Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 27(6): 433 – 443 (2023)
www.ejabf.journals.ekb.eg

Identification, Isolation and Antioxidant Activity of Pigments from *Sargassum polycystum* from Sumbawa, Indonesia

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ARTICLE INFO

Article History:
Received: Oct. 26, 2023
Accepted: Nov. 29, 2023
Online: Dec. 7, 2023

Keywords:
Pigmets,
Isolation,
Antioxidant,
Sargassum polycytum

ABSTRACT

Brown seaweed (*Sargassum polycytum*) is a type of seaweed with the potential to serve as a source of nutrition and medicine. The composition of the pigments contained in it varies greatly. Seaweed samples were collected from the coast of Sumbawa, Indonesia. The seaweed was initially extracted by soaking it in a mixture of acetone:methanol (7 : 3, v/ v). To separate the pigment content in seaweed, after obtaining a dry extract, isolation and identification were performed using Thin Layer Chromatography (TLC) and High-Performance Liquid Chromatography (HPLC) methods. Isolation was performed through column chromatography followed by identification using UV-Vis Spectrophotometry. Antioxidant activity was carried out by DPPH method. The results of pigment identification using TLC showed the formation of 6 pigment spots, namely β-carotene, pheophytin, chlorophyll *a*, chlorophyll *c*, fucoxanthin and xanthophyll. Further identification was conducted using HPLC equipped with a photodiode array detector (PDA) which resulted in 5 peaks: Peak 1 corresponds to chlorophyll $_{c1}$, peak 2 to chlorophyll $_{c2}$, peak 3 to fucoxanthin, peak 4 to xanthophyll, and peak 5 to carotenoid. The isolation process with column chromatography produced 6 isolates: Isolate 1 is β-carotene, isolate 2 is pheophytin, isolate 3 is chlorophyll *a*, isolate 4 is chlorophyll *c*, isolate 5 is fucoxanthin, and isolate 6 is xanthophyll, as determined by spectrum pattern identification. The antioxidant activity of β-carotene IC_{50}= (476± 0.32) $mg.L^{-1}$, pheophytin IC_{50}= (720± 0. 32) $mg.L^{-1}$, chlorophyll a IC_{50}= (683± 0. 32) $mg.L^{-1}$, chlorophyll c IC^{50}= (582± 0. 32) $mg.L^{-1}$, fucoxanthin IC_{50}= (413± 0. 32) $mg.L^{-1}$, and xanthophyll IC_{50}= (521± 0. 32) $mg.L^{-1}$.

INTRODUCTION

Indonesia is an archipelago, with significant portion of its territory consisting of water bodies that can serve as a source of income for coastal communities. One such resource is seaweed, which is categorized as a type of aquatic plant and falls under the classification of macroalgae (**Kadi, 2005**).

According to **Hegazi *et al.* (1998**) seaweed can be categorized into three types based on the color of their thallus, namely red seaweed (*Rhodophyceae*), brown seaweed (*Phaeophyceae*), and green seaweed (*Chlorophyceae*). In this study, a specific type of

brown seaweed (*Phaeophyceae*), namely *Sargassum polycystum*, is used, which is known to have a varied pigment composition. *Sargassum* algae is one of the *Sargassum* genera included in the *Phaeophyceae* class. *Sargassum polycystum* is one type of brown macro algae that grows in Indonesia and is spread in many aquatic environments. *Sargassum polycystum* is widely exported due to its polysaccharide content, namely alginate. Alginate serves as a thickener and emulsifier **(Megayana *et al.*, 2011)**. Apart from its alginate content, *Sargassum* sp. contains several pigments, namely chlorophyll *a*, chlorophyll *c*, β-carotene, xanthophyll, violaxanthin, fucoxanthin, flavoxanthin, and neoxanthin A and B **(Yunizal, 2004)**.

