α-Glucosidase, Angiotensin-converting Enzyme, and Acetylcholinesterase Inhibitory Activities of a Marine Red Alga *Galaxaura oblongata*

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Abstract

Seaweeds have gained interest from the pharmaceutical industry due to their diverse secondary metabolites with potential applications in the prevention and treatment of lifestyle diseases. In spite of the abundance of seaweeds in the coastal area of Iligan Bay in the Philippines, there has been limited investigation into their pharmacological properties. Therefore, this study aimed to evaluate the in vitro α-glucosidase inhibition, angiotensin-converting enzyme (ACE) inhibition, and acetylcholinesterase (AChE) inhibition properties of various fractions obtained from the marine red alga Galaxaura oblongata. The methanol extract of G. oblongata was sequentially partitioned into hexane, dichloromethane, ethyl acetate, butanol, and methanol fractions. All assays were performed in vitro and microplate based. The results of α-glucosidase inhibition activity assay showed that the n-hexane fraction and dichloromethane fraction exhibited greater than 50% inhibitory activity at 200 ppm. Furthermore, ACE inhibition activity assay revealed that dichloromethane fraction, ethyl acetate fraction, and butanol fraction displayed ACE inhibition activity above 50% at 250 ppm. In addition, the hexane fraction, dichloromethane fraction, and ethyl acetate fraction demonstrated potent AChE inhibitory activity at 250 ppm. Overall, the findings show that G. oblongata has antidiabetic, anti-hypertensive, and neuroprotective potentials. This is the first report of the in vitro α-Glucosidase, ACE, and AChE inhibition activities of G. oblongata. Further investigation and purification of the highly potent fractions in each assay is highly suggested to identify and characterize the compounds responsible for the observed bioactivities, which could serve as possible leads for drug discovery efforts in the management of various lifestyle diseases.

Keywords: *α-glucosidase inhibitor; ACE inhibitor; AChE inhibitor; Galaxaura oblongata*

Introduction

Lifestyle diseases pose a significant global public health concern, with projections indicating that by 2030, they will account for 70% of total global deaths and 56% of the global burden of disease (Mathers and Loncar, 2006). To address this issue, various studies across different fields have been conducted over the years to identify pharmacologically active agents that can prevent and treat lifestyle diseases. In recent times, there has been an increasing interest in marine natural products for drug discovery, with seaweeds being explored for their potential as bioactive leads due to their chemical defense mechanisms and diverse array of secondary metabolites (Antony and Chakraborty, 2019).

As of 2019, published research has identified approximately 100 species of seaweeds with pharmacological activities against lifestyle diseases, accounting for about 30% of marine-derived medicines obtained from marine resources (Rengasamy *et al.*, 2020). However, there is limited information on pharmacologically active compounds associated with seaweeds from the Philippines, in spite of the country having a total of 1,065 seaweed taxa, including 600 red seaweed taxa, 272 green seaweed taxa, and 193 brown seaweed taxa (Lastimoso and Santiañez, 2021). For instance, although Iligan Bay in the Philippines is known for its rich diversity and abundance of seaweeds (Elecho *et al*., 2020) most of the species found in this area have not been thoroughly investigated for their pharmacological and biological activities. Furthermore, there is a lack of previous studies on the effects of fractions from *Galaxaura oblongata*, a marine red alga found in Iligan Bay, as anti-diabetic, anti-hypertensive, and neuroprotective agent, as this species is relatively rare compared to other red algae. Hence, this study is part of ongoing efforts to explore the potentials of *G. oblongata* from Iligan Bay in terms of its *in vitro* pharmacological properties.

Materials and Methods

Collection of G. oblongata and solvent partitioning

G. oblongata was collected from the intertidal rocky shores of Iligan Bay in the Philippines. The collected sample was placed in an ice box and

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brought to the Chemistry Department of Mindanao State University–Iligan Institute of Technology (MSU-IIT). Morphological identification was made by a marine biologist collaborator. In the laboratory, the sample was washed, pat-dried, and weighed. Voucher specimen of *G. oblongata* was kept in the laboratory for future reference. The seaweed sample was then steeped in methanol at room temperature for at least two weeks. After filtration, some amount of filtrate was concentrated *in vacuo* at 40°C.

