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ACETYLCHOLINESTERASE INHIBITORY ACTIVITY OF FATTY ACIDS ISOLATED FROM BROWN SEAWEED *SARGASSUM MCCLUREI* SETCHELL

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Summary

Acetylcholinesterase Inhibitory Activity of Fatty Acids Isolated from Brown Seaweed *Sargassum mcclurei* Setchell

The 80% methanol extract of *Sargassum mcclurei* (brown seaweed) showed a significant acetylcholinesterase (AChE) inhibitory activity. The total extract at a concentration of 25 µg/ml inhibited 47 ± 11% of AChE activity. By using organic solvents with increasing polarity, four fractions (*n*-hexane, dichloromethane, *n*-butanol and water) were obtained. *n*-Hexane fraction showing 42% inhibition at 5000 µg/ml was then fractionated by column chromatography in *n*-hexane-ethyl acetate to obtain two pure compounds, identified as palmitic acid and oleic acid. Especially palmitic acid at a concentration of 300 µg/ml could inhibit 81 ± 3% AChE activity (IC_{50} = 100 ± 7 µg/ml).

Keywords: *Sargassum mcclurei, Acetylcholinesterase inhibitory activity, Palmitic acid, Oleic acid.*

1. Introduction

Alzheimer's disease is the most common form of dementia, which is one of the four leading causes of death in developed nations. The disease affects memory, thinking, and behavior, thereby interfering with the patient's daily life [1]. One of the drug classes indicated for people with Alzheimer's disease is the acetylcholinesterase enzyme inhibitor [2].

The acetylcholinesterase inhibitors on the market, such as donepezil, galantamine, and rivastigmine [3], are mainly imported at high cost and have many notable side effects such as: hepatotoxicity, nausea, confusion, sleep disturbances, tachycardia [4],[5]. Therefore, active compounds that improve memory but have few side effects from natural medicinal sources are of interest to research.

One of the important sources of medicinal herbs comes from marine life. Vietnam has more than 3000 km of coastline with many reefs, along with the development of many species of algae, especially brown seaweed, promising great therapeutic potential. In 2019, Moodie et al. reported some compounds from brown seaweed which had a significant acetylcholinesterase inhibitory (AChE) activity [6]. However, there have been no published studies on acetylcholinesterase inhibitory activity on brown seaweed *Sargassum mcclurei* [7], which is commonly found in Nha Trang, Khanh Hoa.

The aim of this study was to investigate the extraction, isolation, and structural determination of compounds with potential acetylcholinesterase inhibitory activity from brown seaweed *Sargassum mcclurei*.

2. Materials and methods

2.1. Seaweed material

The brown seaweed *Sargassum mcclurei* was collected in Song Lo (Phuoc Dong commune, Nha Trang city, Khanh Hoa province) in July 2020. The sample was identified by Anh-Duy Do (Research Institute for Marine Fisheries, Hai Phong city, Vietnam).

2.2. Chemicals

Enzyme AChE type EC 3.1.1.7, acetylthiocholine iodide (ATCI), 5-5'-dithiobis-2-nitrobenzoic acid (DTNB), and Tris base were purchased from Sigma-Aldrich (USA). Berberine chloride standard (QT122 071119, 86.2%) was purchased from the Institute of Drug Quality Control Ho Chi Minh City (HCM, Vietnam) and galantamine standard (17FP01166, 100.2%) was purchased from Aurobindo Pharma (India). Organic solvents, including dichloromethane, ethyl acetate, *n*-butanol were purchased from Xilong (Chinese); methanol from Scharlau (Spain); *n*-hexane from GHTECH (Chinese), and formic acid from VWR Chemicals (USA).

2.3. Equipments

Thin-layer chromatography was carried out on precoated Merck Kieselgel 60 F_{254} plates (0.25 mm, Merck, USA). Spots were detected by ultraviolet light (254 and 366 nm) in a CN-15 darkroom cabinet (Vilber Lourmat Deutschland

GmbH, Eberhardzell, Germany) and by spraying with the vanillin-sulfuric acid reagent (vanillin/H_2SO_4 5%), followed by heating to 105°C. Column chromatography was carried out using *silica gel* 60 (40 - 63 µm, Merck, USA). Nuclear magnetic resonance (NMR) ^1H and ^{13}C (600 MHz) spectra were recorded on a Brucker spectrometer (USA). HPLC- MS analyses were performed with an ACQUITY Arc System - ACQUITY QDa Mass Detector (Waters, USA). The absorbance was measured with iMark microplate reader Biorad 1681130 (CA, USA) at 415 nm.

