlefish and squid ink are bioactive marine byproducts rich in melanin, proteins, polysaccharides, and catecholamine derivatives such as L-DOPA, all of which are closely associated with antioxidant activity. These compounds contribute to free radical scavenging (e.g., DPPH and ABTS), ferric reducing antioxidant power (FRAP), and metal-chelating properties (Derby, 2014). Both melanin-free and melanin-containing fractions of squid ink have shown strong antioxidant effects in seafood systems (Vate and Benjakul, 2013). Additionally, squid ink polysaccharides can mitigate oxidative damage at the cellular level (Chen et al., 2020). Water-soluble melanin extracted from squid ink has recently been identified as a potential natural antioxidant with applications in the food industry (Liu et al., 2023). Aqueous cuttlefish ink extract has been shown to inhibit lipid oxidation in heated fish muscle, underscoring its potential as a natural additive to preserve seafood quality (Trigo et al., 2023).

Furthermore, the nutritional value and biological activities of squid ink contain great potential for use as a functional ingredient in aquafeeds. However, its physicochemical characteristics and efficacy depend heavily on the extraction method and solvent used. Previous studies have shown that solvent polarity affects the yield and bioactive composition of the extract, influencing its functional performance (Nadarajah et al., 2017; Le et al., 2024). Effective extraction of compounds, such as melanin, alkaloids, and peptides, is essential for maximizing their bioactivity. Hence, the goal of this study is to assess the biofunctional properties of cuttlefish ink extracted with different solvents, with a focus on antimicrobial efficacy, physicochemical characteristics, and potential nutritional composition, to determine its suitability as a functional feed additive.

## Materials and Methods

**Source of cuttlefish:** All samples were collected

using 1×1.5 m green nylon cuttlefish traps from the fishermen at the Andaman coast, Trang Province, southern Thailand. Specimens were stored in iceboxes at 4°C and transferred to the laboratory. The specimens were identified using the key basics of species identification of commercially important cephalopods of Thailand (Sukhsangchan and Sunthornket, 2015). After being identified, all specimens were kept deep-frozen in a refrigerator (-15°C).

**Ink collection:** All fresh specimens were stored in foam boxes and transported to the laboratory via a cold chain vehicle, which maintained temperatures between -15 and -20°C during transportation and took approximately 24-36 hours. After all specimens arrived in the laboratory, they were washed twice with clean water. The specimens' ink sacs were surgically removed using sterile scissors, and then rinsed twice with clean water, and disinfected with ethanol (Ebenezer et al., 2022; Sumi et al., 2023). Following that, the ink was extracted from the ink sac during freezing and collected in sterile vials. The ink was stored at -80°C until analysis (Ebenezer et al., 2022). The ink was dried in a hot air oven during processing to remove any remaining water, and an extract was made utilizing the dry ink powder. A blender was used to grind the dried ink into a fine powder, which was kept at 4°C for further analysis (Jeyasanta and Patterson, 2020).

**Ink extraction:** Cuttlefish inks were extracted using different solvents: methanol, ethanol, petroleum ether, IPA, and distilled water. Five grams of cuttlefish ink powder were mixed with 15 mL of different solvents and stored in a sterile glass bottle (Islamy, 2019; Jeyasanta and Patterson, 2020; Ebenezer et al., 2022). After that, it was homogenized using a shaker at 140 rpm for 7 days at room temperature (Affandi et al., 2019; Halimatul et al., 2019; Girija et al., 2014). Ink extracts in different solvents were mixed well using a vortex (IKA, Vortex 3, Thailand). All samples were collected and used for the well diffusion method (Nathan et al., 1978). Then, the remaining crude extract was filtered through Whatman No. 1 filter paper and evaporated to dryness. The resulting dried extract was stored for future use in experiments (Nithya et al., 2011).

**Antimicrobial properties assay**

***Bacterial cultures*:** Aeromonas veronii strain NT-03 (Dong et al., 2017) was provided by the Centex Shrimp laboratory. The bacterium was cultured in nutrient broth and incubated at 37°C for 24 h. Bacterial growth was confirmed, and the optical density (OD) values were measured spectrophotometrically at 600 nm (Bertani, 1951).

