

Malaysian Applied Biology
Biologi Terapan Malaysia



Published by the Malaysian Society of Applied Biology

ISSN 0025-7718

Malaysian Applied Biology
Biologi Terapan Malaysia



Published by the Malaysian Society of Applied Biology

ISSN 0025-7718

Volume 1, No. 1, 2001

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Vol 49(3) October 2020



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ISSN 0126-8643

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Subject Area and Category [Agricultural and Biological Sciences](#)
[Agricultural and Biological Sciences \(miscellaneous\)](#)

Publisher [Malaysian Society of Applied Biology](#)

Publication type [Journals](#)

ISSN [01268643](#)

Coverage [1980, 1986-1987, 2012-2019](#)

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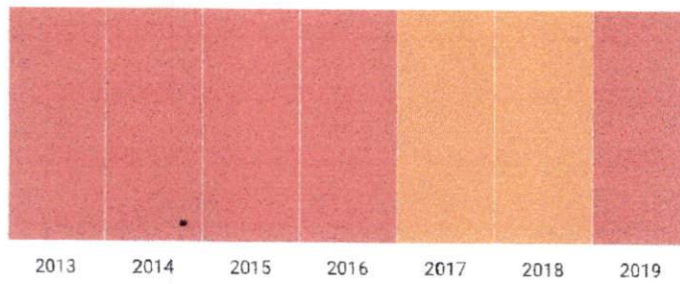
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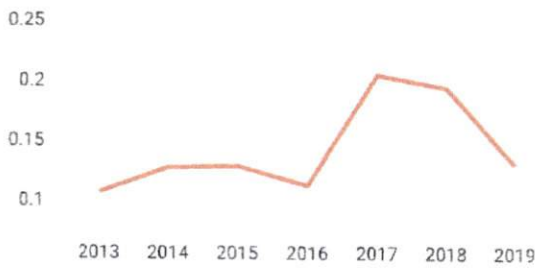
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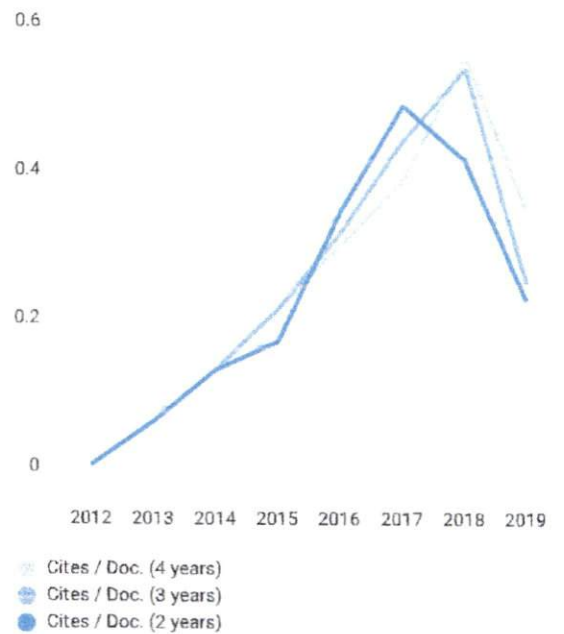
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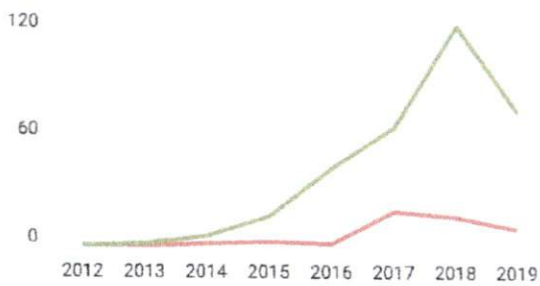
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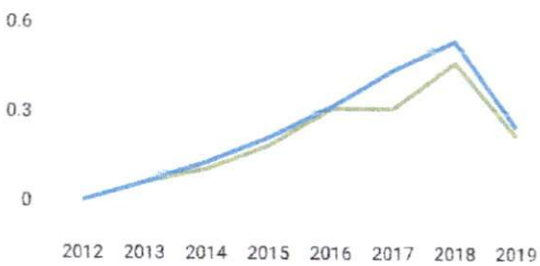
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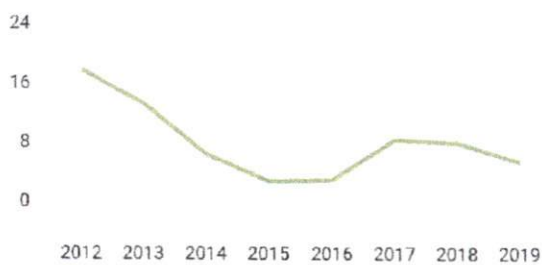
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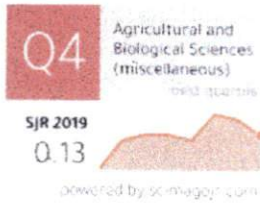
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ENZYME INHIBITORY ACTIVITIES OF MARINE SPONGES AGAINST CHOLINESTERASE AND 5 α -REDUCTASE