Some of *Sargassum polycystum*'s pigments are known to possess potential antioxidants properties. β-carotene and its derivatives exhibit strong antioxidant activity **(Mueller & Boehm, 2011)**. **Xia *et al.* (2013)** conducted a study to assess the antioxidant activity of fucoxanthin using various methods. The results obtained from these different methods indicated that fucoxanthin has high antioxidant activity.

MATERIALS AND METHODS

Sample collection

The main material is *Sargassum polycystum* (Fig. 1) obtained from Montong Beach, Kecamatan Utan, Kabupaten Sumbawa, Sumbawa Besar, Provinsi Nusa Tenggara Barat, Indonesia. The chemicals used were methanol, acetone, diethyl ether, hexane, diethyl ether, *2,2-diphenyl-1-picrylhydrazyl* (DPPH), silica GF_{254} and silica gel 60 (Merck).

Fig. 1. *Sargassum polycystum* from Sumbawa, Indonesia

Pigment extraction

One hundred grams of sample were grounded and extracted by using a mixture solution of acetone : methanol (7 : 3, v/ v). During the extraction, calcium carbonate was added as a neutralizing agent and sodium ascorbate as an antioxidant to prevent further oxidations. The extraction was performed as quickly as possible to avoid further oxidations or enzymatic degradations. Afterward, the extract was filtered and the residue was re-extracted until the color of residue became pale as an indicator of a complete pigment extraction. The resulting

extract was partitioned with diethyl ether. Moreover, the diethyl ether layer was dried with nitrogen gas, following the method of **Britton (1995)** and **Kusmita *et al.* (2015)**.

Thin-layer chromatography (TLC)

The pigment composition of *Sargassum polycystum* fraction was analyzed with hexane : diethyl ether : aceton (6 : 3 : 2, v/ v/ v) as a mobile phase and silica gel GF_{254} (merck) as a stationary phase, accoroding to the guidelines of **Wang *et al.* (1995)**. The colour of each spot on the TLC plate was observed, and the Rf value was calculated. Suibsequently,it was compared with the literature.

High-performance liquid chromatography (HPLC)

HPLC analysis was performed with Shimadzu HPLC equipped with photodiode array (PDA) detector and column temperature controller (Shimadzu, Kyoto, Japan) using a Shim-pack vp-ods C18 column (250 × 4.6 mm i.d.) (Shimadzu, Kyoto). Pigment was eluted with a gradient system using the flow speed of 1.0mL per min at 25ºC. The solution used was methanol : acetonitrile (70 : 30, v/ v). The pigments were detected with a PDA detector and evaluated at a wavelength of 420nm.

Isolation

The dry pigment fraction was dissolved in a small amount of mobile phase, then separated using column chromatography with a silica gel 60 F254 stationary phase and a hexane : ether : acetone (6 : 3 : 2, v/ v) mobile phase, following the method of **Wang *et al.* (1995)**. Each isolate produced was collected in vials and dried from the solvent using nitrogen gas. The isolate was then viewed for the spectral pattern with a spectrophotometer at a wavelength of 350- 800nm, according to the method of **Gross (1991)**.

Antioxidant activity test

The pigment isolate was dissolved in methanol and various concentration series were made. The blank was a methanol solution, while the sample solution consisted of 4ml DPPH plus 1ml extract. Both the blank and the sample were incubated for 30 minutes in a dark room, then their absorbances were measured at a wavelength of 517nm using a single UV-visible spectrophotometer Shimadzu 1240, following the guidelines mentioned by **Samanta *et al.* (2016)**, as follows:

$$\% \text{ Inhibitory} = \frac{[DPPH]_0 - [DPPH]_s}{[DPPH]_0} \text{X}100\%$$

$[DPPH]_0$ = Initial DPPH concentration

$[DPPH]_s$ = Final DPPH concentration remaining

RESULTS

Initial identification of pigments in the *Sargassum polycystum* fraction was carried out by TLC method. Based on the TLC results, 6 pigment spots with their Rf values were generated (Fig. 2).