***Total plate count*:** To determine total plate count, samples were serially diluted with sterile saline (0.85% NaCl). 100 µL of each dilution was plated on nutrient agar and spread evenly using a sterilized glass spreader. Plates were incubated at 37°C for 24 hours, and colony-forming units (CFU) were counted. CFU/mL was calculated using only plates with 30-300 colonies (Baird-Parker, 1976).

***Media preparation for antimicrobial activities*:** Mueller-Hinton Agar (38 g/L) was dissolved in distilled water, sterilized at 121°C for 15 minutes, and poured into sterile petri dishes under aseptic conditions. Plates were allowed to solidify at room temperature to ensure a uniform surface (Girija et al., 2014).

***Antimicrobial activity assay*:** The agar well diffusion method was used following the protocol of Nathan et al. (1978). The agar surface was inoculated with 100 µL of A. veronii suspension using a sterile swab. Wells (7 mm diameter) were punched into the agar using sterile pipette tips. Cuttlefish ink extracts (200 µL) prepared in different solvents were loaded into each well. Ampicillin (10 mg/L) was used as a positive control, and wells containing only solvent served as negative controls (Ismail and Riad, 2010; Jeyasanta and Patterson, 2020). Each solvent was made in four replications. The plates were incubated at 37°C for 24 hours. Zones of inhibition were measured in mm, and activity levels were classified as follows: high (>15 mm), moderate (10-14 mm), low (5-9 mm), and none (<4 mm) (Rios et al., 1988; Nithya et al., 2011).

**Biochemical component:** The highest antimicrobial activity was selected to determine the biochemical component. The Fourier transform infrared (FTIR)

technique is commonly used to determine and evaluate the biochemical content and structural changes of biomolecules, as reported by Rodriguez-Saona and Allendorf (2011), Glass et al. (2012), and Wang and Wang (2021). The region of the infrared spectrum was set at mid-infrared, which ranges from 4000 to 700 $cm^{-1}$. The cuttlefish ink powder, 4 mg, was mixed with potassium bromide (KBr), 1400 mg. Then, the ground material was pressed into pellets using a mortar and pestle for measurement and analysis. The ink powder was placed on the crystal cell and then attached to the mount of the FTIR spectrophotometer. The signal was collected at a resolution of 4 $cm^{-1}$ from a range of 4000-700 $cm^{-1}$ using a LUMOS II FT-IR microscope.

**Proximate composition:** The ink extracted from the best solvent was selected and compared to the ink powder for analysis of the composition. The extracted ink and ink powder were collected in three replications. The estimation was performed according to the standard protocol of AOAC (1990). Crude protein was determined by the micro-Kjeldahl method. The FOSS Kjeltec 8100 apparatus was used for analysis (FOSS Analytical AB, Hoganas, Sweden). Crude lipid was tested by the Soxhlet method using a FOSS Soxtec 2043 apparatus (FOSS Scino Co. Ltd., Suzhou, China). Moisture and ash were estimated by an air oven Lab Tech (LDO-100E, Daihan Labtech Co., Ltd., Namyangju City, Korea). Ash was estimated by using the muffle furnace method. The Lab Tech Model LEF-115S-1 muffle furnace was used to analyze the ash in the feed.

**DPPH radical scavenging assay**

***Sample extraction***: Based on the best effective solvent screening result, the selected solvent was used to extract the ink to evaluate the activity of the DPPH radical scavenger. For comparison, the control ink sample was extracted with distilled water. Approximately 1 g of ink powder was weighed and mixed with 2 ml of solvent, resulting in an ink-to-solvent ratio of 1:2 (w/v). After homogenization, the mixtures were transferred to 15 mL centrifuge tubes for centrifugation at 4,000 rpm for 30 minutes at 4°C to separate the soluble antioxidant compounds. The resulting supernatants were carefully decanted into amber vials to minimize light exposure and immediately stored at –80°C in darkness until subsequent antioxidant activity assays. The extraction method was adapted based on established procedures described in previous studies (Zancan et al., 2023; Waterhouse, 2002; Gajula et al., 2009; Chalamaiah et al., 2018; Ratanasiriwat and Pienchob, 2018).

