ASTAXANTHIN DIESTER AND MONOESTER FROM FRESHWATER PRAWN WASTE AND THEIR ANTIOXIDANT ACTIVITY

Yin Yin Myint[1], Pa Pa Lin[2], Myint Myint Khine[3]

Abstract

The major carotenoids present in prawn waste include astaxanthin complexes. Astaxanthin is a lipid-soluble, and orange-reddish carotenoid pigment. Two astaxanthin compounds, AS-I (astaxanthin diester, an orange oil, 1.84%) and AS-III (astaxanthin monoester, a yellow oil, 1.13%), were isolated from the ethanol crude astaxanthin extract of freshwater prawn waste using silica gel column chromatography with *n*-hexane-acetone eluents. The chemical structures of these isolated compounds were confirmed by UV-Vis spectroscopy, FT-IR spectroscopy, GC-MS spectrometry, and visualization reagents, as well as solubility and R_f values. The antioxidant activity of the isolated astaxanthin compounds was evaluated using IC_{50} values. AS-III (astaxanthin monoester, IC_{50}: 36.84 µg/mL) exhibited greater antioxidant activity than standard ascorbic acid (IC_{50}: 47.48 µg/mL) and AS-I (astaxanthin diester, IC_{50}: 263.53 µg/mL) as determined by the DPPH free radical assay using a UV-Vis spectrophotometer at a wavelength of 517 nm.

Keywords: astaxanthin diester, astaxanthin monoester, prawn wastes, antioxidant activity

Introduction

Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione), a prominent carotenoid, is also found in microorganisms, insects, crustaceans, and micro-green algae (*Haematococcus pluvialis*). Astaxanthin, a red pigment, exhibits a shift in light absorbance when complexed with different proteins, resulting in a colour spectrum ranging from green, yellow, blue, to brown in crustaceans. The red colour is due to the conjugated double bonds at the center of the compound. Astaxanthin protects the neurons of the retina, is a cancer-protective agent that inhibits cancer metastasis, and can prevent various types of cancer. No toxic effects were found, although excessive astaxanthin consumption leads to yellow to reddish pigmentation of the skin in animals. Astaxanthin is a natural antioxidant with some advantageous properties are prevention of cardiovascular and immune system, anti-cancer, anti-aging, Alzheimer disease, athletic performance, muscle soreness, cosmetics, protecting eyesight, and fodder (Hu, *et. al*., 2019). The potency of astaxanthin's antioxidant activities has been reported the efficacy of astaxanthin to be 65 times more than vitamin C (Bayan, *et. al*., 2020). Myanmar name of freshwater prawn is Pujawn and common name is giant river prawn. The scientific name of *Macrobrachium rosenbergii*, belongs to Palaemonidae**.** The colour of *Macrobrachium rosenbergii* can vary according to where the prawns are found but the body can be greenish-grey (Brown, 2019). They are found in most inland freshwater areas including lakes, rivers, swamps, irrigation ditches, canals and ponds, as well as in estuarine areas. It is indigenous in the whole of the South and Southeast Asian including Myanmar as well as in northern Oceania and in the western Pacific islands (FAO, 2009). This research focuses on isolating astaxanthin compounds from freshwater prawn waste and determining their antioxidant activity.

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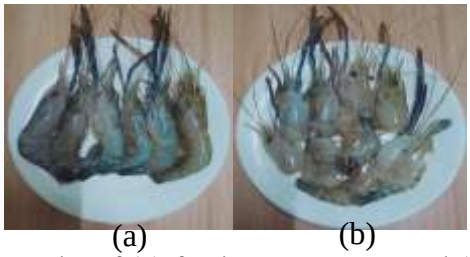


(a) (b)

Figure 1. Photographs of (a) freshwater prawn and (b) prawn wastes

Materials and Methods

Collection, Identification and Preparation of Freshwater Prawn

The freshwater prawn (exoskeletons including shell, claw duo and pincers) were collected from markets nearby, Hinthada Township, Ayeyawady Region. The scientific name was identified at the Zoology Department, Hinthada University. The freshwater prawn waste was cleaned with water. The waste was boiled with 2.0 % aqueous acetic acid for about 15 minutes and dried at room temperature and ground into fine powder. The crude astaxanthin extract was prepared by macerating 200 g of dried powder in 600 mL of ethanol, stirring for one hour on a magnetic stirrer. This procedure was repeated three times, followed by filtering and drying.