Fractionation of crude methanol extract of G. oblongata

The concentrated methanol extract was then sequentially partitioned with solvents of increasing polarity: n-hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc), and n-butanol (BuOH), to obtain five respective fractions, namely Go-Hex, Go-DCM, Go-EtOAc, Go-BuOH, and Go-MeOH.

α-Glucosidase inhibitory activity assay

Antidiabetic activities of *G. oblongata* fractions were assessed using the protocol of Li (2005) with some modifications. In a 96-well plate, reaction mixture containing 60 µL phosphate buffer (100 mM, pH=6.8), 20 µL α-glucosidase (0.5 $U.mL^{-1}$), and 20 µL of fractions were preincubated at 37°C for 15 min. Acarbose was used as the positive control. As control, wells without test substance were set up in parallel. Then, 20 µL of 5mM *p*-nitro-phenyl-α-D-glucopyranoside (*p*-NPG) was added as a substrate. The plates were further incubated at 37°C for 15 min. The reaction was stopped by adding 80 µL of 200 mM Na_2CO_3 solution. All determinations were done in two trials with three replicates each trial. The absorbance of the released *p*-nitrophenol was measured at 405 nm using a microplate reader (CLARIOstar® Plus). α-Glucosidase inhibition was calculated as follows (Li, 2005):

$$\alpha - Glucosidase\ Inhibition, \% = \frac{Absorbance\ of Control - Absorbance\ of\ Sample}{Absorbance\ of\ Control} \times 100$$

Angiotensin-converting enzyme (ACE) inhibitory activity assay

ACE inhibitory activity was evaluated using ACE activity assay kit (Sigma-Aldrich CS0002). Briefly, all reagents were diluted in the assay buffer, according to the manufacturer's instructions. A total of 10 µL of sample and positive control (Lisinopril) and negative control (assay buffer) were pipetted into the wells of a 96-well black opaque plate. Then, 40 µL of ACE, provided in the kit, was added to sample and control wells. Sample blanks consisted of 50 µL of assay buffer. A standard curve was prepared in 100 µL assay buffer per well ranging from 0 to 8 nmol for calculating enzymatic activity. Subsequently, 50 µL of fluorogenic substrate warmed to 37°C was added to experimental, control and blank sample wells. The reaction was carried out at 37°C and fluorescence was read every minute for 5 min in a microplate reader (CLARIOstar® Plus) at 320 nm (excitation) and 405 nm (emission). The absorbance of excitation and emission were set at 320 nm and 405 nm, respectively. All determinations were done in two trials with two replicates each trial. The results were expressed as %ACE Inhibition from the equation (Alvarenga *et al.*, 2016):

$$ACE\ Inhibition\ \% = \frac{Slope\ of\ control - Slope\ of\ Sample}{Slop\ of\ Control} x100$$

Acetylcholinesterase (AChE) inhibitory activity assay

AChE inhibitory was analyzed using an AChE activity assay kit (Abcam ab138871). Briefly, all reagents were diluted in the assay buffer, according to the manufacturer's instructions. The AChE reaction mixture (50 µL) was added to a 96-well plate, mixed with 50 µL of AChE standard solution or sample, and reacted at room temperature for 30 min. The absorbance was measured at 410 nm using a microplate reader (CLARIOstar® Plus). The results were expressed as %AChE Inhibition from the equation (Mamun *et al.*, 2021):

$$AChE\ Inhibition\ \% = \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} x\ 100$$

Statistical analysis

Results are reported as mean values and significant differences between the negative control and the sample fractions were determined by T-test at a 95% confidence level.

Result and Discussion

α-Glucosidase inhibitory activity evaluation

Diabetes is a chronic metabolic disorder affecting millions of people worldwide. One approach to controlling blood glucose levels is the use of α-glucosidase inhibitors, which inhibit the breakdown of carbohydrates and delay their absorption in the small intestine, resulting in lower postprandial blood glucose levels (Gericke *et al.*, 2017). Several studies are being done to identify constituents from natural sources possessing anti-diabetic properties, with

emphasis on the inhibition of α-glucosidase and α-amylase. In this study, rapid microplate-based colorimetric assay was used to screen for the presence of α-glucosidase inhibitors in various fractions of seaweed samples.