2.4. Extraction and isolation of Sargassum mcclurei

The seaweed was washed with seawater then dried in a convection drying oven at 40°C until the moisture content reached about 8%. Dried seaweed samples were ground and sifted into powder with the size of 0.75 mm and stored in PE vacuum bags (500 g/bag) under vacuum at -20 °C.

Dried seaweed powder (2 kg) was extracted three times during 60 min with MeOH 80% in an ultrasonic bath. The extract obtained was filtered, evaporated, freeze-dried, and stored at -20 °C.

The freeze-dried methanol extract (211.93 g) of brown seaweed *Sargassum mcclurei* was extracted by solid-liquid extraction technique, using organic solvents with increasing polarity as follows: *n*-hexane, dichloromethane, *n*-butanol, and distilled water. The *n*-hexane extract was fractionated by column chromatography following the gradient: CH_2Cl_2/*n*-hexane to afford 23 fractions (Fr. 1-23). Fractions 8 and 23 yielded two pure compounds identified as palmitic acid (24.7 mg) and oleic acid (13.7 mg). Each compound was analyzed by NMR (^1H and ^{13}C) and by HPLC-MS in both positive and negative ion modes.

2.5. Acetylcholinesterase (AChE) inhibitory assay

The microplate assay for AChE inhibition activity was based on Ellman's method. In a 96-well plate (LOT: A901354, Aptaca, Italy): 140 µL Tris-HCl buffer pH 8, 20 µL control sample or test sample, and 20 µL enzyme AChE 0.25 U/ml were mixed and incubated at 25 °C for 15 min. The reactions were then initiated with the addition of 10 µL of DTNB 2.5 mM and 10 µL of ATCI 2.5 mM, which were mixed and incubated at 25°C for 15 min. The absorbance was measured with a wavelength of 415 nm [8],[9]. Carry out each sample on 3 wells. The percentage of inhibition was calculated by comparing the rates for the samples to the blank (without inhibitors). Galantamine and berberine chloride were used as positive controls.

The samples, including crude and fractionated extracts, compounds **1** and **2,** controls galantamine and berberine chloride, were dissolved in methanol 20% at stock solution concentration (50000 µg/ml or 500 µg/ml, via extracts or compounds, respectively) and stored at -20°C. Before AChE inhibitory assay, the stock solutions were de-frozen and then diluted in distilled water to examination concentrations.

The percentage of AChE inhibition (% I) was calculated by the followed formula:

$$I\% = (1 - \frac{A_{S-E} - A_S}{A_{O-E} - A_O}) \times 100\%$$

I %: Percentage of AChE inhibition

A_S: Absorbance of a sample without enzyme AChE

A_{S-E}: Absorbance of a sample with enzyme AChE

A_O: Absorbance of the blank without enzyme AChE

A_{O-E}: Absorbance of the blank with enzyme AChE

Value IC_{50} was calculated using the graph of log (dose) vs. % I.

3. Results

3.1. Study on extraction conditions targeting AChE inhibitory activity

Different factors affecting the extraction were performed in this research, such as extraction method, extraction solvent, extraction duration time, and extraction temperature. Extracts from these conditions were all evaluated for AChE inhibitory activity (**Table 1**).

Table 1. AChE inhibitory activity of extracts from *Sargassum mcclurei* in different extraction conditions

Factor	Condition	Average inhibitory activity (%) ± SD	Average extraction efficiency (%) ± SD
	Magnetic stirring + reflux	21 ± 11	21 ± 4
Method	Ultrasonic	28 ± 2	17 ± 4
	Reflux	22 ± 6	18 ± 1

	Methanol 100%	5 ± 9	16 ± 1
Solvent	Methanol 80%	28 ± 2	17 ± 1
	Water	15 ± 3	18 ± 1
	30 °C	24 ± 13	17 ± 4
Temperature	45 °C	22 ± 15	17 ± 1
	60 °C	28 ± 2	18 ± 1
	15 min	9 ± 5	17 ± 1
Duration	30 min	20 ± 14	17 ± 4
	60 min	28 ± 2	18 ± 1

AChE inhibitory activities in the presence of extracts (5000 µg/ml in methanol 20%) were mesured by using Ellman's method. Data are mean ± SD (n=3) expressed in % of inhibitory activity, compared with the blank (methanol 20%). Extraction efficiencies following different extraction conditions are percentage of the mass of the extracts and the one of initial dried seaweed powder (weight method). Data are mean ± SD (n=3).