SUCIATI^{1,2*}, KARMA RABGAY³, YUNDA FACHRUNNIZA³, TONGCHAI SAESONG³,
 TRI ARYONO HADI⁴, TUTIK SRI WAHYUNI^{1,2}, ATY WIDYAWARUYANTI^{1,2}
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Accepted 10 April 2019, Published online 30 June 2019

ABSTRACT

Marine sponges have been the source of various metabolites with potent biological activities. In this study fifteen methanolic extracts of marine sponges, collected off the coast of Tabuhan Island, Banyuwangi, East Java, Indonesia were evaluated in relation to their cholinesterase and 5 α -reductase inhibitory activities. The results revealed that the extract of *Petrosia* sp. inhibited the 5 α -reductase enzyme at 100 μ g/mL, with 61.21% inhibition, which is slightly lower than the positive control, finasteride, of 76.70%. The results of the cholinesterase inhibitory screening showed that three marine sponges namely, *Callyspongia* sp., *Niphates olemda*, and *Agelas nakamurai* presented notable cholinesterase inhibitory activities. The highest potency was found in *A. nakamurai*, with an IC₅₀ value of 1.05 μ g/mL. All three samples inhibited both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), however, the extract of *N. olemda* showed a higher inhibition against AChE compared to BuChE. The chemistry of the *Callyspongia* sp., *N. olemda* and *A. nakamurai* were investigated using thin layer chromatography and ¹H NMR methods. The results suggested the presence of terpenes and alkaloids in the samples. Further study is needed to determine the metabolite responsible for cholinesterase inhibitory activity.

Key words: Marine sponge, acetylcholinesterase inhibitor, 5 α -reductase inhibitor

INTRODUCTION

Oceans, which cover almost two-thirds of the Earth's surface, have been the habitat of various living creatures, including algae, sponges, cnidarians, mollusks, bryozoans, ascidians and echinoderms as well as microorganisms (Costello *et al.*, 2010). This species diversity makes the marine environment one of the most prolific sources of natural products. More than 20,000 natural compounds have been discovered from various marine organisms (El Gamal, 2010). Many of these marine creatures produce unique and biologically active compounds,

which are not found in the terrestrial ecosystem (Thakur *et al.*, 2005; Harvey, 2008; Molinski *et al.*, 2009).

