

Antioxidant Phlorotannin from Brown Algae *Sargassum duplicatum*: Enzyme-assisted Extraction and Purification

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To cite this article:

Vu Ngoc Boi, Nguyen Thi My Trang, Dang Xuan Cuong, Hoang Thai Ha. Antioxidant Phlorotannin from Brown Algae *Sargassum duplicatum*: Enzyme-assisted Extraction and Purification. *World Journal of Food Science and Technology*. Special Issue: Marine Bio-Polymer: Bio-Activity, Extraction and Application. Vol. 4, No. 2, 2020, pp. 62-68. doi: 10.11648/j.wjfst.20200402.17

Received: April 8, 2020; Accepted: April 26, 2020; Published: May 19, 2020

Abstract: The study focused on the enzyme-assisted extraction and the purification of antioxidant phlorotannin content from brown algae *Sargassum duplicatum* commonly grown in Vietnam. Antioxidant activities were evaluated, consisting of total antioxidant activity and reducing power activity for the enzyme-assisted extraction. Antioxidant purified phlorotannin was analyzed total antioxidant activity, reducing power activity, DPPH free radical scavenging activity, and lipoxxygenase inhibition activity. The treating condition of brown algae by enzyme consisting of enzyme kinds (termamyl enzyme, cellulose enzyme, viscozyme enzyme), the enzyme concentration (2.5, 5.0, 7.5, 10.0, 12.5, and 15.0% (v/v)), and the treating time of algae (1, 2, 3, 4, and 5 hours). Brown algae were then soaked in 96% ethanol with the ethanol-to-brown algae ratio of 30/1 (v/w) for 24 hours at the room temperature for antioxidant phlorotannin extraction that purified by using the liquid-to-liquid method and the gel chromatography of Sephadex LH 20. Antioxidant phlorotannin purification was determined to base on DEPT and ¹H NMR spectrum. The results showed that 7.5% cellulase enzyme destroyed brown algae cell for 3 hours leading the best efficiency of antioxidant phlorotannin extraction from brown algae for 24 hours by 96% ethanol with the ethanol-to-brown algae ratio of 30/1 (v/w) at the room temperature. Phlorotannin content, total antioxidant activity, and reducing power activity corresponded to 4.45±0.11 mg phloroglucinol equivalent/g DW, 11.17±0.28 mg ascorbic acid equivalent/g DW, and 11.09±0.24 mg FeSO₄ equivalent/g DW, respectively. Two fractions of antioxidant phlorotannin (17 to 28 and 36 to 45) were collected after purification, possessed 4.1 to 177.3 and 8.3 to 112.2 mg phloroglucinol equivalent per 1087.56 mg glue, respectively. Total antioxidant activity, reducing power, and DPPH free radical scavenging corresponded to 12.48 to 585.52 and 28.08 to 371.28 mg ascorbic acid equivalent/1087.56 mg glue, 15.6 to 765.44 and 33.28 to 486.72 mg FeSO₄ equivalent/1087.56 mg glue, and 72.23 to 100% and 76.02 – 100%, respectively. Lipoxxygenase enzyme inhibition activity got 62.83 to 87.4 μM and 66.19 to 87.09 μM linoleic acid equivalent/ 100μl extract, respectively. DEPT and ¹H NMR spectrum showed phloroglucinol exist in two over fractions.

Keywords: Antioxidant, Brown Algae, Enzyme, Extraction, Lipoxxygenase, Phlorotannin, Purification

1. Introduction

Brown algae contain many bioactive substances consisting of a phlorotannin, fucoidan, alginate, and laminarin [1-3]. Inside, phlorotannins are the most interesting because of their antioxidant activity [4]. Antioxidant activity help

phlorotannin inactive free radicals that cause different diseases in the human [5-8]. Phlorotannin is the diversity of the structure and the bioactivities with phloroglucinol basic units. The bioactivities of phlorotannin are demonstrated, for

example, antioxidant, antibacterial, antiviral, anticancer, and cardiovascular disease prevention [1, 9, 10]. Phlorotannins have most the molecular weights in the range from 10 to 100 kDa and the content from 20 to 250 mg/g dry algae.