Chemicals

All chemicals and organic solvents were used laboratory grade.

Apparatus

UV-Vis (UV 1800, Shimadzu), FT IR spectrophotometer (Perkin Elmer, Spectrum II) and GC-MS_QP2010_Ultra spectrometer (Shimadzu) were used for structural identification of isolated compounds. The spectra were measured at the Department of Chemistry at Pathein University and Mandalay University.

Separation of Astaxanthin Compounds from Ethanol Crude Astaxanthin Extract

To separate the astaxanthin compounds, pet ether and ethyl acetate soluble materials were firstly extracted by liquid-liquid extraction from ethanol extract (7.53 g). Pet ether (4.25 g) and ethyl acetate (1.19 g) soluble organic constituents were primarily TLC checking carried out to isolated specific compounds. Both soluble extracts were applied to separate astaxanthin compounds by column chromatography.

Separation from pet ether soluble extract

Gradient elution was performed successively with *n*-hexane only (F-1); *n*-hexane: acetone,10:1 (F-2); *n*-hexane: acetone, 5:1 (F-3) and *n*-hexane: acetone, 3:1 (F-4), *n*-hexane: acetone, 1:1 (F-5) v/v and totally of five fractions were collected. All separated fractions were checked on TLC chromatograms under UV Lamp (254 and 365 nm) as well as using silver nitrate in methanol and 5 % sulphuric acid reagents. Fractions F-1 and F-5 were obtained as oil mixtures, F-2 was directly isolated as orange oil, F-3 as colourless needle crystal, and F-4 as yellow oil. The isolated compound from fraction F-2, named AS-I, was obtained as an orange oil (0.14 g), R_f value 0.80. The colorless needle crystal from fraction F-3 washed with *n*-hexane,

correspond to compound AS-II, a fatty acid (0.28 g), with an R_f value 0.55. From fraction F-4, a yellow oil (0.08 g), named AS-III, was obtained with an R_f value 0.53. All R_f values were determined using *n*-hexane: acetone 3:1 v/v as the eluent.

AS-I (0.0034 g), AS-II (0.0860 g), and AS-III (0.0231 g) were isolated from ethyl acetate soluble extract. In this isolation, the eluents were similarly carried out as described in the isolation of pet ether soluble extract.

Determination of Physicochemical Characteristic and Structural Identification

The isolated compounds AS-I and AS-III were characterized by structural identification as UV chromophores and functional groups using UV-Vis and FT IR spectroscopy, as well as fatty acid esters using GC-MS spectrometry. In this study, solubility comparisons, reported R_f values, and spectral data were used to determine the chemical structure.

Screening of Antioxidant Activity of Isolated Compounds: AS-I and AS-III

The antioxidant activity of the isolated compounds (astaxanthin compounds) from prawn waste was screened by 2,2-diphenyl-1-picrylhydrazyl **(**DPPH) free radical scavenging assay using the UV- Vis spectrophotometer according to Baliyan, *et. al*., 2022 with slight modification. DPPH powder (2 mg) and methanol (100 mL) were thoroughly mixed by a vortex to obtain the 20 µg/mL DPPH solution which was freshly prepared in a brown colored bottle. The standard ascorbic acid (100 µg/mL) solution was two-fold serially diluted with methanol to get the concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 µg/mL, respectively. The solution of isolated compounds (400 µg/mL) were two-fold serially diluted with methanol to get the concentrations of 400, 200, 100, 50, 25, and 12.5 µg/mL, respectively. The blank solution of tests with desired concentrations were prepared by mixing 2 mL of each concentrations of test solutions with 2 mL of methanol. The control solution was prepared by mixing 2 mL of 20 µg/mL DPPH solution with 2 mL of methanol in the bottle.