The results in Figure 1 demonstrate notable α-glucosidase inhibition activity of hexane and dichloromethane fractions throughout the concentration range in a dose-dependent manner; their inhibition activity decreases to less than 50% at 100 ppm. Methanol fraction also exhibited inhibition activity in a dose-dependent manner, but the values are less than 10%. Ethyl acetate fraction only showed activity at 400 ppm while butanol fraction showed no measurable activity at all concentrations.

Seaweeds are widely recognized as a significant source of natural bioactive secondary metabolites, specifically polyphenolic compounds characterized by unique linkages, known as phlorotannins (Van Alstyne *et al.*, 2001; Torres *et al.*, 2008). Previous research by the groups of Heo (2009) and Lee (2010) on seaweeds has elucidated the presence of diverse derivatives of phlorotannins, which have been found to inhibit α-glucosidase and exhibit a protective effect against oxidative stress induced by high glucose in human umbilical vein endothelial cells. These findings suggest that the α-glucosidase inhibitory activities of seaweeds can be attributed to the presence of phlorotannins. α-Glucosidase inhibitory activity of other red seaweed species had been reported elsewhere (Seung-Hong and You-Jin, 2013; Chan *et al.*, 2015).

Angiotensin-converting enzyme (ACE) inhibitory activity evaluation

Hypertension is a leading cause of cardiovascular disease and is a major public health concern worldwide. ACE (angiotensin converting enzyme) inhibitors are commonly prescribed drugs for treating hypertension by blocking the conversion of angiotensin I to angiotensin II, which is a potent vasoconstrictor. However, these drugs can cause side effects and are not always well tolerated. As such, there is growing interest in identifying natural alternatives to ACE inhibitors that may be safer and more effective.

Figure 2 shows that Lisinopril was more efficient in inhibiting ACE, as shown by its higher %ACE inhibition value. Among the fractions, Go-DCM exhibited the highest ACE inhibition with a value of 77.24%. Meanwhile, Go-EtOAc, and Go-BuOH demonstrated ACE inhibition activity greater than 50% ACE inhibition while Go-Hex and Go-MeOH exhibited around 40% ACE inhibition.

As previously hypothesized, it is plausible that phlorotannins are present in fractions of seaweeds. It has been proposed that phlorotannins exhibit inhibitory activity against ACE (angiotensin-converting enzyme) by sequestering the enzyme's metal cofactor, zinc ion (Zn^{2+}), and forming complexes with glycoproteins and proteins, owing to their ability to bind to metal ions and precipitate proteins (Li *et al.*, 2011; Nagappan *et al.*, 2017). Tannins have been reported to form robust complexes with proteins,