The results obtained above suggested the optimal extraction conditions to receive the extract with the highest percentage of inhibition activity (I = 28 ± 2%): extraction with methanol 80% by ultrasonic method at 60°C for 60 minutes. We also determined that three times could give a complete extraction. Since the 4th time of extraction, only salt was obtained and detected on TLC.

3.2. Preparation of extract and isolation pure compounds from Sargassum mcclurei

With the selected conditions, dried seaweed powder (2 kg) of *Sargassum mcclurei* was extracted using 20 L of methanol 80%. The extract was later evaporated with a rotary evaporator under reduced pressure at 50 °C and lyophilized, to obtain 211.93 g of lyophilized powder.

The lyophilized powder was sequentially fractionated with *n*-hexane (14.10 L), dichloromethane (15.60 L), *n*-butanol (9.50 L), and finally water (0.20 L), separately concentrated to dryness under vacuum, and obtained four respective fractions: *n*-hexane (1.46 g), dichloromethane (0.50 g), *n*-butanol (61.08 g) and water (105.57 g). In the water fraction, 13.26 g of crystallized salt was removed (productivity 6.72%). The extraction and isolation processes are illustrated in **Fig. 1**.

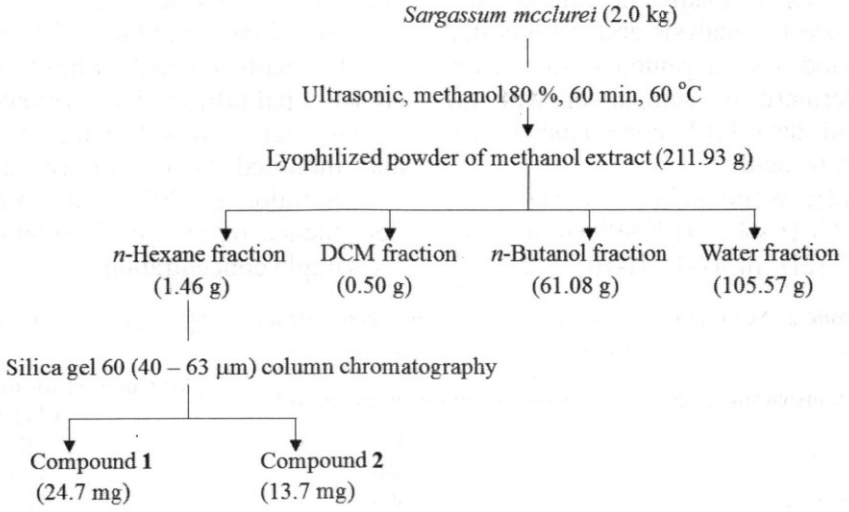

Fig. 1. Flow diagram for the isolation of fatty acid from the aqueous methanol extract of *Sargassum mcclurei*

Results on AChE inhibitory activity (IC_{50}) of four fractions *n*-hexane, dichloromethane, *n*-butanol, and water showed that the *n*-hexane fraction possessed a potential AChE inhibitory activity (**Table 2**). This fraction was then loaded on column chromatography and eluted with *n*-hexane-ethyl acetate (at changed ratios) to obtain 23 secondary fractions. From the 8th and 23rd fractions, by recrystallization, we obtained two pure compounds, which were analyzed by TLC and HPLC-MS.

The chemical structures of isolated compounds were elucidated by analyzing their MS and NMR data and by comparing with those in the literature. We, therefore, determined two fatty acids from *Sargassum mcclurei*: palmitic acid (1) and oleic acid (2).