DPPH free radical scavenging assay

The standard ascorbic acid and each of the test solutions were separately prepared by mixing 2 mL of 20 µg/mL DPPH solution with 2 mL of each test solution in a brown bottle. The solutions were mixed using a vortex and incubated at room temperature for 30 minutes. The absorbance of each concentration of solution was measured at λ_{max} 517 nm using a UV-Visible spectrophotometer (BK-V1000, Biobase). The experiment was carried out in triplicate for each concentration. The percentage of radical scavenging activity (% RSA) was calculated by plotting the absorbance against the concentration of the test solutions shown in Tables 2 and 3 and Figures 7.

$$\%RSA = \frac{\text{Absorbance of DPPH- (Absorbance of Sample-Absorbance of blank)} \times 100}{\text{Absorbance of DPPH}}$$

The IC_{50} (half-maximal inhibitory concentration) value was calculated using a linear regressive excel program. The standard deviation was also calculated.

Results and Discussion

Isolation of Astaxanthin Compounds and Structural Identification

The solubilities, R_f values, and chemical characteristics of the isolated compounds AS-I and AS-III were determined and compared with reported data for astaxanthin diester, astaxanthin monoester, and free astaxanthin. The isolated compounds were soluble in petroleum ether, *n*-hexane, ethyl acetate, and acetone, but showed poor solubility in ethanol, methanol, and water. Their solubility in moderately polar solvents (ethyl acetate and acetone) suggests the presence of some polar functional groups; however, their overall low polarity indicates a predominantly lipophilic character. Based on these observations, AS-I and AS-III are likely lipid-like compounds, possibly carotenoid esters rather than free fatty acids.

Based on the R_f values provided, it may deduce some preliminary inferences about the polarity and potential identities of the compounds: AS-I appears to be the least polar, AS-III is the most polar compound. The reported R_f values of astaxanthin diesters (0.75–0.85) and astaxanthin monoesters (0.6) were compared with the observed R_f values of AS-I and AS-III by same solvent system (Minyuk and Solovchenko, 2018) described in Table 1. The R_f values of astaxanthin diester and monoester are consistent with the observed R_f values of AS-I and AS-III. However, additional analysis is required to definitively identify these compounds.

Determination of chemical characteristic of isolated compounds

When examined under a UV lamp at 254 nm, AS-I and AS-III appeared as dark spots against a green background, suggesting the presence of conjugated systems typical of carotenoids. Both compounds were charred upon spraying with 5% sulfuric acid followed by heating, indicating the presence of carbon-based organic matter (Jork et al., 1990).

Table 1. Comparing the R_f values: AS-I, AS-III and Literature Astaxanthin diester, Astaxanthin Monoester and Astaxanthin Free Ester

Isolated compound	R_f value in *n*-hexane: acetone (3:1 v/v)		Remark
	Observed	*Literature	
AS-I	0.80	0.75-0.85	Astaxanthin diester
AS-III	0.53	0.60	Astaxanthin monoester
-	-	0.33	Astaxanthin free ester

* Minyuk and Solovchenko, 2018

On TLC developed with n-hexane–acetone (3:1 v/v) and visualized with silver nitrate in methanol, AS-I appeared orange, while AS-III exhibited a yellow coloration. Such differences in colouration are consistent with the behaviour of keto-carotenoids, which typically display orange to red shades depending on the number and position of keto groups. Moreover, free keto-carotenoids bearing hydroxyl groups at the 3, 3′-positions and keto groups at the 4, 4′-positions are known to display low R_f values in hexane-based solvent systems due to their stronger interaction with silica gel (Minyuk and Solovchenko, 2018).