Phlorotannins are useful for the field of foods, functional foods, and pharmaceuticals [1, 4]. Especially, phloroglucinol is a drug for symptomatic treatment of painful manifestations due to dysfunction of the gastrointestinal tract and bile ducts, treatment of pain or spasm in the urinary tract (renal colic and urethral pain), treatment of manifestations of pain or spasms in gynaecology (dysmenorrhea, labour difficulties, postpartum uterine pain), and treatment support contractions during pregnancy, accompanied by rest. Therefore, there are many methods of phlorotannin extraction and its purification (liquid-to-liquid, and chromatography) from brown algae, for example, microwave, supersonic, supercritical CO₂, reflux, radiation (infrared and gamma Co60), and enzyme [11]. However, the enzyme-assisted extraction of phlorotannin has only found in the notice of Maya [12] on brown algae *Sargassum aquifolium*, *Sargassum ilicifolium*, *Sargassum muticum*, and *Sargassum polycystum*. Enzyme-assisted extraction of phlorotannin is environmentally friendly and highly selective when combined with ethanol solvents.

Brown algae *Sargassum duplicatum* distribute commonly in numerous areas in the world, such as the Pacific ocean [13], Indian ocean [14], and along with the coast of Vietnam.

Thus, the study focused on the enzyme-assisted extraction and the purification of active phlorotannin from brown algae *Sargassum duplicatum* grown in Vietnam.

2. Material and Methods

2.1. Material

Brown algae *Sargassum duplicatum* found commonly grown in Vietnam were collected, classified, and cleaned by seawater. Brown algae were then transformed into the laboratory under 10°C and dried until 19% of moisture [3, 15]. Dried algae were continuously ground and stored in PE bag at the room temperature for further studies.

All chemicals using in the current study were from Sigma – Aldrich. Distilled water and 96% ethanol was in Vietnam.

2.2. Sample Preparation

2.2.1. The Enzyme-assisted Extraction

Brown algae powder was soaked in other enzyme kinds (enzyme termamyl, enzyme cellulose, enzyme viscozyme) with the enzyme concentration (2.5, 5.0, 7.5, 10.0, 12.5, and 15.0% (v/v)) for time (1, 2, 3, 4, and 5 hours) and filtered for collecting the residue through the Whatman No4 membrane. The residues were continuously macerated in 96% ethanol for 24 hours with the ethanol-to-brown algae ratio of 30/1 (v/w) at the room temperature and filtered the membrane for the supernatant collection. The surveyed method was a factor run and other factors fixation. Evaluation of phlorotannin content and antioxidant activity (total antioxidant activity and reducing power activity) was on all supernatants for finding

the suitable condition of enzyme-assisted extraction.

2.2.2. Purification of Antioxidant Phlorotannin

The filtrate was vacuum concentrated at 45°C with 120 psi until glue form and segmented by the liquid-to-liquid method. The glue was segmented by n-hexane, chloroform, ethyl acetate, and n-butanol, in turn. Ethyl acetate fraction was concentrated and segmented by chloroform. The supernatant was continuously segmented by n-butanol for collecting the fraction that did not dissolve in n-butanol, named fraction ECN. The fraction ECN was concentrated and segmented by 96% ethanol. Ethanol fraction was concentrated and run on Sephadex LH20. 10 ml of filtrate for each tube after the column was collected and evaluated phlorotannin content, antioxidant activity (total antioxidant activity, reducing power activity, and DPPH free radical scavenging), and lipoxygenase inhibition activity. The mobile phase was chloroform: methanol: formic acid (90: 9: 1, respectively). DEPT and ¹H NMR analysis were on the most fraction of activity phlorotannin.

2.3. Quantification of Phlorotannin Content

Quantification of phlorotannin content was to base the description of Vu et al. [3]. Phlorotannin reacted Folin Ciocalteus reagent in alkaline pH (10% of Na₂CO₃) for forming phenol-MoW₁₁O₄₀)⁴⁻ blue that was measured the absorbance at the wavelength of 750 nm with phlorotannin standard.

2.4. Evaluation of Antioxidant Activity

2.4.1. Total Antioxidant Activity

Evaluation of total antioxidant activity was according to Prieto et al. [16], basing to the metabolism of Mo⁶⁺ to Mo⁵⁺ for the absorbance measurement of the compound at the wavelength of 695 nm with the ascorbic acid standard.