***DPPH radical scavenging activity*:** The antioxidant activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method, as described by Brand-Williams et al. (1995) with slight modifications. A DPPH solution was prepared by weighing 0.0039 g of DPPH and adding 100 mL of methanol to achieve a 100 µM concentration. The antioxidant extraction was put in the test tube, and methanol was added, followed by a series of dilutions. Then, DPPH was added 1 mL (1:1), and the mixture was incubated in the dark at room temperature for 30 minutes. The absorbance was measured at 517 nm using a T60UV spectrophotometer (PG Instruments Limited, Leicestershire, UK), and the results were compared to a methanol blank (Ratanasiriwat and Pienchob, 2018; Zaharah and Rabeta, 2018; Rajesh et al., 2021).

***Calculation of % DPPH radical scavenging activity***: The scavenging activity was calculated using the equation (Rajesh et al., 2021) of DPP Scavenging Activity (%=[1-(ABS sample/ABS control)] x 100.

**Yield:** Based on the best result against microbial and biochemical components, yield refers to the amount of ink obtained from a solvent reaction compared to the amount of starting ink. Yield was calculated based on the ratio of ink powder weight after extraction to the ink powder weight before extraction, and then multiplied by 100 (Chamidah et al., 2013).

**Water solubility index and water absorption index:** To compare the efficiency of the water solubility index (WSI) and water absorption index (WAI), experiments were conducted in three replications with two factors: 24 hours and 30 minutes. The water solubility index (WSI) was determined using the method described by Anderson et al. (1969) and Amza et al. (2011), with slight modifications.

Table 1. Diameter of antibacterial inhibition zones for different solvents.

| Solvents | Clear zone (mm.) | Classified activity |
|---|---|---|
| Methanol | 13.75±0.48$^{c}$ | Moderate activity |
| Ethanol | 16±0.41$^{b}$ | High activity |
| Petroleum ether | 11.25±0.48$^{d}$ | Moderate activity |
| Distill water | 9±0.41$^{e}$ | Low activity |
| IPA | 27±0.41$^{a}$ | High activity |
| Ampicillin | 9.5±0.29$^{e}$ | No activity |
| Negative | 9.5±0.29$^{e}$ | No activity |

Table 2. FTIR transmittance (%) of the ink powder and ink extracted.

| Treatment | FTIR transmittance (%) | Number of peaks |
|---|---|---|
| Ink powder | 76.2±0.05$^{a}$ | 6 |
| Ink extracted by IPA | 83.81±0.03$^{a}$ | 7 |
| Ink extracted by methanol | 100.9±1$^{b}$ | 11 |
| Ink extracted by ethanol | 102±0.01$^{b}$ | 7 |

Approximately 2 g of the sample was combined with 30 mL of distilled water in a test tube and thoroughly mixed using a vortex mixer (IKA, Vortex 3, Thailand). The sample was incubated at 60°C for 24 hours and 30 minutes. Subsequently, the samples were centrifuged at 4,000 rpm for 10 minutes using an OHAUS FC 5816R centrifuge (Germany). The supernatant was carefully collected into a pre-weighed beaker, while the residue was dried at 105°C for 24 hours to remove any remaining water. The dried residue was weighed, and the water solubility index was calculated using the equation: WSI (%) = (Weight of residue after drying (g) / Initial sample weight (g)) ×100.

The water absorption index (WAI) was calculated as the weight of precipitate (in grams) per gram of the initial weight of ink powder, providing a quantitative measure of water absorption capacity as follows: WAI (%) = (Weight of sediment (g)/Weight of dry sample (g)) ×100

**Statistical analysis:** The results were expressed as the average ± standard error (SE) for four replications in antimicrobial analysis and triplicate determinations in others. Homogeneity was used as a test for normality distribution. Analysis of variance and the significant differences between mean values were determined using the Tukey test at a significance level of $P<0.05$. Data were analyzed using a one-way ANOVA test in IBM SPSS Statistics (Version 23).

## Results

**Antimicrobial properties:** The results showed that antibacterial effectiveness varied depending on the solvent used for extraction. This study highlights the potential of *S. pharaonis* ink as a source of antimicrobial compounds, with its efficiency influenced by the choice of solvent. The colony count was $5.5*10^{8}$ CFU/mL, and OD was 1.186. The largest zone of inhibition was observed with IPA as the solvent, exhibiting a diameter of 27±0.41 mm. This was followed by ethanol, which produced a zone of 16±0.41 mm. The smallest clear zone was observed with distilled water (9±0.41 mm) (Table 1).