The observed TLC characteristics suggest that AS-I and AS-III may contain two keto groups in their structures, possibly resembling astaxanthin derivatives.

Further spectroscopic analyses, including UV-Vis and FT-IR spectroscopy as well as GC-MS, were conducted to characterize the functional groups of AS-I and AS-III.

Isolated compound AS-I

The UV-Vis spectrum of AS-I in methanol (see in Figure 2) displayed absorption in the range of 265–600 nm when measured using a UV-Vis spectrophotometer (UV-1800, Shimadzu). Two distinct absorption maxima (λ_{max}) were observed at 275 nm and 449 nm. The major band at 449 nm is characteristic of $\pi \rightarrow \pi$ electronic transitions in conjugated polyene systems typical of carotenoids, while the additional band at 275 nm may correspond to higher-energy electronic transitions associated with the extended conjugated structure (Field et al., 2008). On TLC, AS-I appeared as an orange spot, which is consistent with carotenoid-type compounds possessing extended conjugated systems (Minyuk and Solovchenko, 2018).

The FT-IR spectrum of AS-I was compared with reported spectra of free astaxanthin (Nemani et al., 2024) and astaxanthin diesters (Subramanian et al., 2013) see in Figure 4 (a),(b) and (c). The FT-IR spectral profile of AS-I provides strong evidence for its structural similarity to astaxanthin derivatives. The presence of two distinct absorption bands at 1737 cm^{-1} and 1715 cm^{-1} indicates both esterified carbonyl groups and the characteristic carbonyl vibration at the 4, 4′-positions of the β-ionone rings. The additional ν_{C-O} stretching absorptions at 1185 and 1082 cm^{-1} further support the presence of aliphatic ester functionalities. Importantly, the absence of a broad ν_{O-H} stretching band in the 3550–3200 cm^{-1} region suggests that the hydroxyl groups at the 3, 3′-positions have undergone esterification, consistent with fatty acid ester derivatives of astaxanthin (Subramanian et al). The absorption band at 1222 cm^{-1} indicates υ_{C-C-C} and $\delta_{C-C(=O)-C}$ for the C-C-C of the ketone (Silverstein, *et. al*., 2005). Moreover, the absorption at 3007 cm^{-1}, assigned to ν_{C-H} of alkenes, together with the CH_2 and CH_3 stretching and bending vibrations at 2924, 2854, 1438, and 964 cm^{-1}, indicates the presence of long hydrocarbon chains, characteristic of esterified carotenoids (Pretsch et al., 1989 and Silverstein et al., 2005). These observations, taken together, suggest that AS-I is not a free astaxanthin but rather an esterified form, most likely a diester, in agreement with previously reported spectral features of astaxanthin diesters (Nemani et al., 2024).

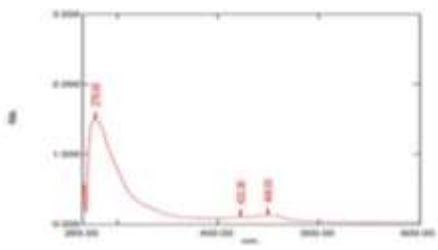
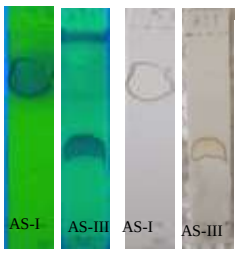


Figure 2. UV-Vis spectrum of isolated compound AS- I



(a) (b)

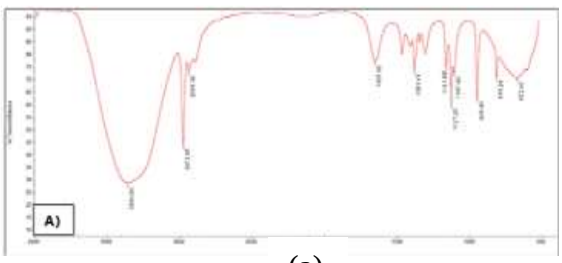
Figure 3 TLC chromatograms of AS-I and AS-III
(a) under 254 nm of UV lamp
(b) after spraying with $AgNO_3$