2.4.2. Reducing Power Activity

Evaluation of reducing power activity was to base on the metabolism of Fe³⁺ to Fe²⁺ for the absorbance measurement at the wavelength of 655 nm with FeSO₄ standard [2].

2.4.3. DPPH Free Radical Scavenging Activity

Evaluation of DPPH free radical scavenging activity based on the describe of Dang et al. [2] The form of yellow colour from the reaction between DPPH and antioxidants exhibited the numbers of DPPH radicals were inactive. All sample measurement of absorbance was at a wavelength of 550 nm.

2.5. Evaluation of Lipoxygenase Inhibition Activity

150 µL of extract added to 2850 µL of the compound (0.2M citrate-phosphate buffer (pH 9.0), 0.25% of Tween 20, 0.125mM linoleic acid, and lipoxygenase enzyme (57µg protein)) for forming 03 ml of the compound that was measured the absorbance at the wavelength of 234 nm with a linoleic acid standard [17].

2.6. Data Analysis

All experiments were triplicated ($n=3$). Statistic and ANOVA analysis were by using the software MS. Excel 2013. Unnormal value movement was by the method of Duncan.

3. Results and Discussion

3.1. Effect of the Enzyme-assisted Extraction on Phlorotannin Content and Bioactivity

3.1.1. Effect of Enzyme Kinds

Enzyme kinds affected strong phlorotannin content extracting from brown algae *Sargassum duplicatum* ($p<0.05$). Phlorotannin content corresponded to 3.26 ± 0.08 , 3.57 ± 0.11 , and 3.34 ± 0.07 mg phloroglucinol equivalent/g DW as using termamyl enzyme, cellulase enzyme, and viscozyme enzyme for treating the algae, respectively (Figure 1). Phlorotannin content of cellulase enzyme-treated algae was the highest, compared to other enzyme-treated algae. The thing showed that cellulase enzyme destroyed cell membrane of brown algae better than viscozyme enzyme and termamyl enzyme leading to the difference in phlorotannin content.

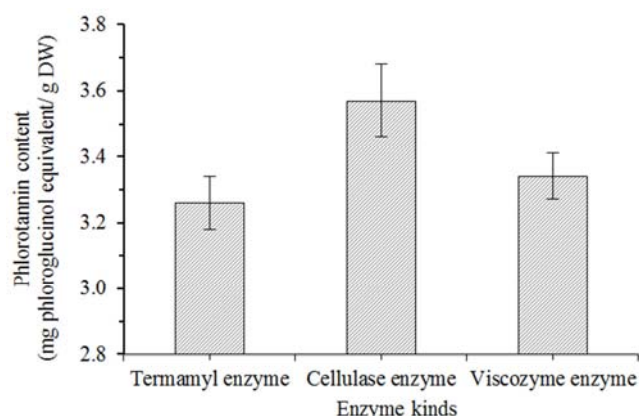


Figure 1. Effect of enzyme kinds on phlorotannin content.

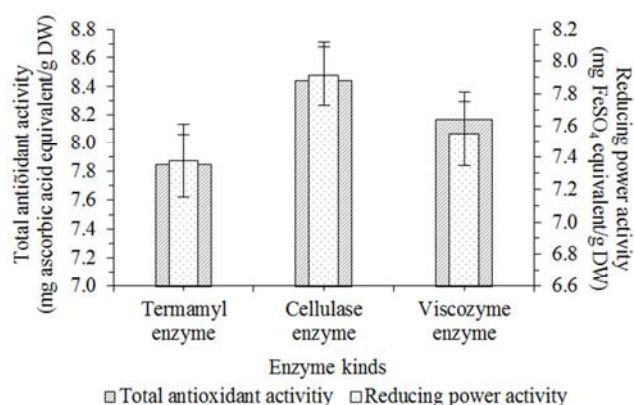


Figure 2. Effect of enzyme kinds on antioxidant activity.

Enzyme kinds using for the treatment of algae also strongly affected total antioxidant activity and reducing power activity ($p<0.05$). Total antioxidant activity and reducing power activity got the highest value as cellulase enzyme-treated

algae in comparison to other enzyme-treated algae, corresponding to 8.44 ± 0.27 mg ascorbic acid equivalent/g DW, 7.91 ± 0.18 mg FeSO₄ equivalent/g DW, respectively (Figure 2). Total antioxidant activity of extract from viscozyme enzyme-treated algae and termamyl enzyme-treated algae was 96.8 and 93 times of cellulase enzyme-treated algae, respectively. Reducing power activity of viscozyme enzyme-treated algae was 102.3% and 95.5% of termamyl enzyme-treated algae and cellulase enzyme-treated algae, respectively. Phlorotannin content has a positive correlation to antioxidant activities.