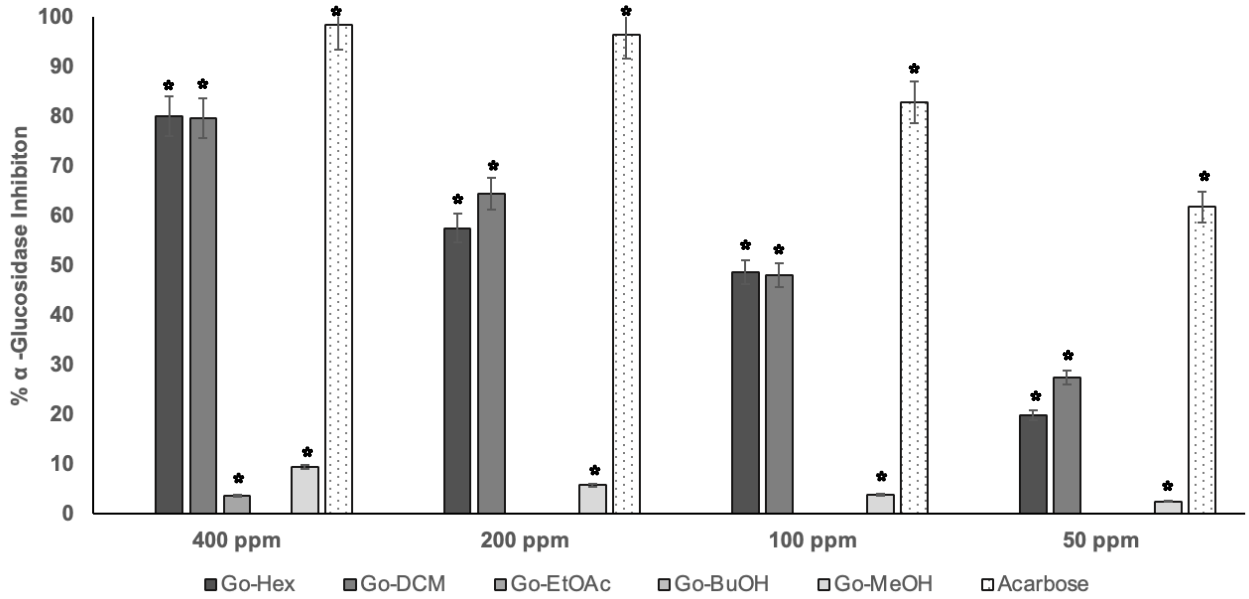

Figure 1. α-Glucosidase inhibition activities at various concentrations of *G. oblongata* fractions and Acarbose. Results are presented as mean with SD as error bar. Test samples with asterisk (*) show significantly higher percent inhibition activity than the negative control based on T-test ($P \leq 0.05$).

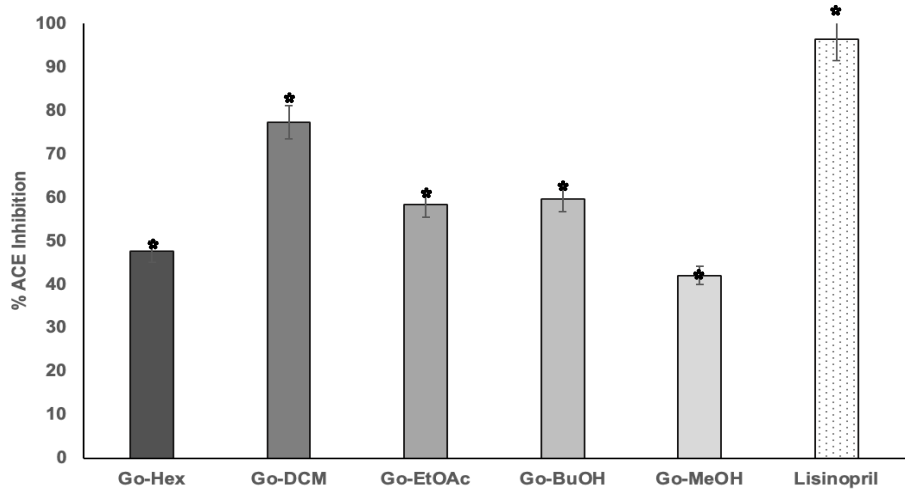

Figure 2. ACE inhibition activities at 250 ppm of *G. oblongata* fractions and Lisinopril. Results are presented as mean with SD as error bar. Test samples with asterisk (*) shows significantly higher percent inhibition activity than the negative control based on T-test ($P \leq 0.05$).