Identification of fatty acids of astaxanthin diester by GC-MS

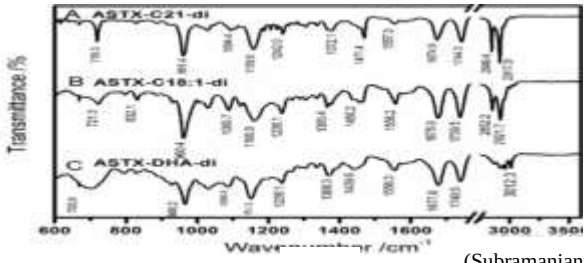
The fatty acid esters of AS-I were identified by GC-MS spectrometry. The analysis revealed a total of nine fatty acid esters, comprising both saturated and unsaturated derivatives. Among these, five unsaturated fatty acid esters were detected: ethyl 9-hexadecenoate (RT 15.825 min), 9,12-octadecadienoic acid (*Z, Z*)-, methyl ester (RT 19.046 min), 9-octadecen oic acid (*Z*)-, methyl ester (RT 19.226 min), *trans, trans*-9,12-octadecadienoic acid, propyl (RT 21.334 min) and (*E*)- 9- octadecenoic acid ethyl ester (RT 21.552 min). Typically, the hydroxyl groups at the 3- and 3'-positions of the β-ionone rings in astaxanthin can form ester bonds with various fatty acids, such as oleic or linoleic acid, resulting in diester derivatives (Brotosudarmo et al., 2020). The relatively high R_f value of AS-I compared to another isolated compound, AS-III, in the same solvent system further supports the presence of ester groups at these positions. Based on the combined spectral and physicochemical data suggest that AS-I is an astaxanthin unsaturated fatty acid diester.