3.1.2. Effect of the Enzyme Concentration

The different enzyme concentration caused a difference in phlorotannin content and antioxidant activities ($p<0.5$). Phlorotannin content got the highest value as using 7.5% of the enzyme for the algae treatment, corresponding to 4.28 ± 0.13 mg phloroglucinol equivalent/g DW. The change of phlorotannin content had a trend according to the non-linear model with the maximum peak as an increase of enzyme concentration from 2.5% to 15%. Phlorotannin content was 2.94 ± 0.06 mg phloroglucinol equivalent/g DW as 2.5% of enzyme-treated algae, corresponding to 68.69% of the maximum peak (Figure 3). Cell membrane destroy of brown algae increased according to the increase of enzyme concentration. When protein in cell membrane structure released, they combined to phlorotannin for forming the precipitate of phlorotannin-protein [4, 18]. Therefore, phlorotannin content decreased when enzyme concentration was over 10%.

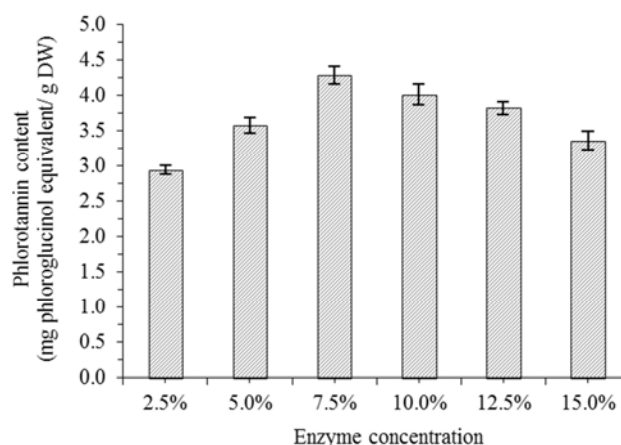


Figure 3. Effect of enzyme concentration on phlorotannin content.

Total antioxidant activity and reducing power increased when using enzyme concentration was from 2.5% đến 7.5%, and decreased as enzyme concentration using over 7.5% that caused the increase of the phlorotannin-non solute consisting of protein (Figure 4). Therefore, total antioxidant activity and reducing power activity decreased as using enzyme concentration was from 10% to 15%. Total antioxidant activity and reducing power activity changed according to the model of level 2 with a maximum peak at 7.5% of enzyme concentration. The correlation between phlorotannin and antioxidant activities was high ($R^2>0.8$).

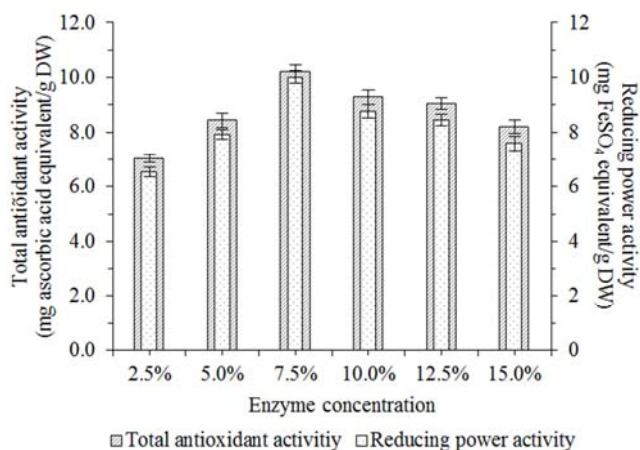


Figure 4. Effect of enzyme concentration on antioxidant activities.