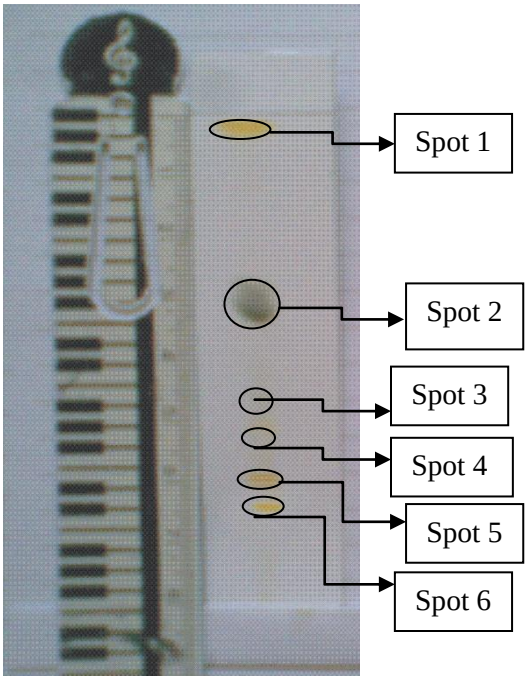

Fig. 2. TLC chromatogram of pigment fractions from *Sargassum polycystum* showing: β-caroten (spot 1), pheophytin (spot 2), chlorophyll *a* (spot 3), chlorophyll *c* (spot 4), fucoxanthin (spot 5), and xanthophyll (spot 6)

Table 1. Retardation factor (Rf) of pigment fractions from *Sargassum polycystum*

No.	Spot color	Rf	Identification	Literature
1	Yellow	0.97	β caroten	**Jeffrey *et al.* (1997)**
2	Gray	0.63	Pheophytin	**Wang *et al.* (1995)**
3	Bluish green	0.43	Chlorophyll *a*	**Wang *et al.* (1995)**
4	Yellowish green	0.36	Chlorophyll *c*	**Strain *et al.* (1943)**
5	Orange	0.25	Fucoxanthin	**Strain *et al.* (1943)**
6	Yelow	0.18	Xanthophyll	**Strain *et al.* (1943)**

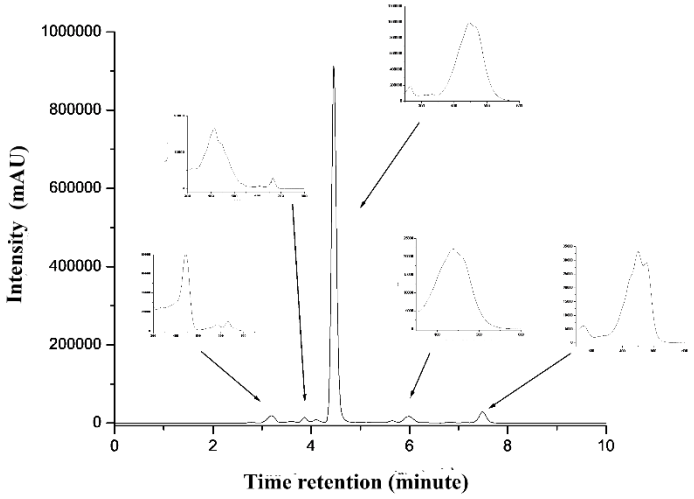

Fig. 3. HPLC profile of *Sargassum polycystum* ether fraction. The detection was carried out at 420nm with a flow rate of 1mL per min^{-1}

To further isolate the ether fraction, column chromatography was employed. The results of the chromatogram for the separation of the dry extract using column chromatography is shown in Fig. (4).

From the separation results by column chromatography method, 6 isolates were obtained, namely yellow, orange, yellow green, blue green, gray, and yellow. The isolates were identified by UV-Vis spectrophotometer spectra pattern. The spectra pattern results are shown in Fig. (5).