Enzyme inhibitors have played important roles in the development of natural compounds for pharmacological and agricultural applications. Terrestrial and marine organisms have been reported as the source of enzyme inhibitors (Ruocco *et al.*, 2017). Examples of target enzymes for biological screening include cholinesterase and 5 α -reductase. Cholinesterase is present in the human central nervous system and is involved in the hydrolysis of acetylcholine (ACh) to choline and ethanoic acid. ACh is a neurotransmitter, produced in the nerve ending of the presynaptic nerve, which is asso-

* To whom correspondence should be addressed.

ciated with memory and cognitive functions (Houghton *et al.*, 2006; Dev & Maurya, 2017). ACh deficiency is a common feature in a patient with Alzheimer's disease (AD), therefore, the inhibition of cholinesterase is one of the strategies for the treatment of AD. Example of approved AChE drugs, including tacrine, rivastigmine, physostigmine and galantamine, however, there is the limitation of these drugs, for example, short half-life, side effect such as hepatotoxicity as well as the loss of drug efficacy (Nukoolkarn *et al.*, 2008; Farrokhnia & Nabipour, 2014). Therefore, the search for more potent and safe cholinesterase inhibitor has been desirable.

Galantamine and rivastigmine are examples of naturally derived acetylcholinesterase inhibitors, which have inspired more research on more potent AChE inhibitors from natural sources, including marine organisms. Several marine sponge compounds have also been reported to inhibit AChE enzymes, such as a steroidal alkaloid from *Corticium* sp. (Langje *et al.*, 2007), pyridoacridine alkaloid petrosamine, isolated from *Petrosia* sp., which was predicted to bind to the TcACHE enzyme (Nukoolkarn *et al.*, 2008), pyridinium alkaloids from *Reniera sarai* and from a genus *Plakortis* (Alonso *et al.*, 2005; Turk *et al.*, 2007; Kubota *et al.*, 2010), a bis-indole alkaloid from *Fascaplysinopsis* sp. (Bharate *et al.*, 2012), and a bromopyrrole alkaloid isolated from *Agelas oroides* (Erdogan-Orhan *et al.*, 2012).

A 5 α -reductase is an enzyme responsible for the conversion of testosterone, the main androgen in humans, to dihydrotestosterone (DHT), a more potent androgen. The excessive production of DHT may cause androgen dependent disorders. The characteristics of these diseases include androgenic alopecia (male pattern baldness), acne, benign prostate hyperplasia (BPH) and female hirsutism (Tsukamoto *et al.*, 1998; Suphrom *et al.*, 2012; Srivilai *et al.*, 2016). An antiandrogen is a compound that can block or suppress the action of the male sex hormone, such as testosterone or DHT. One of the antiandrogen mechanisms is the inhibition of the 5 α -reductase enzyme so that the conversion of testosterone to DHT is blocked. This 5 α -reductase inhibitor acts selectively inside its target cells, therefore, it does not lower the serum concentration of testosterone and causes almost no adverse reactions, such as the reduction of libido (Grant & Ramasamy, 2012). The use of 5 α -reductase inhibitors, such as finasteride which has been approved by the United States FDA, for treatment of BPH, however, may cause side effect i.e gynecomastia, impairment of muscle growth, and severe myopathy (Aggarwal *et al.*, 2010). Therefore, the search for a more potent and selective antiandrogen is needed.

Several plant metabolites have been reported as 5 α -reductase inhibitors (Seo *et al.*, 2002; Suphrom *et al.*, 2012; Srivilai *et al.*, 2016; Grant *et al.*, 2012), however, there are limited reports of marine derived compounds that target this enzyme. The objective of the current study is to investigate the inhibitory activity of fifteen marine sponges, derived from Tabuhan Island, Banyuwangi, East Java, Indonesia, against cholinesterase and 5 α -reductase enzymes.

MATERIALS AND METHODS

Reagents

Acetylcholinesterase from electric eel (AChE type VI-S, *EeAChE*), human recombinant acetylcholinesterase (hrAChE), horse-serum butyrylcholinesterase (BuChE), acetylthiocholine iodide (ATCI), 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB), and bovine serum albumin (BSA), trisma base-HCl buffer (Tris buffer), galantamine and finasteride, reagents were obtained from Sigma-Aldrich. Crude enzyme 5 α -reductase was prepared from androgen-dependent LNCaP cells (CRL-1740TM from American Type Culture Collection, VA, USA). The cell was cultured in a medium RPMI-1640, supplemented with 10% fetal bovine serum, 100 U/mL penicillin G and 100 μ g/mL streptomycin (Gibco, Paisley, Scotland).