**Biochemical component:** The current study aims to explore the functional groups of *S. pharaonis* ink, which is extracted with methanol, ethanol, and IPA, by using FTIR. According to the extracts of ink in different solvents, they exhibited varying absorption ranges. The percent of transmittance was shown to be significantly highest in the ink powder (76.2±0.04%) and in the ink extracted with IPA (83.81±0.03%). The lowest transmittance was observed in the extracts prepared with ethanol (102±0.01%) and methanol (100.9±0.0%). Strong absorbance was shown in the control (ink powder), followed by the ink extracted by IPA, compared to other solvents.

The strong absorbance peaks in ink extracted by IPA observed at 3344.40 $cm^{-1}$ and 3299.58 $cm^{-1}$ correspond to the N-H and O-H groups, which are characteristic of alcohols, phenols, and amines. N-H and O-H overlap indicated the presence of a carboxylic acid group. A second medium-intensity absorption peak was observed at 2934.88 $cm^{-1}$,

Table 3. Chemical composition of ink powder and ink extract.

| Proximate composition | Treatments | |
|---|---|---|
| | Ink powder | Ink extract |
| Moisture (%) | 17.24±0.24[b] | 23.21±0.16[a] |
| Crude protein (%) | 45.99±0.72[a] | 42.25±0.32[b] |
| Crude lipid (%) | 0.21±0.00[a] | 0.18±0.00[b] |
| Ash (%) | 16.27±0.05[a] | 15.80±0.1[b] |
| Fiber (%) | 0.46±0.00[a] | 0.43±0.01[b] |
| NFE (%) | 37.07±0.88[b] | 41.33±0.3[a] |

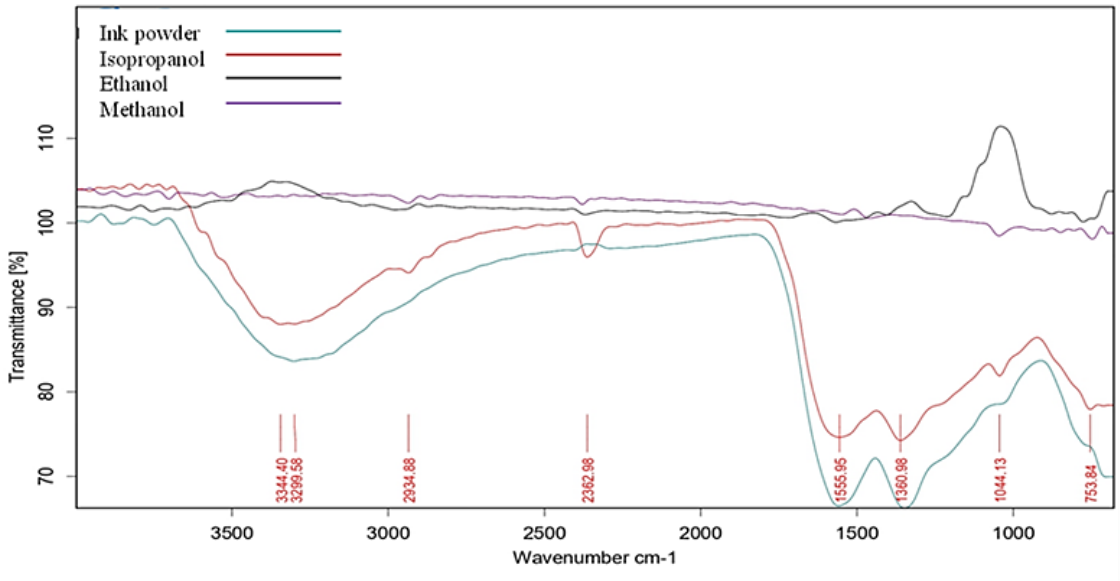


Figure 1. FTIR peak of ink powder and ink extract in different solvents.

indicating the presence of the C-H group, which is typically found in alkanes (Pavia et al., 2013). Further absorption peaks at 2362.96 $cm^{-1}$ were attributed to the O-H group of carboxylic acids. Peaks at 1555.95 $cm^{-1}$ correspond to the N-H group, associated with aliphatic amines. Additional peaks at 1360.98 $cm^{-1}$ were ascribed to nitrogen-containing groups, specifically aliphatic nitro compounds.