Isolated compound AS-III

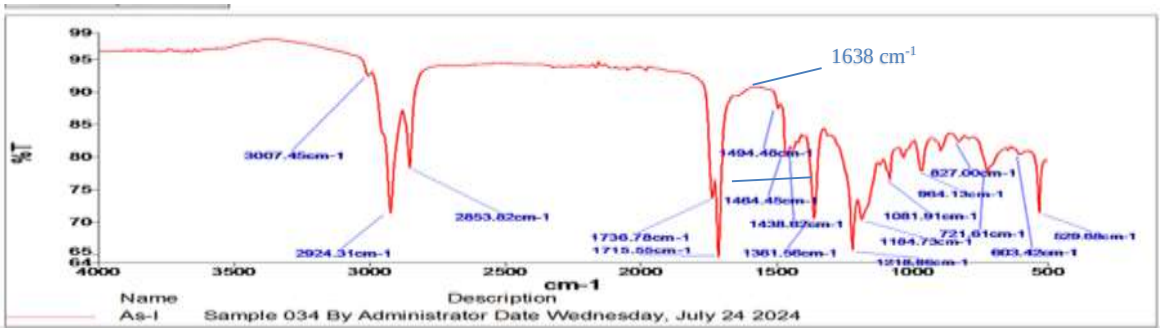
AS-III exhibits a more polar nature than AS-I, as indicated by their respective R_f values. The R_f value of AS-III aligns with the reported value for R_f astaxanthin monoester in an *n*-hexane: acetone (3:1 v/v) solvent system (Minyuk and Solovchenko, 2018). The FT IR spectrum of AS-III clearly differentiates between mono- and diester of astaxanthin through distinct spectral shifts. A board absorption band of observed at 3505 cm^{-1} corresponds to υ_{O-H} stretching vibration, suggesting the presence of a free hydroxyl group at either the ~~in~~ 3- or 3′-position of β-ionone rings. A very strong absorption band at 1707 cm^{-1} is attributed to the $\upsilon_{C=O}$ stretching of the 4,4'-β-ionone rings carbonyl groups. This band exhibits greater intensity and higher energy compared to the stretching C=O vibration at 1657 cm^{-1} reported for free astaxanthin esters (Subramanian, *et. al*., 2013), indicating potential overlap of carbonyl signals from both ester and keto functionalities. A strong absorption band at 1092 cm^{-1} confirms the presence of aliphatic esters through C-O stretching (Pretsch, *et. al*., 1989). The absorption at 1222 cm^{-1} is consistent with stretching υ_{C-C-C} and bending ($\delta_{C-C(=O)-C}$) vibrations characteristic of ketone group (Silverstein, *et. al*., 2005). The band at 3006 cm^{-1} arises from the C–H stretching of alkene groups (–C=C–H), influenced by the fatty acid environment on one end of the astaxanthin molecule.Additional absorption bands at 2924 cm^{-1} and 2855 cm^{-1} corresponds to aliphatic C-H stretching, while those at 1438 cm^{-1}, 1362 cm^{-1} and 904 cm^{-1} are associated with C-H bending vibrations of -CH_2 and -CH_3 groups. In monoester, esterification typically occurs at only one end of the astaxanthin molecule, leaving the –OH group at the other end unmodified. The presence of stretching O-H band at 3505 cm^{-1} supports this structural arrangement. Comparison of the observed FT IR spectrum of AS-III with reported spectra reveals consistency, particularly in the absorption bands associated with O-H, C=O, C=C, C-C(=O)-C, and the fingerprint region as illustrated in Figure 5 (a), (b) and (c) (Subramanian, *et al*., 2013). The lower R_f value of AS-III (0.53) compared to AS-I (0.80) in the same solvent system further supports its higher polarity, suggesting the presence of a free hydroxyl group at the 3- or 3′-position of the β-ionone ring. Based on the collective spectral and physicochemical data, the isolated compound AS-III can be considered an astaxanthin monoester. However, the specific fatty acid moiety attached to the esterified end of AS-III cannot be identified using the FT IR data alone. Further structural elucidation using techniques such as NMR or mass spectrometry, is required for complete characterization.



(a) (Nemani, *et. al*., 2024)



(b) (Subramanian, *et. al*., 2013)



(c)

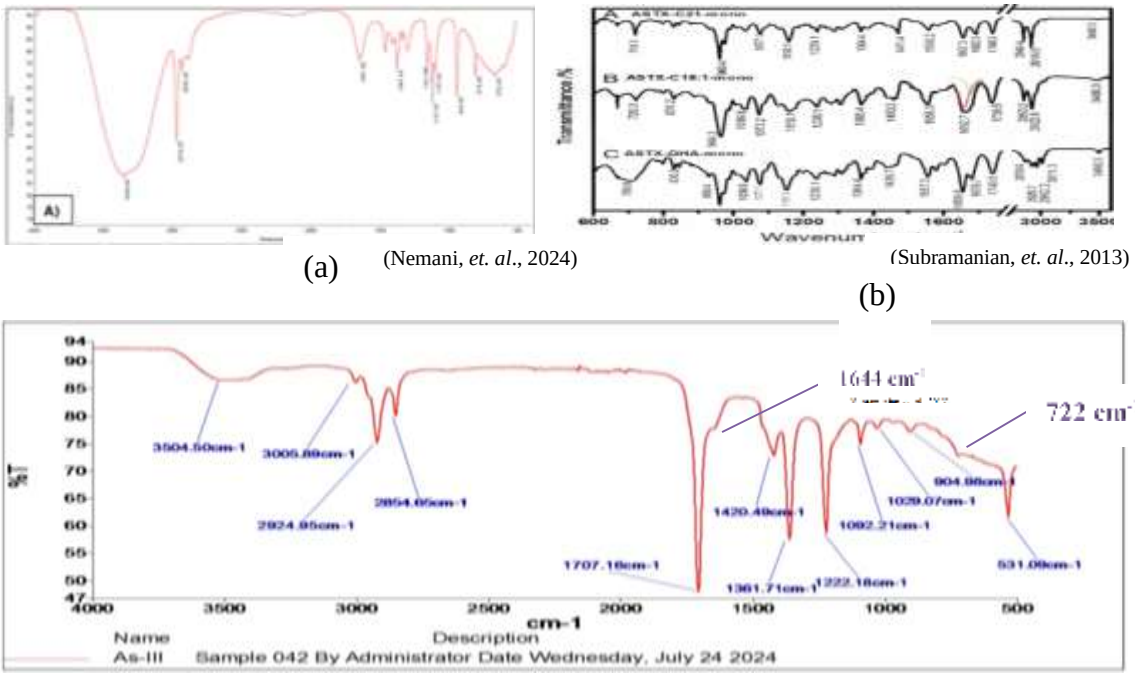
Figure 4 Comparing FT IR spectra of (a) pure astaxanthin (b) astaxanthin diester and (c) isolated compound AS-I