3.1.3. Effect of the Extraction Time

Extraction time affected strong phlorotannin content and antioxidant activities ($p < 0.05$). At three hours of extraction, phlorotannin content, total antioxidant activity, and reducing power activity got the highest value, corresponding to $4.45 \text{ mg} \pm 0.11 \text{ mg}$ phloroglucinol equivalent/g DW, $11.17 \pm 0.28 \text{ mg}$ ascorbic acid equivalent/g DW, and $11.09 \pm 0.24 \text{ mg}$ FeSO₄ equivalent/g DW, respectively (Figure 5). Phlorotannin content got the lowest value ($3.17 \pm 0.09 \text{ mg}$ phloroglucinol equivalent/g DW) as the extraction time of one hour. The extraction time had a positive correlation to the solutes in the extract from brown algae, but the solutes mainly contained phlorotannin-non solutes that caused the difficulty in purification processing of active phlorotannin. The things explained for the results of phlorotannin decrease when the extraction time was over three hours.

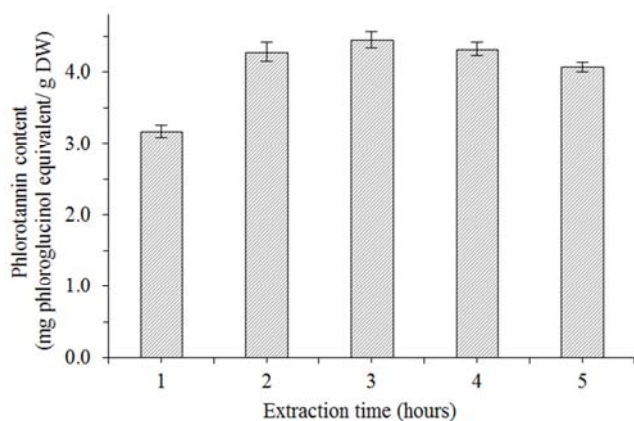


Figure 5. Effect of extraction time on phlorotannin content.

Total antioxidant activity and reducing power activity was affected by the extraction time and changed according to the model of level 2 with the maximum peak. Total antioxidant activity and reducing power activity got the lowest value when the extraction time was one hour, corresponding to $7.25 \pm 0.21 \text{ mg}$ ascorbic acid equivalent/g DW and $7.04 \pm 0.17 \text{ mg}$ FeSO₄/g DW, respectively (Figure 6). When the extraction time was 5 hours, total antioxidant activity and reducing power activity was 87.02% and 82.6% of extracted algae at three hours,

respectively. Phlorotannin content was a positive correlation to antioxidant activities according to the change of extraction time.

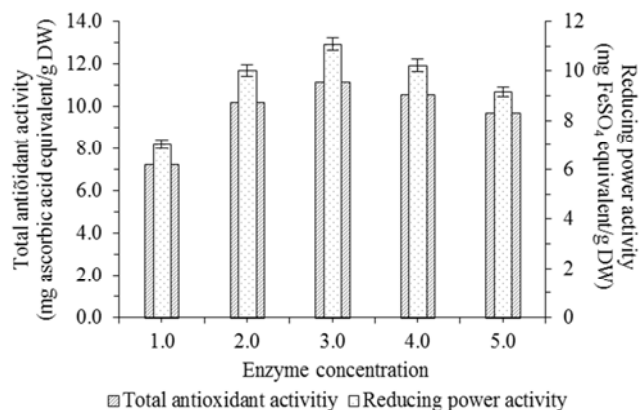


Figure 6. Effect of extraction time on antioxidant activities.

3.2. Activity Phlorotannin Purification

3.2.1. Phlorotannin Content

The results showed that phlorotannin existed in fraction 17 to 28 and 36 to 45 in sixty fractions collecting after the chromatography. Phlorotannin content of fraction 17 to 28 and 36 to 45 corresponded to 4.1 to 177.3 mg phloroglucinol equivalent/ 1087.56 mg glue (glue using for the Sephadex LH 20 run) and 8.3 – 112.2 mg phloroglucinol equivalent/ 1087.56 mg glue (Figure 7).

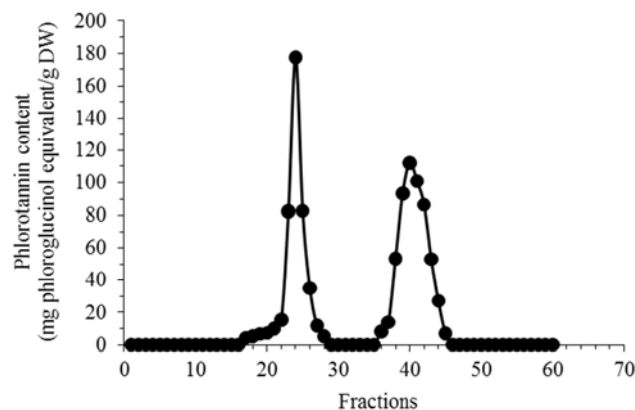


Figure 7. Phlorotannin fractions after the column Sephadex LH 20.