Weak peaks between 1044.13 $cm^{-1}$ were attributed to the C-O group, typically found in alcohols and phenols. Finally, a weak peak at 735.84 $cm^{-1}$ was assigned to the C-Cl stretch, characteristic of primary chlorides. *The Fourier* transform infrared spectroscopy peak is shown in Table 2 and Figure 1.

**Proximate composition:** Based on the comparison of the chemical composition between the ink powder and the ink extract using IPA (Table 3), the crude protein, lipid, ash, and fiber contents in ink powder were measured at 45.99±0.72, 0.21, 16.27±0.05, and 0.46, respectively, which were significantly higher than in the ink extract. In contrast, the moisture and nitrogen-free extract (NFE) in the ink extract were 23.21±0.16 and 41.33±0.3, respectively, both of which were significantly higher than the values observed in the ink powder.

**DPPH radical scavenging activity:** The antioxidant mechanism is primarily recognized as radical scavenging; this test measures the ability of antioxidants to inhibit the capacity of DPPH. The antioxidant capacity of cuttlefish ink powder and ink extracted using IPA was evaluated using the DPPH radical scavenging assay. Ink powder extracted with IPA showed a scavenging activity of 52.82±0.97% at the tested concentration of 1000 mg/mL. To consider the properties of pure ink powder, distilled water was chosen as the solvent for ink extraction. The DPPH radical scavenging activity shown was 66.21±0.12% at the concentrate test at 12.5 mg/mL. The DPPH radical scavenging assay in the ink powder was shown to be significantly higher than in the IPA-extracted

Table 2. UV-visible spectral analysis result of S-CSNPs.

| Ink powder | Treatments | |
|---|---|---|
| | 30 minutes | 24 hrs. |
| Water solubility index | 17.78±0.04[b] | 22.09±0.4[a] |
| Water absorption index | 2.32±0.03[a] | 2.3±0.02[a] |

ink.

**Yield:** The calculation results for the final yield of the extracted ink showed an average of 101.31±0.51%. This indicates that the yield of ink extracted using IPA increased.

**Water solubility index and water absorption index:** The results indicated that drying conditions and mixing time had a significant effect on WSI ($P \leq 0.05$). The highest WSI value (22.09±0.4) was observed in cuttlefish ink powder mixed for 24 hours, while the lowest value (17.78±0.04) was recorded in the 30-minute treatment. Conversely, WAI did not show a significant difference between the two treatments. The values were 2.32±0.03 for the 30 minutes of mixing and 2.30±0.02 for the 24 hours of mixing. A summary of the WSI and WAI values for cuttlefish ink powder under different mixing conditions is presented in Table 4.

## Discussions

Similar results regarding solvent-dependent extraction efficiency have been reported in studies on marine-derived bioactive compounds (Trigo et al., 2023; Casula et al., 2025).

**Antimicrobial activity:** The present study demonstrated that *S. pharaonis* ink extracts exhibited significant inhibitory effects against *A. veronii*, consistent with previous reports (Nithya et al., 2011; Girija et al., 2014; Ismail and Riad, 2018; Islamy, 2019; Zakaria et al., 2019; Hossain et al., 2019; Sari et al., 2019; Fitrial and Khotimah, 2021; Ebenezer et al., 2022). The antimicrobial efficacy varied according to the solvent used, indicating that the chemical composition and extraction efficiency of bioactive compounds were solvent dependent. Extracts prepared with organic solvents generally produced larger inhibition zones than the positive control (ampicillin), whereas extracts prepared with distilled water resulted in the smallest inhibition zones, likely due to their poor ability to dissolve active compounds (Ismail and Riad, 2018). Methanol-extracted ink showed inhibition zones comparable to those in prior studies, such as 19±0.9 mm (Zakaria et al., 2019; Islamy, 2019) and 14±0.8 mm (Ismail and Riad, 2018). However, variations may be due to differences in solvent polarity, pathogen strain, and inoculum density. Although methanol and ethanol are widely used to break hydrogen and ionic bonds in biomolecular systems (Eisenberg and King, 1977), they demonstrated lower antibacterial activity and weaker FTIR absorbance in this study compared to IPA. IPA proved to be the most effective solvent, exhibiting stronger antibacterial activity and greater FTIR absorbance, which suggests an efficient extraction of bioactive compounds (Yilgor et al., 2003). In contrast, petroleum ether and distilled water showed minimal or no antibacterial activity. These findings confirm that IPA is superior for extracting functional compounds from cuttlefish ink, particularly those with antimicrobial potential.