Palmitic acid (1): white solid; ESI-MS (*m/z*) 255.2 [M - H]$^-$ $C_{16}H_{32}O_2$; ^{1}H-NMR (600 MHz, CD_3OD): δ_H 2.29 (2H, t, J = 7.5 Hz, H-2), 1.62 (2H, h, J = 7.0 Hz, H-3), 1.33 (24H, m, H-4 - H-15), 0.92 (3H, t, J = 7.1 Hz, H-16); ^{13}C-NMR (150 MHz, CD_3OD): δ_C 177.8 (C-1), 35.1 (C-2), 30.2 - 33.1 (C-4 - C-14), 26.1 (C-3), 23.7 (C-15), 14.4 (C-16).

Compound **1** was obtained as a white solid. The molecule formula of compound **1** was identified as $C_{16}H_{32}O_2$ by ESI-MS at *m/z* 255.2 [M - H]$^-$. The ^{1}H-NMR data of compound **1** showed signals of one methyl group at δ_H 0.92 (3H, t, J = 7.1 Hz, H-16), an α-carbonyl methylene group at δ_H 2.29 (2H, t, J = 7.5 Hz, H-2) and a β-carbonyl methylene group at δ_H 1.62 (2H, h, J = 7.0 Hz, H-3). The ^{1}H-NMR spectrum of compound **1** showed a broad signal of a long chain of methylene groups at 1.33 (24H, m, H-4 - H-15). When CD_3OD is used as a solvent, no carbonyl signal can be observed in the ^{1}H NMR spectrum. The ^{13}C-NMR spectrum had the signal of a carbonyl ester group at δ_C 177.8 (C-1) and the signal of a methyl group at δ_C 14.4 (C-16). All the other signals at δ_C 35.1 (C-2), 30.2 - 33.1 (C-4 - C-14), 26.1 (C-3), and 23.7 (C-15) were due to methylene carbons in a long chain. The spectra analysis above suggested that this compound was a saturated long-chain fatty acid. Furthermore, by comparison with the published spectral data [10], compound **1** was identified as palmitic acid.

Oleic acid (2): white solid; ESI-MS (*m/z*) 281.9 [M - H]$^-$ $C_{18}H_{34}O_2$; ^{1}H-NMR (600 MHz, CD_3OD): δ_H 5.37 (2H, m, H-9 - H-10), 2.21 (2H, t, J = 7.5 Hz, H-2), 2.05 (4H, m, H-8, H-11), 1.63 (2H, m, H-3), 1.34 (20H, m, H-4 - H-7, H-12 - H-17), 0.92 (3H, t, J = 7.2 Hz, H-18); ^{13}C-NMR (150 MHz, CD_3OD): δ_C 179.3 (C-1), 130.9 (C-10), 130.8 (C-9), 36.5 (C-2), 30.2 - 33.1 (C-4 - C-7, C-12 - C-16), 28.1 (C-8), 28.1 (C-11), 26.9 (C-3), 23.7 (C-17), 14.4 (C-18).

Compound **2** was obtained as a white solid. The ESI-MS of 2 showed a peak at *m/z* 281.9 [M - H]$^-$ corresponding to the molecular formula $C_{18}H_{34}O_2$. The ^{1}H-NMR and ^{13}C-NMR spectral data of compound **2** were similar to those of compound **1** with the presence of a methyl group (δ_H 0.92 (3H, t), δ_C 14.4), an α-carbonyl methylene group (δ_H 2.21 (2H, t), δ_C 36.5) and a β-carbonyl methylene group ((δ_H 1.63 (2H, m), δ_C 26.9). NMR spectra of compound **2** compound had the signal of a double bone (δ_H 5.37 (2H, m), δ_C 130.9 and 130.8), the signal of two α-methylene groups (δ_H 2.05 (4H, m), δ_C 28.1 and 28.1) and the signal of methylene in a long chain (δ_H 1.34 (20H, m), δ_C 30.2 - 33.1 and 23.7). Based on the above spectra data analysis and comparison with the published spectral data [10], compound **2** was identified as oleic acid.

3.3. Evaluation of AChE inhibitory activity of isolated fatty acids and organic solvent fractionated extracts

In the AChE inhibitory activity assay, both pure compounds and extracts from *Sargassum mcclurei* exhibited moderate AChE inhibitory activities (**Table 2**). The inhibitory concentrations ranged from 5 to 5000 µg/ml.