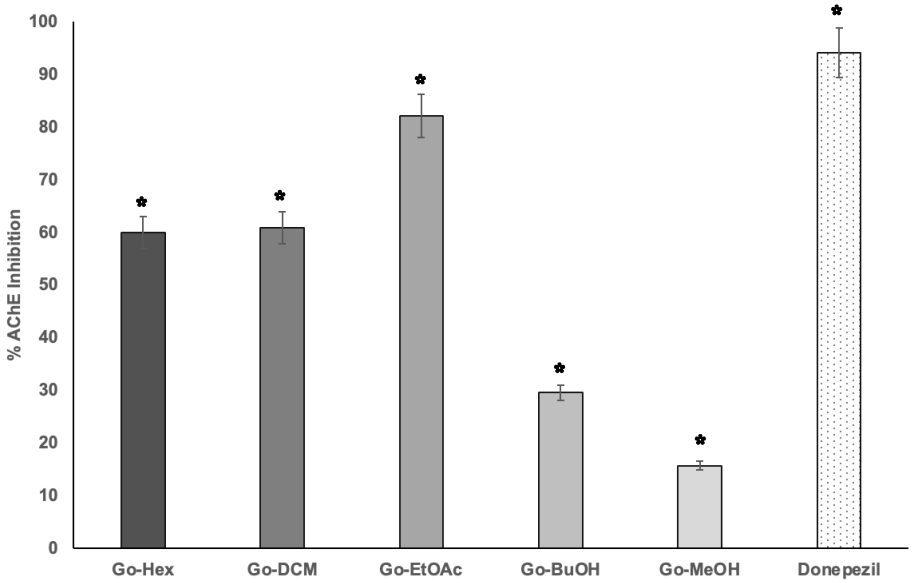

Figure 3. AChE inhibition activities at 250 ppm of *G. oblongata* fractions and Donepezil. Results are presented as mean with SD as error bar. Test samples with asterisk (*) shows significantly higher percent inhibition activity than the negative control based on T-test ($P \leq 0.05$).

either through reversible hydrogen bonding via peptide or amide linkages, or through irreversible covalent condensations (Wijesinghe *et al.*, 2011). Given that ACE, like all enzymes, is a type of protein, it is plausible that the inhibition of ACE could be intricately associated with the phlorotannins' capacity to interact with proteins. The ACE inhibitory activity of other red seaweed species had been reported by the groups of Beaulieu (2016) and Makkar (2018).

Acetylcholinesterase (AChE) inhibitory activity evaluation

Alzheimer's disease is a degenerative and progressive neurological disorder that affects key brain regions, particularly the hippocampus and neocortex, which are responsible for cognitive functions and neurotransmitter acetylcholine (Ach) (McNamara *et al.*, 2014). It can manifest as memory impairment, behavioral abnormalities, and cognitive disturbances. According to the cholinergic theory, the significant reduction of AChE, the enzyme responsible for breaking down ACh, in the central nervous system is associated with the symptoms of the disease (McNamara *et al.*, 2014). The primary therapeutic approach is inhibition of acetylcholinesterase activity to increase the availability of ACh in the brain. Based on a criterion by the group of Vinutha (2007), the inhibitory activity of plant extracts against AChE can be categorized into three classes based on their

potency, which includes potent inhibitors (inhibition>50%), moderate inhibitors (inhibition ranging from 30% to 50%), and weak inhibitors (inhibition<30%).

As shown in Figure 3, Go-Hex (59.86%), Go-DCM (60.89%), and Go-EtOAc (82.07%) are potent AChE inhibitors while Go-BuOH (29.55%) and Go-MeOH (15.73%) are weak AChE inhibitors. Seaweed extracts that contain mixtures of monoterpenes typically exhibit significant inhibition of AChE (Machado *et al.*, 2015). These monoterpenes act as reversible competitive inhibitors of AChE (Keane and Ryan, 1999) and the observed AChE inhibitory effect in seaweeds is hypothesized to be the result of the combined actions of these multifunctional compounds, which can exert their activities through various mechanisms (Koroch *et al.*, 2007). The AChE inhibitory activity of other red seaweed species had been reported by the group of Ismail (2020).

Conclusion

The n-hexane and dichloromethane fractions of *G. oblongata* exhibited greater than 50% *in vitro* α-glucosidase inhibitory activity at 200 ppm. Moreover, the dichloromethane, ethyl acetate, and butanol fractions demonstrated greater than 50% in vitro ACE inhibition at 250 ppm, while the n-hexane, dichloromethane, and ethyl acetate fractions showed potent in vitro AChE inhibitory activity at 250 ppm. These findings suggest that the bioactive extract and fractions of *G. oblongata* may contain promising molecules with potential therapeutic applications against lifestyle diseases. Further purification and analysis of the chemical components of this marine red alga could lead to the discovery of new anti-diabetic, anti-hypertensive, and neuroprotective agents.

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