Basing on the characterization of gel Sephadex LH 20, phlorotannin of fraction 17 to 28 possessed the molecular weight higher than that of fraction 36 to 45. The peak area of fraction 17 to 28 was 79.68% of fraction 36 to 45. The molecular weight of phlorotannin depended on brown algae species, habitation, and harvest seasons. The method of the extraction and the purification affected the numbers of phlorotannin fractions that collected after the column Sephadex LH20. The results of the current study were different in comparison to the previous studies [15, 19, 20].

3.2.2. Total Antioxidant Activity

Total antioxidant activity of fraction 17 to 28 and 36 to 45

corresponded to 12.48 to 585.52 mg ascorbic acid equivalent and 28.08 to 371.28 mg ascorbic acid equivalent per 1087.56 mg glue (Figure 8). Total antioxidant activity of different fractions had a strong correlation to phlorotannin content ($R^2=0.99$). It meant that phlorotannin had a role in the decision of total antioxidant activity. The change of total antioxidant activity depended on phlorotannin content change.

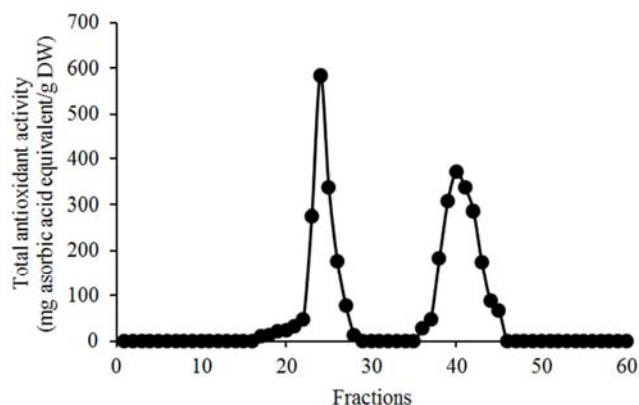


Figure 8. Total antioxidant activity of different fractions after the column Sephadex LH 20.

3.2.3. Reducing Power Activity

Reducing power activity of fractions 17 to 28 and 36 to 45 got 15.6 to 765.44 and 33.28 to 486.72 mg FeSO_4 equivalent/1087.56 mg glue. Reducing power activity of fractions 17 to 28 was higher than fractions 36 to 45 (Figure 9).

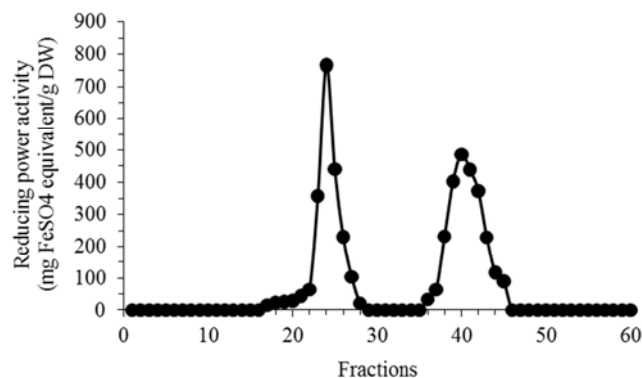


Figure 9. Reducing power activity of different fractions after the column Sephadex LH 20.

The correlation between reducing power activity of different fractions and phlorotannin content was strong ($R^2=0.99$). It meant that phlorotannin possesses reducing power activity. The results showed that it is suitable for the results and the discussion in the enzyme-assisted extraction in the current study and the previous notices on antioxidant phlorotannin [15, 21].

3.2.4. DPPH free Radical Scavenging Activity

DPPH free radical scavenging activity of fraction 17 to 28 and 36 to 45 varied from 72.23 to 100% and 76.02 to 100%, respectively, and correlated to phlorotannin content, respectively ($R^2=0.99$) (Figure 10). Fraction 17 to 28

possessed DPPH free radical scavenging activity larger than fraction 36 to 45. The things showed that higher molecular weight of phlorotannin exhibited the better DPPH free radical scavenging activity than the lower molecular weight of phlorotannin. Phlorotannin of two fractions 17 to 28 and 36 to 45 had DPPH free radical scavenging activity, suitable for the previous notices [22].