The half-maximal inhibitory concentration (IC_{50}) of palmitic acid, determined from the dose-activity curve, was 100 µg/ml. This compound also inhibited AChE activity into 81 ± 3% at a concentration of 300 µg/ml. **Fig. 2** shows the dependence of the AChE inhibitory activity on the sample concentration.

Table 2. AChE inhibitory activity of the extracts and constituents of *Sargassum mcclurei*
Results are presented as mean ± SD (n=3)

Extracts/ fractions/compounds	Concentration range (µg/mL)	Maximal AChE inhibitory activity (I %) ± SD
Crude extract	5 - 5000	**40** ± 9
n-Hexane fraction	5 - 5000	**42** ± 16
Dichloromethane fraction	5 - 5000	**84** ± 25
n-Butanol fraction	5 - 5000	**73** ± 16
Water fraction	5 - 5000	**34** ± 6
Compound **1** (Palmitic acid)	0.3 - 300 (IC_{50} = **100** ± 7)	**81** ± 3
Compound **2** (Oleic acid)	0.3 - 300	**50** ± 6
Berberine chloride*	0.1 - 50 (IC_{50} = **0.45** ± 0.05)	**98** ± 3
Galantamine*	0.1 - 50 (IC_{50} = **1** ± 0.06)	**99** ± 3

* Berberine chloride and galantamine were used as positive controls for AChE inhibitory effect.