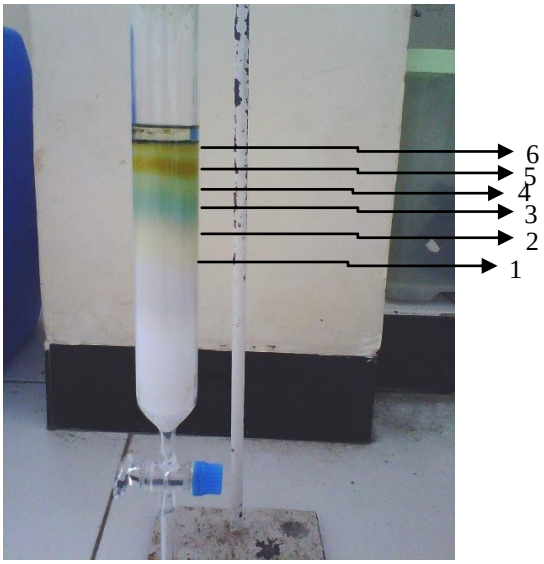

Fig. 4. Isolation using column chromatography. The results show 6 layers with different colors

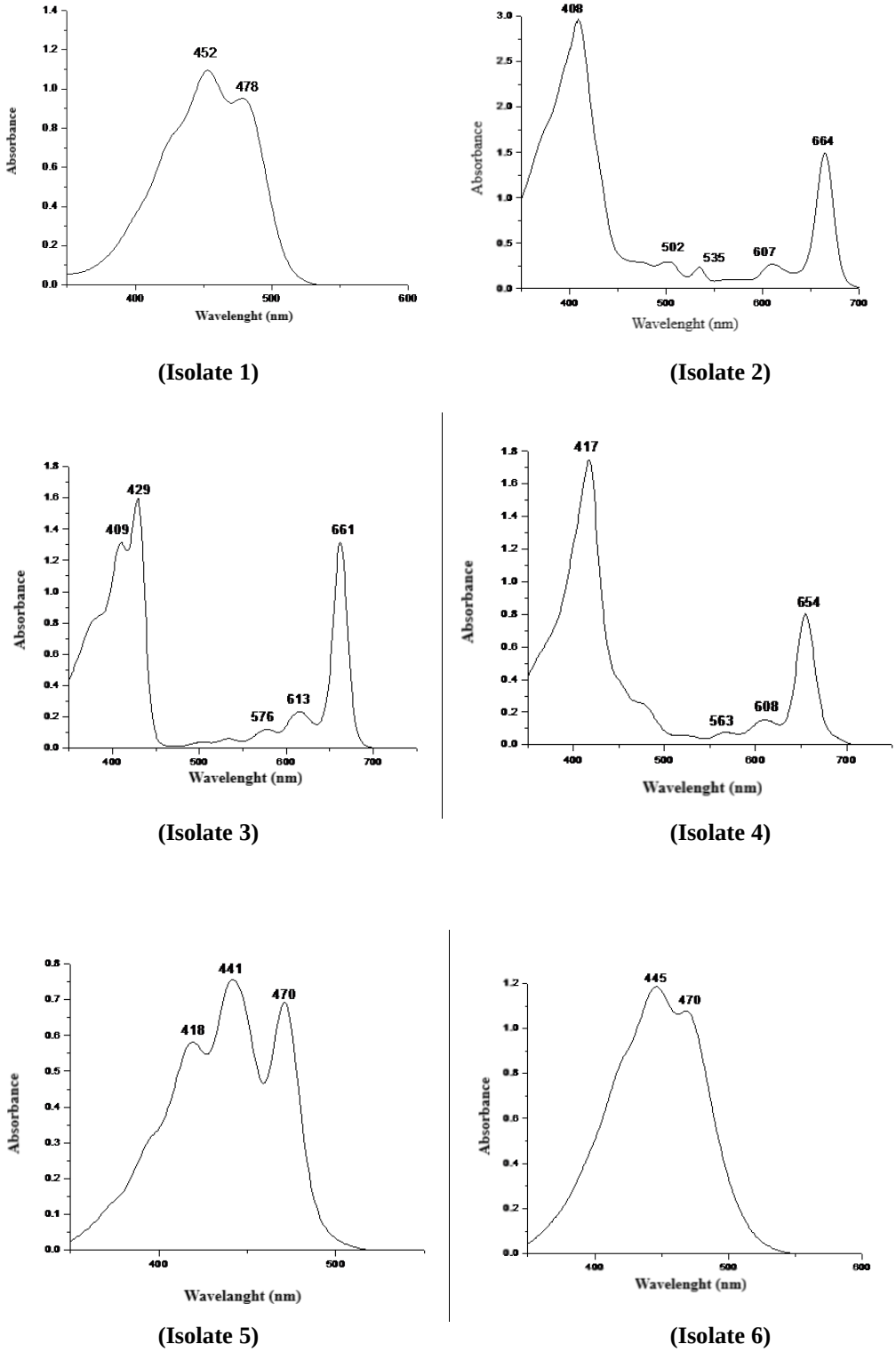

Fig. 5. Spectrum of isolate 1 (β-karoten), isolate 2 (pheophytin), isolate 3 (chlorophyll *a*), isolate 4 (chlorophyll *c*), fraksi 5 (fucoxanthin), and fraksi 6 (xanthophyll)

The isolates were then assessed for antioxidant activity using the DPPH method. The antioxidant activity are shown in Fig. (6).

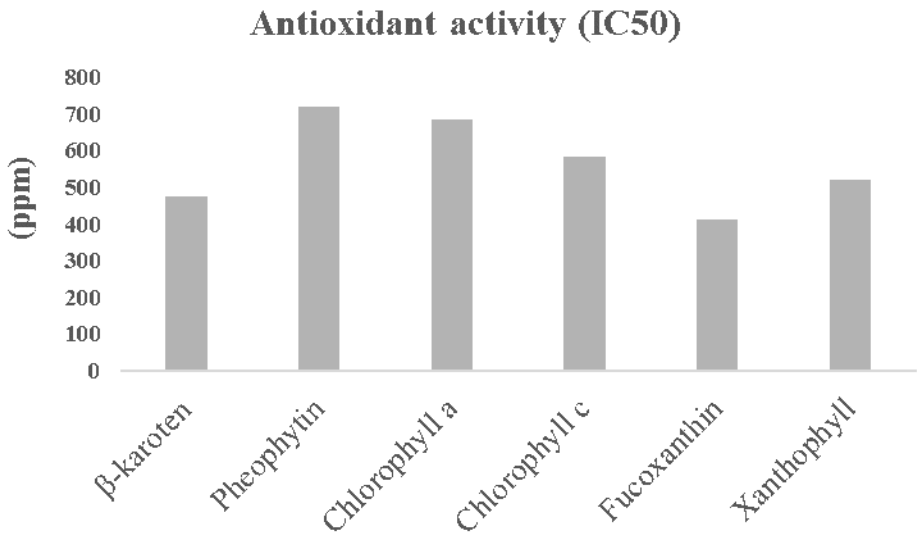

Fig. 6. The histogram of IC50 concentration of isolate pigments from *Sargassum polycytum* using the DPPH method

DISCUSSION

Spots 1 and 6 of the TLC results correspond to carotenoids (Fig. 2.). According to **Gross (1991)** carotenoid pigments are typically yellow-red in color. Carotenoids can be divided into two groups, namely non-polar carotenoids/carotene and polar carotenoids/xanthophyll. The mobile phase used in this TLC is hexane : ether : acetone (6: 3: 2) and the stationary phase used is silica gel GF 60. The stationary phase is polar, due to the presence of oxygen atoms on its surface, while the mobile phase is more non-polar so that the movement of carotene is faster than other pigments, influencing the resulting retardation factor (Rf) value. The Rf value of carotene is greater than xanthophyll and other pigments. In the research conducted by **Britton *et al.* (1995)** the Rf value for β-carotene falls within the range of 0.8- 1.0. The Rf value obtained in this study, which is 0.97, aligns with the Rf value mentioned in that research. Based on the Rf value, spot 1 was identified as β-carotene.