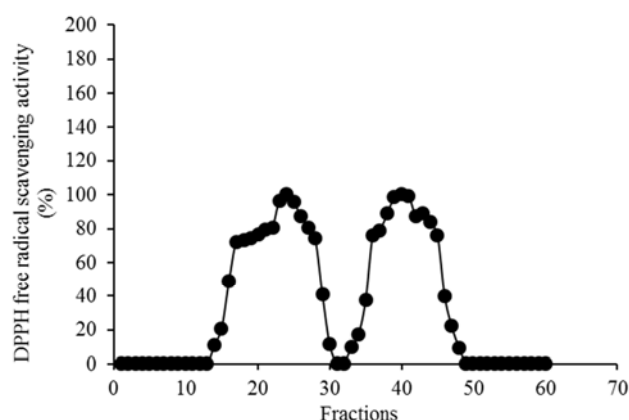


Figure 10. DPPH free radical scavenging activity of different fractions after the column Sephadex LH 20.

3.2.5. Lipoygenase Enzyme Inhibition Activity

Lipoygenase enzyme inhibition activity of fraction 17 to 28 and 36 to 45 corresponded to 62.83 to 87.4 and 66.19 to 87.09 μM linoleic acid equivalent/100 μl (Figure 11). Lipoygenase enzyme inhibition activity correlated to phlorotannin content ($R^2=0.99$), similar antioxidant activities. The thing showed that phlorotannin possesses both antioxidant activities and lipoygenase enzyme inhibition activity that changed according to the phlorotannin content change.

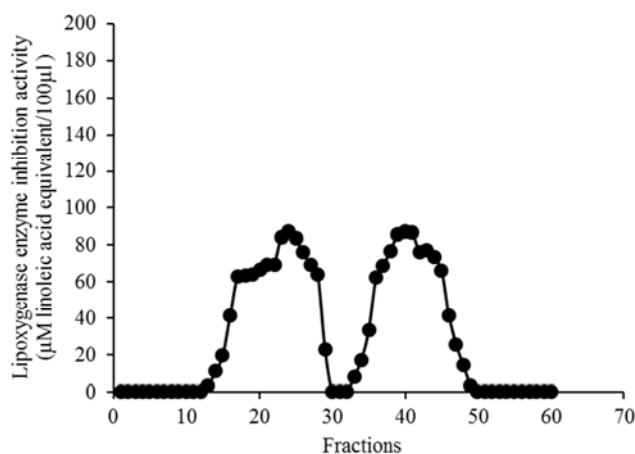


Figure 11. Lipoygenase enzyme inhibition activity of different fractions after the column Sephadex LH 20.

3.2.6. Structure Characterization of Purified Phlorotannin

^1H - NMR and ^{13}C - NMR spectrum noticed two signals, ^1H - NMR ($\delta = 4.790$ and 6.033), ^{13}C - NMR ($\delta = 157.79$ and 95.18), respectively (Figures 12 and 13). Proton spectrum exhibited the hydro signal of aromatic ring group -CH. DEPT

spectrum only had carbon of group –CH and –CO (Figure 14). Therefore, it could identify that low molecular weight phlorotannin exists in segments 36 to 45 is phloroglucinol (monomer of phlorotannin), suitable for the previous notices [23, 24]. However, the isolation method of phloroglucinol from brown algae *Sargassum duplicatum* was different in the current study.

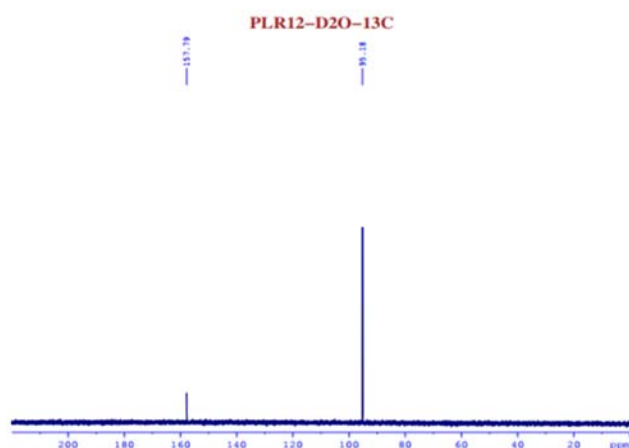


Figure 12. ^1H -NMR spectrum of fraction 36 to 45.