Sponge collection and extraction

Marine sponges were collected from the Tabuhan Island dive site, Banyuwangi, East Java, Indonesia using SCUBA at a depth of 10-20 m on 23 April 2017. Fresh sponges were taken to the laboratory and stored at -20°C until extraction. Voucher specimens were preserved in alcohol and deposited at the Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia. Identification of the sponges was conducted by Dr. Tri Aryono Hadi of Research Center of Oceanography, Indonesian Institute of Sciences, Jakarta, Indonesia. Fresh sponges were diced and lyophilized. The dry sponge materials were then grounded and extracted exhaustively with methanol to produce the crude methanolic extracts.

Cholinesterase inhibitory assay

The assay was carried out according to the modified Ellman's method (Ellman *et al.*, 1961; Ingkaninan *et al.*, 2003; Sirimangkalakitti *et al.*, 2015). Sponge extracts were dissolved in 50 mM Tris buffer containing not more than 10% MeOH to obtain a 1 mg/mL concentration, and these were further diluted in the microplate well to a final test concentration of 100 μ g/mL. Sample solutions were

added to a 96-well microplate, followed by the addition of 1.5 mM ATCI (25 μ L), 3 mM DTNB (125 μ L), and Tris buffer (50 μ L). The substrate was then hydrolyzed by the addition of 25 μ L of 0.28 U/mL of either *EeAChE*, *hrAChE* or BuChE. The solutions were shaken for 30 s in a microplate reader (Bio-Tek Instrument, USA) before measurement. The product, 5-thio-2-nitrobenzoate, indicated by a yellow color was measured at 405 nm every 17 s for 2.29 min. Every experiment was carried out in triplicate. Galantamine was used as a positive control, and 10% methanol was used as a negative control. For the measurement of IC_{50} , serial concentrations of the samples were prepared. The enzyme activity was calculated as a percentage of the velocity of the test sample, compared with that of the non-treated control. The inhibitory activity was calculated as:

$$\% \text{ Inhibition} = [(\text{Mean velocity of control} - \text{Mean velocity of sample}) / \text{Mean velocity of control}] \times 100$$

5 α -Reductase assay

The 5 α -reductase inhibitory activity was carried out by the method of Srivilai *et al.* (2016). The enzymatic assay was performed in a 96-well plate. The final test solution (200 μ L) contained 10 μ L of test samples dissolved in DMSO, 34.74 μ M testosterone, 1 mM NADPH, homogenized crude enzyme (75 μ g protein) and Tris buffer pH 7.4. The mixture was incubated at 37°C for 60 min. Then, 300 μ L hydroxylamine (10 mg/mL in 80% ethanol) was added and further incubated at 60°C for 60 min. The plate was then centrifuged at 1700 g for 10 min. The supernatant was collected and injected into LC-MS for the determination of DHT production.

The DHT production was determined by LC-ESI-QTOF-MS (Agilent 1260 infinity series connected with Agilent 6540 UHD accurate-mass Q-TOF LC/MS). The Phenomenex Luna[®] C-18 (150 x 4.6 mm, 5 μ m) column was used as a stationary phase. The mobile phase was 0.1% v/v formic acid in purified water (solvent A) and 0.1% v/v formic acid in acetonitrile (solvent B), gradient elution starting from 40% solvent A and 60% solvent B, then solvent B increase to 80% (8 mins), followed by isocratic 80% solvent B for 4 mins, with a flow rate of 0.5 mL/min. The column temperature was set to 35°C, and the sample injection volume was 20 μ L. The extracted ion chromatogram of the derivatized-DHT (m/z [M+H]⁺ 306.2428) was observed. The peak area was used to calculate the inhibitory activity as shown in the equation below:

$$\text{Enzymatic inhibition (\%)} = [1 - (\text{sample-control}_{0\text{min}}) / (\text{control}_{60\text{min}} - \text{control}_{0\text{min}})] \times 100$$

Phytochemical Screening

Thin layer chromatography was performed on silica gel 60 F₂₅₄ plates as a stationary phase, and ethyl acetate: methanol (1:1) as mobile phase. Visualization was carried out using anisaldehyde-H₂SO₄ (terpene) or Dragendorff (alkaloid). The ¹H NMR spectra were recorded in a JEOL 400 MHz NMR instrument using CDCl₃ as a solvent.