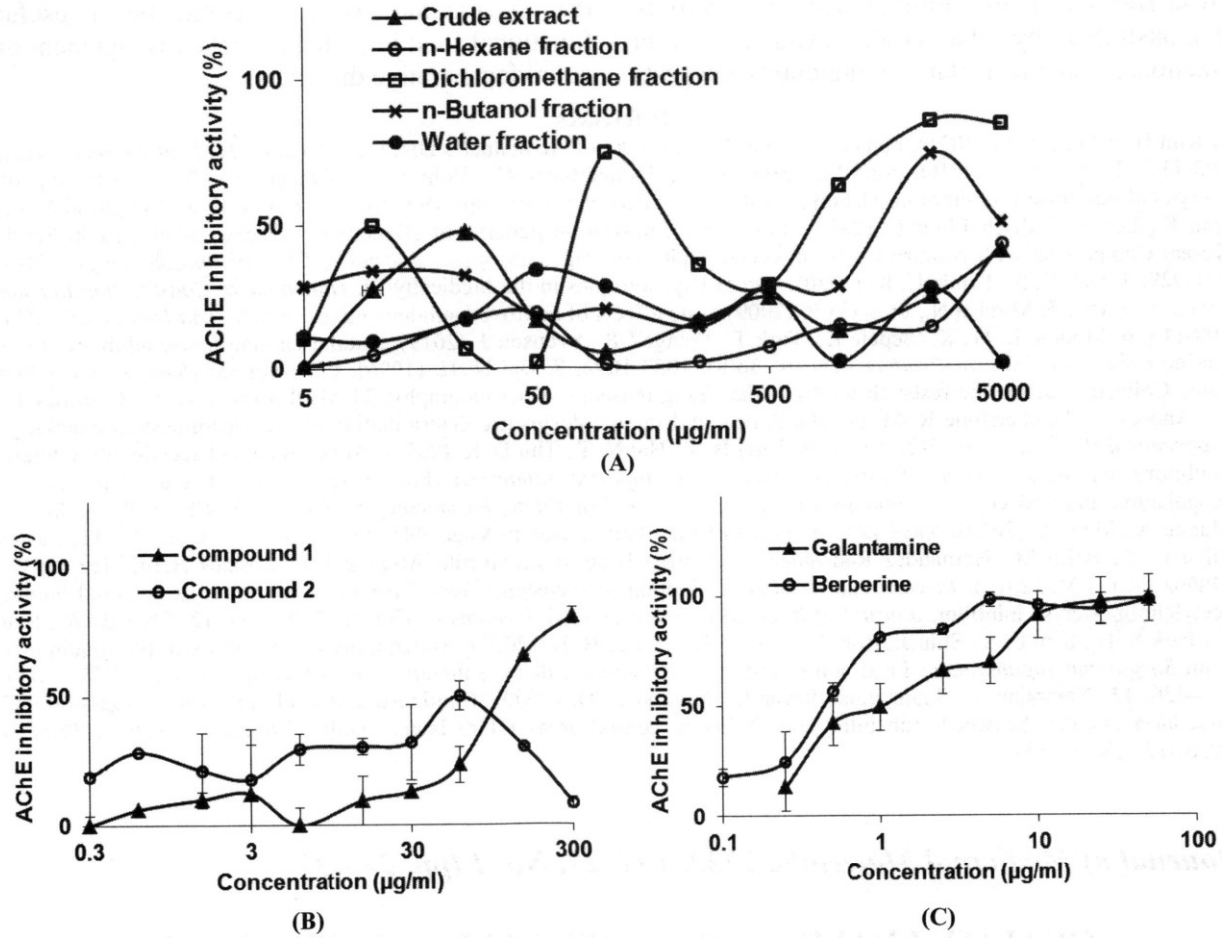

Fig. 2. Dose-dependent effect of extracts (**A**), of compounds **1** and **2** (**B**) from *Sargassum mcclurei* and of the controls galantamine and berberine (**C**) on the AChE inhibitory activity. Data are mean ± SD (n=3) expressed in % of inhibitory activity, compared with the blank (methanol 20%).

4. Discussion

Evaluation for the AChE inhibitory activity of the extracts and constituents of *Sargassum mcclurei* showed that the dichloromethane and *n*-butanol fractions presented the highest activity, I% = 84 ± 25 and 73 ± 16 respectively, in the performed concentration range (5-5000 µg/mL). These fractions were further chosen for the isolation and purification of AChE inhibitors.

Currently, many compounds isolated from *Sargassum* have been described as capable to inhibit AChE, such as fucosterol isolated from *Sargassum horridum* with higher AChE inhibitory activity than neostigmine [11]; two plastoquinones sargaquinoic acid and sargachromenol were isolated from *Sargassum sagamianum* and *Sargassum serratifolium* (IC_{50} = 23.2 and 32.7 µM, respectively) [12],[6]; fucoxanthin from *Sargassum horneri* also presented anticholinesterase activity in mice [6]. Another study on screening of 11 seaweeds from South Indian coastal areas for the AChE inhibitory showed that *Sargassum* was the mos t active genus among all the different genera tested [13]. Most of the compounds isolated from the genus *Sargassum* had moderate inhibitory activity. However, no concerning data of *Sargassum mcclurei* has been published till now. Palmitic acid isolated from *Sargassum mcclurei* also showed moderate inhibitory activity with IC_{50} of 100 ± 7 µg/mL (corresponding concentration of 398 ± 27 µM). Oleic acid did not show activity linearity to concentration in the performed range.

These seaweed extracts and their active components could emerge as natural and alternative anticholinesterase drugs or serve as starting points for synthesizing more effective AChE inhibitors.

5. Conclusion

This is the first study to investigate cholinesterase inhibitory properties of the extracts from *Sargassum mcclurei*. Two fatty acids, palmitic acid (1) and oleic acid (2), were isolated and determined AChE inhibitory activity

(IC_{50} 100 µg/mL for palmitic acid). The activity demonstrated by the crude extract, solvent fractions, and the isolated compounds suggests that *Sargassum mcclurei* could be a useful functional food ingredient for the management of neurodegenerative disorders.