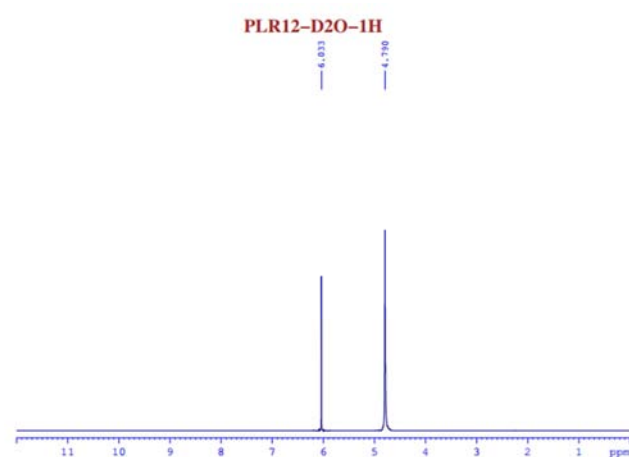


Figure 13. ^{13}C -NMR spectrum of fraction 36 to 45.

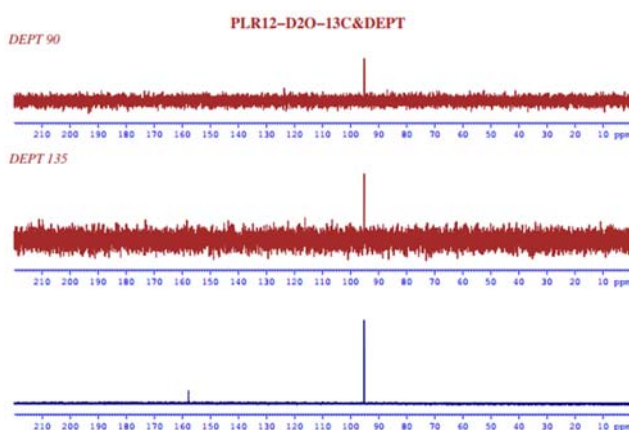


Figure 14. DEPT spectrum of fraction 36 – 45.

4. Conclusion

Antioxidant phlorotannin and antioxidant activities (total antioxidant activity and reducing power activity) got the highest value, corresponding to $4.45 \text{ mg} \pm 0.11 \text{ mg}$ phloroglucinol equivalent/g DW, and $(11.17 \pm 0.28 \text{ mg})$ ascorbic acid equivalent/g DW, and $11.09 \pm 0.24 \text{ mg}$ FeSO₄ equivalent/g DW), respectively as the enzyme-assisted extraction at the condition consisting of 7.5% enzyme concentration for 3 hours and then the residues soaking in 96% ethanol for 24 hours at the room temperature with the ethanol-to-brown algae ratio of 30/1 (v/w). Phloroglucinol purification from brown algae *Sargassum duplicatum* was in the following order: (1) extract concentration at 45°C with 120 psi until glue form; (2) The glue segment by n-hexane, chloroform, ethyl acetate, and n-butanol, in turn; (3) The ethyl acetate segment concentration and the extraction by chloroform for the upper liquid layer collection; (4) the supernatant segment by n-butanol and the n-butanol-non fraction collection; (5) The concentration and the segment of the insoluble fraction in n-butanol fraction by 96% ethanol; (6) the concentration of the ethanol fraction and run on Sephadex LH20 for collecting fraction 36 to 45. Fraction 17 to 28 contained phlorotannin that had the molecular weight, the antioxidant activity, and the lipoxygenase enzyme inhibition activity were higher than phlorotannin in fraction 36 to 45.

Acknowledgements

Thank Dr. Hoang Ngoc Cuong, Bsc. Pham Thi Hoang Oanh (Binh Duong University) for help in the experiment processing. Thank Nha Trang University, Department of Science and Technology (Ninh Thuan province), and Nha Trang Institute of Technology Application and Research for funding.

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