Data analysis

The 50% inhibitory concentration (IC_{50}) was determined using GraphPad Prism 6.0 software by plotting log concentrations as axis and % inhibition as ordinate. The inhibition data of the samples against AChE and BuChE were analyzed using independent-sample T tests in IBM SPSS statistics 21 software.

RESULTS AND DISCUSSION

Cholinesterase inhibitory activity

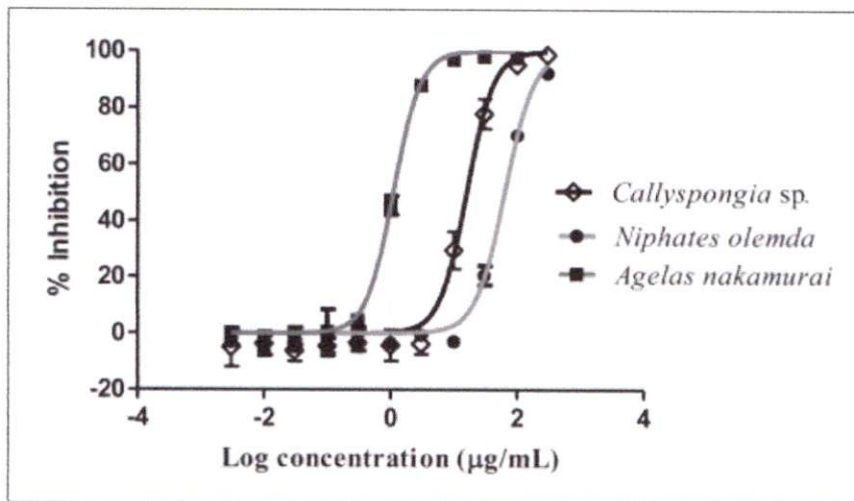
Fifteen marine sponges were collected from the Tabuhan Island dive site, Banyuwangi, Indonesia. The methanolic extracts of the sponges were subjected to both cholinesterase and 5 α -reductase inhibitory assays. The results presented in Table 1 showed that at 100 μ g/mL three sponges, namely, *Callyspongia* sp., *Niphates olemda* and *Agelas nakamurai*, strongly inhibited the AChE enzyme, with a percentage inhibition of 97.16, 91.89 and 85.67%, respectively. The methanolic extracts of *Axynissa* sp. and *Stylissa carteri* showed 30-50% inhibitory activity, while the rest of the extracts presented an inhibition of below 10%. The dose-dependent inhibitory activity of the three active extracts (*Callyspongia* sp., *N. olemda*, and *A. nakamurai*) were further investigated using the human recombinant AChE (*hrAChE*) enzyme. The results are presented in Figure 1, and the IC_{50} values are tabulated in Table 3. It was found that among the three samples tested, the methanolic extract of *A. nakamurai* had the highest potency with an IC_{50} value of 1.05 μ g/mL, while the other two extracts, *Callyspongia* sp. and *N. olemda*, presented IC_{50} values of 14.69 and 56.78 μ g/mL, respectively. The IC_{50} value of the *A. nakamurai* extract is slightly higher than that of the positive control galantamine (0.63 μ g/mL).