**Biochemical component:** Fourier Transform Infrared (FTIR) spectroscopy was used to characterize the functional groups in *S. pharaonis* ink extracted with methanol, ethanol, and IPA. FTIR is a widely accepted analytical method that identifies molecular structures and functional groups in complex biological matrices (Kumosinski and Farrell, 1993; Patrick et al., 1993; Palaniappan and Vijayasundaram, 2008).

The spectral profiles showed that IPA-extracted ink had the highest absorbance, reflecting its superior efficiency in extracting bioactive compounds. Major absorption peaks included 3344.40 and 3299.58 $cm^{-1}$ (N–H and O–H groups in amines, amides, alcohols, and phenols), 2934.88 $cm^{-1}$ (C–H stretch of alkanes), and 2362.96 $cm^{-1}$ (O–H in carboxylic acids). Other notable peaks were 1555.95 and 1360.98 $cm^{-1}$ (aliphatic amines and nitro compounds), along with weaker signals at 1044.13 and 735.84 $cm^{-1}$

corresponding to alcohols, phenols, and chlorinated compounds (Nadarajah et al., 2017; Affandi et al., 2019; Elangovan and Arumugam, 2023; Le et al., 2024).

These findings confirm the complex biochemical nature of cuttlefish ink. The prominent bioactive pigment melanin, specifically eumelanin, was identified, supported by functional group markers of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA), both derived from the amino acid tyrosine (Ito and Wakamatsu, 1998; Pezzella et al., 1997; Simon et al., 2009). These results validate IPA as an effective solvent for extracting biologically active components from cephalopod ink.

**Proximate composition:** In this study, a comparative analysis of the proximate composition between cuttlefish ink powder and IPA-extracted ink revealed that the ink powder contained significantly higher levels of crude protein, lipid, ash, and fiber. This is likely due to the higher concentration of melanin, a natural pigment composed of carbon, hydrogen, oxygen, and nitrogen ($C_{18}H_{10}N_2O_4$; 318 g/mol), found in the dried form (Nauen, 2003; Mourad et al., 2015; Kipalahi et al., 2024). The moisture content was relatively low in the powder (17.24±0.24%) but higher in the extract (23.21±0.16%), exceeding the values reported in previous studies (Puttongsiri and Phuruen, 2017; Zaharah and Rabeta, 2018; Kipalahi et al., 2024; Salsabila et al., 2024). This difference may result from cold storage conditions (4°C), which limit evaporation and affect free water retention (Zaharah and Rabeta, 2018). The increased levels of moisture and non-fat extract (NFE) in the extract might also result from the solvent extraction process, which leads to the dissolution of bioactive substances such as melanin, proteins, lipids, and minerals (Islamy, 2019; Zakaria et al., 2019; Sari et al., 2019; Abidin et al., 2021).

The protein content in the powder (45.99±0.72%) was higher than in the extract (42.25±0.32%), potentially due to reduced protein degradation during the drying process. Extraction conditions, including temperature, pH, and time, significantly impact protein stability (Armand et al., 2014; Kumar et al., 2021; Franca-Oliveira et al., 2021). These values are comparable to those previously reported for cephalopod inks: 62.46% in squid (Zaharah and Rabeta, 2018), 47.17% in octopus (Kipalahi et al., 2024), 43% in cuttlefish (Salsabila et al., 2024), and 43.41-40.12% in *Sepia* (Mai et al., 2006). Similarly, Derby (2014) and Ganesan et al. (2017) found 45.62 and 46.71% protein content in *Loligo duvauceli* and cuttlefish ink, respectively.

The lipid content observed was within the range of 0.2-3.96%, consistent with previous research (Ganesan et al., 2017; Zaharah and Rabeta, 2018; Riyad et al., 2020; Kipalahi et al., 2024). Ash content was slightly higher in the powder (16.27±0.05%) than in the extract (15.80±0.1%). This difference may result from degradation of minerals during solvent exposure or disruption of melanin granules (Neifar et al., 2009; Haile et al., 2015; Ho et al., 2016), with drying methods also influencing the final composition (Salsabila et al., 2024).