The bluish green color observed in stain no.3 has an Rf of 0.43 and it is identified as chlorophyll *a*. Research conducted by **Wang *et al.* (1995)** showed that chlorophyll *a* has an Rf value of 0.40, which closely matches the Rf value found in this study, which is 0.43. These findings are further supported by **Bacon (1964)**, **Minguez-Mosquera *et al.* (1989)** and **Wang *et al.* (1995)** in which they state that chlorophyll *a* is bluish green. Based on the Rf value, the stain no.3 is identified as chlorophyll *a*.

On stain no. 4, yellowish green color with Rf 0.36 is visible, which corresponds to the color of the chlorophyll *c* pigment, as reported in the study by **Strain *et al.* (1943)**, where a nonpolar mobile phase was used. Stain no. 5 on the orange-colored TLC plate has an Rf of 0.25, consistent with the color of the fucoxanthin pigment spot found in study of **Strain *et al.* (1943)**. The gray-green spot 2, with an Rf of 0.63, is believed to be a pheophytin pigment. Additionally, the yellow-colored spot 6, with an Rf of 0.18 and considered a xanthophyll pigment, aligns with findings reported in the research by **Strain *et al.* (1943)**.

Further identification is carried out by HPLC (High Performance Liquid Chromatography). HPLC is an excellent method in analyzing pigments in higher plants and algae as it allows the separation of chlorophyll, carotenoids, and their derivatives with a small sample and a brief analysis **(Gross, 1991)**. The separation of seaweed pigments was performed using a HPLC equipped with a PDA detector. In this HPLC analysis, sample measurements can be made at the desired wavelength interval, specifically at a wavelength of 420nm. The 420nm wavelength can detect the presence of chlorophyll, carotenoids, and their degradation products. Therefore, the spectrum pattern of each separated peak on the HPLC chromatogram can be obtained. This spectrum pattern serves for the precise identification of each pigment peak by comparing it with reference libraries containing similar samples and methods.

Pigment identification was carried out based on the spectrum pattern of each peak and the order of its polarity compared to the results of the study of **Hegazi *et al.* (1998)**, which used almost the same solvent composition and stationary phase to analyze brown seaweed. Based on the results of HPLC, 5 pigments were obtained from the crude extract of *Sargassum* sp., namely chlorophyll $_{c1}$, chlorophyll $_{c2}$, fucoxanthin, xanthophyll and carotenoids (Fig. 3.). The results of this separation are sorted based on the order of elution, namely from pigments that have low solubility to pigments that have high solubility. The advantage of identification using HPLC is able to separate 2 types of pigments that have almost the same polarity, namely chlorophyll $_{c1}$ and chlorophyll $_{c2}$. It is evident that the pigments present in *Sargassum polycystum* are chlorophyll pigments, carotenoids and their derivatives. This aligns with the findings of **Hegazi (1998)**, who emphasized that the most dominant pigments in *Sargassum polycystum* are chlorophyll and fucoxanthin pigments. The prevalence of fucoxanthin in *Sargassum polycystum* contributes to the brown coloration of this type of seaweed **(Yunizal, 2004)**.

The spectra of isolate 1 formed two absorption peaks within wavelength range of 400- 600nm. This spectral pattern, is characterized by the presence of two peaks, and it is consistent with the spectral pattern of carotenoid pigments. The maximum absorbance values of isolate 1 were observed at the wavelengths of 452 and 478nm (Fig 5.). These

values are close to the maximum absorbance values of β-carotene which are at the wavelengths of 451 and 478nm.