The three samples were then screened against butyrylcholinesterase (BuChE) in order to investigate the selectivity. The results shown in Table 2 suggested that the methanolic extracts of *Callyspongia* sp., *N. olemda* and *A. nakamurai* inhibited both the AChE and BuChE enzyme. However, the extract of *N. olemda* showed a lower

Table 1. The inhibitory activities of the methanolic extracts of marine sponges against acetylcholinesterase (AChE) and 5 α -reductase (5 α R)

Samples	% AChE Inhibition	% 5 α R Inhibitor
<i>Callyspongia</i> sp.	97.16 \pm 0.93	57.72 \pm 5.27
<i>Clathria (Thalysias) reinwardtii</i>	< 10	45.20 \pm 2.71
<i>Niphates olemda</i>	91.89 \pm 0.17	57.65 \pm 1.50
<i>Haliclona (Reniera) fascigera</i>	< 10	22.25 \pm 9.12
<i>Clathria (Clathria) basilana</i>	< 10	31.52 \pm 4.45
<i>Acanthella cavernosa</i>	< 10	< 10
<i>Agelas nakamurai</i>	85.67 \pm 0.76	< 10
<i>Niphates</i> sp.	< 10	28.04 \pm 4.00
<i>Petrosia (Petrosia) hoeksemai</i>	< 10	39.18 \pm 4.87
<i>Petrosia (Petrosia) sp.</i>	< 10	61.21 \pm 3.81
<i>Axynissa</i> sp.	31.04 \pm 0.47	27.54 \pm 8.65
<i>Coelocarteria agglomerans</i>	< 10	52.23 \pm 8.81
<i>Ulosa ada</i>	< 10	52.46 \pm 7.86
<i>Stylissa carteri</i>	49.00 \pm 2.34	29.01 \pm 5.63
<i>Cinachryrella</i> sp.	< 10	< 10
Galantamine	98.44 \pm 0.59	
Finasteride		76.70 \pm 8.71

Data presented as mean \pm SD of three independent experiments, each done in triplicate. Samples were tested at 100 μ g/mL. Galantamine and finasteride were tested at 100 μ M (36.83 μ g/mL) and 8 μ M (2.98 μ g/mL), respectively.

**Fig. 1.** Dose-dependent response of selected marine sponges to the inhibition of acetylcholinesterase. Data presented as mean \pm standard deviation of three independent experiments.**Table 2.** Comparison of inhibitory activities of selected marine sponge extracts against acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE)

Samples	% AChE Inhibition	% BuChE Inhibitor
<i>Callyspongia</i> sp.	97.16 \pm 0.93	93.65 \pm 0.13
<i>Niphates olemda</i>	91.89 \pm 0.17	73.30 \pm 0.55
<i>Agelas nakamurai</i>	85.67 \pm 0.76	94.92 \pm 0.93
Galantamine	98.44 \pm 0.59	90.94 \pm 0.15

Data presented as mean \pm standard deviation of three independent experiments, each done in triplicate. Samples were tested at 100 μ g/mL. Galantamine was tested at 100 μ M (36.83 μ g/mL).

Table 3. IC₅₀ values for the selected marine sponge extracts against acetylcholinesterase

Samples	IC ₅₀ (µg/mL)
<i>Callyspongia</i> sp.	14.69 ± 2.95
<i>Niphates olemda</i>	56.78 ± 2.53
<i>Agelas nakamurai</i>	1.05 ± 0.05
Galantamine	0.63 ± 0.05

Data presented as mean ± standard deviation of three independent experiments, each done in triplicate.

inhibition against BuChE, compared to AChE enzyme. The inhibition data of the three samples against AChE and BuChE were then analyzed using IBM SPSS statistics 21 software. A comparison of each sample's activities against AChE and BuChE were conducted using independent-sample T-tests. The results indicated that there is a significant difference observed between the inhibition of the *N. olemda* extract against AChE and BuChE (p -value < 0.001), while there are no significant differences observed between the extracts of *Callyspongia* sp. and those of *A. nakamurai* against AChE and BuChE (p -value >0.001).