References

1. Kim H., Chung J. Y. (2021), Pathobiolgy and Management of Alzheimer's Disease, *Chonnam Medical Journal*, *57*(2), 108-117. **2.** Marucci G., Buccioni M., Ben D. D., Lambertucci C., Volpini R., Amenta F. (2021), Efficacy of acetylcholinesterase inhibitors in Alzheimer's disease. *Neuropharmacology*, 190, 108352. **3.** Lee H. J., Roughead E. E., Han E., Lee J., Kalisch Ellett L. (2021), Post-market utilization patterns of Alzheimer's disease treatments in South Korea: Comparison with countries with universal health coverage, *European Journal of Clinical Pharmacology*, 77(6), 921-929. **4.** Deb K. S., Panda U. K. (2020), Use of Psychotropics in the Medically Ill, *Handbook of Consultation Liaison Psychiatry*, 153. **5.** Mimica N., Presecki P. (2009), Side effects of approved antidementives, *Psychiatria Danubina*, 21(1), 108-113. **6.** Moodie L. W. K., Sepčić K., Turk T., FrangeŽ R., Svenson J. (2019), Natural cholinesterase inhibitors from marine organisms, *Natural Product Reports*, 36(8), 1053-1092. **7.** Dai N. H. (1996), *Chi rong mơ (Sargassum) ở Việt Nam,* Collection of marine research works - Nha Trang museum of oceanography, 31-51. **8.** Ellman G. L., Courtney K. D., Andres V., Featherstone R. M. (1961), A new and rapid colorimetric determination of acetylcholinesterase activity, *Biochemical Pharmacology*, 7(2), 88-95. **9.** Tung B. T., Hai N. T., Thu D. K. (2017), Antioxidant and acetylcholinesterase inhibitory activities *in vitro* of different fraction of *Huperzia squarrosa* (Forst.) Trevis extract and attenuation of scopolamine-induced cognitive impairment in mice, *Journal of Ethnopharmacology*, 198, 24-32. **10.** Di Pietro M. E., Mannu A., Mele A. (2020), NMR Determination of Free Fatty Acids in Vegetable Oils, *Processes*, 8(4), 410. **11.** Castro Silva E. S., Bello M., Hernández Rodríguez M., Correa Basurto J., Murillo Álvarez J. I., Rosales Hernández M. C., Muñoz Ochoa M. (2019), *In vitro* and *in silico* evaluation of fucosterol from *Sargassum horridum* as potential human acetylcholinesterase inhibitor, *Journal of Biomolecular Structure & Dynamics*, 37(12), 3259-3268. **12.** Choi B. W., Ryu G., Park S. H., Kim E. S., Shin J., Roh S. S., Shin H. C., Lee B. H. (2007), Anticholinesterase activity of plastoquinones from *Sargassum sagamianum*: Lead compounds for Alzheimer's disease therapy, *Phytotherapy Research: PTR*, 21(5), 423-426. **13.** Natarajan S., Shanmugiahthevar K. P., Kasi P. D. (2009), Cholinesterase inhibitors from Sargassum and Gracilaria gracilis: Seaweeds inhabiting South Indian coastal areas (Hare Island, Gulf of Mannar), *Natural Product Research*, 23(4), 355-369.

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QUALITY EVALUATION OF *CENTELLA ASIATICA* FROM VARIED GEOGRAPHICAL AREAS IN VIETNAM BY RP-HPLC/DAD AND CHEMOMETRICS

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Summary

Quality Evaluation of *Centella asiatica* from Varied Geographical Areas in Vietnam by RP-HPLC/DAD and Chemometrics

Quality of *Centella asiatica* samples collected from varied geographical areas in Vietnam were evaluated based on non-target peaks and asiaticoside content by a reversed phase HPLC/DAD method. The results indicated that there are differences in the chemical compositions between the samples collected in different regions of Vietnam. The content of asiaticoside, a major bioactive compound of *Centella asiatica*, ranged from 0.07% to 1.19%. The PCA-DA method on the non-target peaks showed a fairly good classification for the samples collected in Red River Delta, Northeast, South and North Central regions.

Keywords: *Centella asiatica, Asiaticoside, A reversed phase HPLC/DAD, Quality evaluation, Chemometrics*

1. Introduction

Centella asiatica (L.) (CA) is an important herb in traditional Vietnamese medicine. This plant is cooling, soporific, cardiotonic, nervine tonic, stomachic, improves appetite, antileprotic, antiseptic, tonic to nerves and memory. It is also used in diseases of skin, nerves and blood [1]. Various compounds, including triterpenoids, saponins, flavonoids, glycosides, alkaloids have been isolated from CA to date. The triterpenoid saponins including asiaticoside, madecassoside, and their aglycones, asiatic acid and madecassic acid are the most abundant pentacyclic triterpenoids in CA [1],[2],[3]. These chemical compounds exhibit numerous biological activities, including gastric ulcer healing, wound healing,