The gray color shown in isolate 2 has been identified as pheophytin which is one of the chlorophyll derivatives known for its loss of magnesium ions. The UV-visible spectra pattern of this compound revealed maximum absorbance wavelengths at 408 and 664nm. These results are close to the maximum absorbance wavelength of pheophytin in the study of **Jeffrey *et al* (1997)**, which were 409.5 and 665.5nm.

In this study, isolate 3 has been identified as chlorophyll *a*, which shows maximum absorption at wavelengths of 409, 429, and 661nm. These results are close to the maximum absorbance wavelength of chlorophyll *a* which are 410, 430, and 662nm **(Gross, 1991)**. These findings are not much different from the spectral pattern of chlorophyll *a* marker and the research conducted by **Jeffrey *et al.* (1997)**, which showed a maximum absorption at 662 and 662.1nm, respectively. This is also supported by chlorophyll pigments showing a major peak of around 400nm, as well as a number of small peaks between 500 and 600nm, and one more major peak above 625nm. For chlorophyll *a* and *b*, the longer wavelength peaks are situated at 630 and 688nm, respectively **(Harborne, 1987)**. Any disparities in the position of the soret and Q peaks of chlorophyll *a* between the experiment and the literature is due to differences in the equipment used for measurement.

Based on the results obtained in this study, isolate 4 is chlorophyll *c* which shows maximum absorption at 417 and 654nm. Chlorophyll *c* is observed in the Soret region (at a wavelength of 400- 450nm) and Qy region (at a wavelength of 650- 680nm), indicating its coexistence with chlorophyll *a* or its derivatives. Being a polar pigment situated in close proximity to chlorophyll *a* and susceptible to degradation, the separation of chlorophyll *c* necessitates a more specific method.

The spectral pattern in isolate 5 is a fucoxanthin pigment. The results of this study show maximum absorption at 445 and 470nm. This maximum absorption value is almost the same as the maximum absorption value of fucoxanthin in the literature with polar solvents, namely 449 and 468nm **(Jeffrey *et al.* 1997)**.

The spectral pattern of isolate 6 shows maximum absorption at 418, 441, and 470nm. The maximum absorption aligns with the maximum absorption of violaxanthin. Since the maximum absorption value is almost the same as the maximum absorption value of violaxanthin in the literature with a polar solvent (acetone), namely 418.7, 442.3, and 471.7nm **(Jeffrey *et al.* 1997)**. Since violaxanthin is classified within the xanthophyll group, it can be inferred from the spectral results of fraction 6 that it is indeed a xanthophyll.

IC50 concentration of β caroten was (476± 0.32) $mg.L^{-1}$, pheophytin (720± 0. 32) $mg.L^{-1}$, chlorophyll *a* (683± 0. 32) $mg.L^{-1}$, chlorophyll *c* (582± 0. 32) $mg.L^{-1}$, fucoxanthin (413± 0. 32) $mg.L^{-1}$, and xanthophyll (521± 0. 32) $mg.L^{-1}$, respectively. The IC50 value that was measured suggests the concentration that works effectively to inhibit the free

radical activity at 50%. If the IC50 is smaller than the coumpound, hence it has high antioxidant activity. The presence of π-conjugated bonds in pigmets enable them to act as antioxidants through electron transfer mechanism.

CONCLUSION

Sargassum polycytum has 6 types of pigments, namely β carotene, pheophytin, chlorophyll *a*, chlorophyll *c*, fucoxanthin, and xanthophyll. Antioxidant activity of fucoxanthin> β carotene> xanthophyll> chlorophyll c> chlorophyll a> pheophytin.

ACKNOWLEDGE

The authors would like the thank Yayasan Pharmasi Semarang for the financial support to this research through ‘‘HIBAH YAYASAN 2023” research grant scheme.

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