Antioxidant activity of Isolated Astaxanthin Compounds

DPPH (2,2-diphenyl-1-picrylhydrazyl) is a relatively stable, hydrophobic free radical. A greater decrease in absorbance indicates more effective DPPH radical scavenging activity. The antioxidant activity of standard ascorbic acid, isolated astaxanthin compounds, AS-I and AS-III, were evaluated by determining their IC_{50} values (half-maximal inhibitory concentration). This was achieved by plotting the % RSA (radical scavenging activity) against compound concentration using linear regression in Excel. The IC_{50} values obtained were 263.53 µg/mL for AS-I (astaxanthin diester) and 36.84 µg/mL for AS-III (astaxanthin monoester) and 7.48 µg/mL for ascorbic acid. These results indicate that AS-III exhibits stronger antioxidant activity than the standard ascorbic acid, while AS-I shows lower activity in comparison. Given that DPPH is a hydrophobic, the lipid solubility of astaxanthin monoester (AS-III) likely enhances its ability to interact with and scavenge DPPH more effectively than the hydrophilic ascorbic acid and the bulkier astaxanthin diester (AS-I). This greater interaction may facilitate a more efficient electron transfer process, resulting in superior antioxidant performance. The percent of radical scavenging activity (%RSA) and IC_{50} values are presented in Tables 2 and 3 and Figure 7. According to the findings, astaxanthin compounds are natural carotenoid pigment with a long polyconjugated chain, which confer unique properties, including strong antioxidant activity due to their ability to delocalize electrons.

Furthermore, astaxanthin monoester also possesses hydroxyl group that can donate hydrogen atoms to neutralize free radicals. This enables it to neutralize more DPPH radicals per molecule than astaxanthin diester. From this study suggest that AS-III (astaxanthin monoester) is important constituents of prawn waste for the antioxidant potential and the natural carotenoid

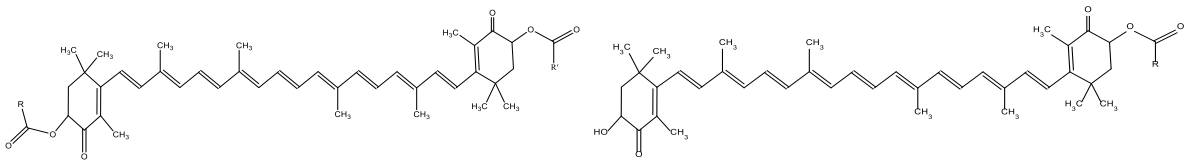
pigment with a long polyconjugate chain prevent oxidative damage that can be therapeutically useful.