**DPPH radical scavenging assay:** The study found that the aqueous extract of ink powder had significantly higher DPPH radical scavenging activity (66.21±0.12% at 12.5 mg/ml) compared to the IPA extract (52.82±0.97% at 1000 mg/mL), highlighting the significance of solvent polarity in antioxidant extraction. Zaharah and Rabeta (2018) found that ink powder extracted with distilled water had significantly higher DPPH scavenging activity (~94.87±4.87%) than ethanol (~67.57 ± 7.55%), and negligible activity when using a non-polar solvent (hexane). Trigo et al. (2023) found that an aqueous ink extract had strong antioxidant properties in a heated fish muscle system and reduced lipid oxidation while preserving unsaturated fatty acids (e.g., polyene index) during thermal treatment.

Research on melanin-free ink from *Loligo formosana* indicates significant antioxidant activity against DPPH and ABTS radicals. This finding suggests that factors beyond melanin, including other soluble phenolic or hydrophilic components, play a role in radical scavenging (Vate and Benjakul, 2023; Sukmiwati et al., 2023) regarding ink extracts of Loligo duvauceli. Taken together, these findings indicate that polar solvents, particularly water, are

more effective than less polar solvents in extracting hydrophilic antioxidant components (e.g., phenols, peptides, and melanin derivatives) from cephalopod inks, which has implications for optimizing extraction protocols for food, nutraceutical, and pharmaceutical applications.

**Yield:** The extraction process may have affected the final weight of the material, as it influences the content, including the biochemical composition present in the extracted ink (Abidin et al., 2021).

**Water solubility index and water absorption index:** Protein solubility is a vital functional property in food systems (Ahamed et al., 2018), and the Water Solubility Index (WSI) quantifies this trait (Sila et al., 2014). In this study, the WSI after 30 minutes was 17.78±0.04, similar to earlier research: Ahamed et al. (2018) (17.12), Puttongsiri and Phuruen (2017) (17.44), and Jeyasanta and Patterson (2020) (16.8), all considered high (Shazwani and Rabeta, 2020). After 24 hours, the WSI increased to 22.09±0.4, which is in line with the time-influenced solubility trends found by Vargas-León et al. (2019), Ikegwu et al. (2013), and Halimatul et al. (2019). High WSI values indicate enhanced dispersibility, which contributes to the formation of stable colloidal systems and improved emulsification and foaming (Sila et al., 2014; Zayas, 1997; Kristinsson and Rasco, 2000). The Water Absorption Index (WAI) values, ranging from 2.30 to 2.32, did not differ significantly over time and are consistent with previous reports (Choi et al., 2012; Puttongsiri and Phuruen, 2017; Vargas-León et al., 2019; Patria et al., 2020). WAI indicates starch degradation and the ink's ability to bind water, which could be due to β-glucan or polysaccharides (Bhatty, 1993; Nakazawa and Wang, 2003). These polysaccharides may also contribute to the ink's antimicrobial properties. Thus, the presence of protein and carbohydrate fractions in the ink may have a synergistic effect on its bioactivity.

## Conclusion

Extracts of ink in different solvents exhibited varying absorption ranges. Strong absorbance was shown in the control (ink powder), followed by the ink extracted by IPA, compared to other solvents. The antimicrobial activity, biochemical composition, nutrition profile, and physical ink property content between *S. pharaonis* ink powder and ink extract emphasize the significant role of the extraction process in determining the final composition of the ink. The biochemical composition of the strong absorption ink was shown in the ink powder, followed by the ink extracted by IPA. Antibacterial effectiveness varied depending on the solvent used for extraction, with the largest antimicrobial zone in IPA solvent. Conversely, some of the proximate compositions may undergo degradation during the extraction process, depending on the solvent and method used. Therefore, to maximize the effectiveness of an extract, it is essential to consider its intended application and select the most appropriate solvent accordingly. When it comes to nutritional value, ink powder may be a more stable alternative, particularly as a source of protein and minerals, making it a promising candidate for further research in food and dietary applications.

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