(a) (Nemani, *et. al*., 2024) (Subramanian, *et. al*., 2013) (b)

(c)

Figure 5 FT IR spectra of (a) pure astaxanthin (b) astaxanthin monoesters and (c) isolated compound AS-III



(a) (b)

Figure 6 Possible chemical structures of isolated compounds (a) AS-I, astaxanthin diester and (b) AS-III, astaxanthin monoester

Table 2. Absorbance Values, Percent Radical Scavenging (% RSA) and IC_{50} Values of Standard Ascorbic Acid

Conc.(µg/mL)	Absorbance at λ_{max} 517 nm	% RSA± SD	IC_{50} (µg/mL)
6.25	0.299	26.53± 0.05	
12.50	0.283	30.46±0.01	47.48
25.00	0.274	32.67±0.04	
50.00	0.194	52.33±0.05	
100.00	0.074	81.81±0.01	

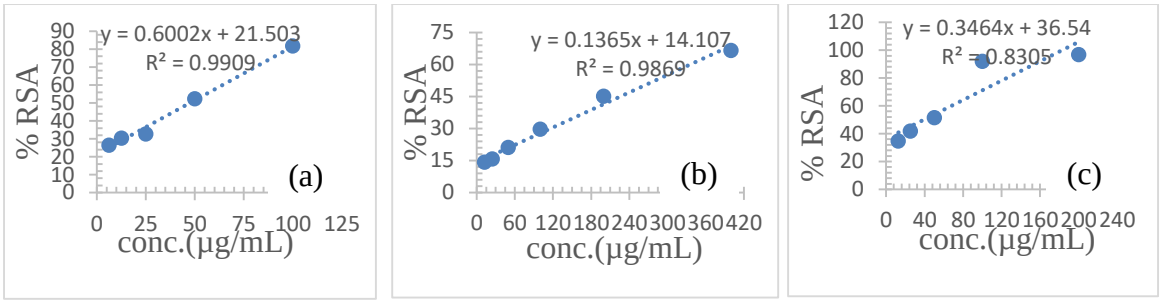


Figure 7 Plot of % Radical Scavenging Activity *versus* concentrations of (a)standard ascorbic acid (b) AS-I and (c) AS-III

Table 3. Absorbance Values, Percent Radical Scavenging (% RSA) and IC_{50} Values of Isolated Compound AS-I and AS-III

Conc. (µg/mL)	Absorbance at λ_{max} 517 nm		% RSA with SD		IC_{50} (µg/mL)	
	AS-I	AS-III	AS-I	AS-III	AS-I	AS-III
12.50	0.471	0.327	14.16 ±0.00	34.74±0.03		
25.00	0.464	0.303	15.64±0.03	41.94±0.08	263.53	36.84
50.00	0.438	0.262	21.14±0.03	51.44±0.17		
100.00	0.401	0.104	29.59±0.02	92.01±0.06		
200.00	0.333	0.077	45.03±0.02	96.80±0.00		
400.00	0.243	0.075	66.59±0.04	97.78±0.01		

Conclusion

The possible chemical structures of isolated compounds AS-I (1.84 %, orange oil) and AS-III (1.13 %, yellow oil) were identified as astaxanthin diester and astaxanthin monoester, respectively, from the ethanol crude extract of prawn waste. Their identification was based on comparisons of R_f values, visual observation tests, and FT-IR and UV-Vis spectral data with those of known astaxanthin esters. Additionally, the presence of fatty acid esters in AS-I (astaxanthin diester) was confirmed through GC-MS analysis. The antioxidant activity of the isolated astaxanthin compounds was determined by their IC_{50} values, which were found to be 36.84 µg/mL for AS-III and 263.53 µg/mL for AS-I. These results indicate that the monoester form (AS-III) exhibits significantly stronger antioxidant activity than the diester form (AS-I). Overall, the extraction and isolation of astaxanthin derivatives from prawn waste represent a sustainable and eco-friendly alternative to conventional sources and methods, offering both environmental and economic benefits.

Acknowledgements

I would like to thank Rector Dr Theingi Shwe, Pro-rectors Dr Ye Ye Than and Dr Aye Lwin and Professor and Head Dr Tin Htwe Mu for their permission to submit the paper.

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