5 α -Reductase inhibitory activity

The methanolic extracts from the fifteen marine sponges were also subjected to a 5 α -reductase inhibitory assay. Samples were tested at a concentration of 100 µg/mL. Finasteride was used as a positive control at a concentration of 8 µM (2.98 µg/mL). The results (Table 1) showed that the extract of *Petrosia* sp. had the highest inhibition at 61.21% compared to other tested samples, however, it was lower than the activity of finasteride which had inhibition of 76.70%. Four extracts, namely, *Callyspongia* sp., *N. olemda*, *C. agglomerans* and *U. ada* showed approximately 50% inhibition, while the rest of the extract presented below 50% inhibition.

Phytochemical screening

The chemical composition of the *Callyspongia* sp. *N. olemda* and *A. nakamurai* were studied since these three samples had a high inhibition against the AChE enzyme. The screening was conducted using thin layer chromatography (TLC) and ¹H NMR methods. The results indicated that the methanolic extract of *N. olemda* contains terpene, as indicated by the anisaldehyde sulphuric acid dye on the TLC plates. This was further supported by ¹H NMR, which showed signals at δ_H 1.0-5.0 ppm which corresponded to the presence of methyl, methylene, and methine groups. The TLC and ¹H NMR spectra of the methanolic extracts of *Callyspongia* sp. and *A. nakamurai* suggested the presence of alkaloid

and terpene. These findings are in agreement with the data reported in the literature for metabolites reported from *Callyspongia* sp., *N. olemda* and *A. nakamurai*. Terpene compounds, such as niphateolide A, which has been isolated from *N. olemda* (Kato *et al.*, 2015). *Callyspongia* sp. has been reported to contain terpene such as polyhydroxy isocoplane (Kurnianda *et al.*, 2017) and alkaloid such as callylactam A and callyimine as well as diketopiperazine compounds (Yang *et al.*, 2013; Yang *et al.*, 2016). *Agelas nakamurai* have been reported to contain several terpenoids, such as nakamurools A-D (Shoji *et al.*, 1996), as well as diterpene alkaloid agelasines (Hertiani *et al.*, 2010; Zhang *et al.*, 2017; Chu *et al.*, 2017). Hertiani *et al.* (2010) have investigated the chemistry of Indonesian *Agelas nakamurai*, collected from Menjangan Island, north of the Bali Sea, and found that a diterpene alkaloid, (-) agelasine D, was a major compound in this marine sponge (Hertiani *et al.*, 2010). The ¹H NMR spectrum of our sample of *A. nakamurai* showed signals at δ_H 10.51, 8.45, 6.95 and 4.05 ppm which suggested the presence of methyladenine moiety, as well as several signals between δ_H 1.5-5.5 ppm corresponded to diterpene moiety. These data are similar to those reported for agelasine D. Further analyses should be undertaken to confirm the presence of this compound in our sample.

Several sponges from the genus *Agelas* have shown anticholinesterase activity. The extract of *Agelas oroides*, as well as the isolated bromopyrrolle alkaloid, oroidin, have been reported to have a moderate AChE inhibitory activity. *Agelas clathrodes* and *Agelas marmarica* have shown a moderate inhibition against AChE (Sepčić *et al.*, 2010; Beedessee *et al.*, 2013). The chemical constituents of *Petrosia* sp. was not investigated in this study due to the limited availability of sample. Several studies reported that this sponge contains polyacetylenes and sterols known to have anticancer activity (Kim *et al.*, 2002; Choi *et al.*, 2004; Park *et al.*, 2007; Pailec *et al.*, 2017). To the best of our knowledge, there is no report on the AChE inhibitory activity of *Callyspongia* sp., *Niphates olemda* and *Agelas nakamurai* and 5 α -reductase inhibitory activity of *Petrosia* sp. Further study should be carried out to investigate the metabolites responsible for this enzyme inhibitory activity of the active samples.

CONCLUSION

Fifteen marine sponges were screened against cholinesterase and 5 α -reductase. The results suggested that three sponge extracts, namely, *Callyspongia* sp., *Niphates olemda* and *Agelas*

nakamurai showed a high inhibition against the AChE enzyme, and *A. nakamurai* had the highest inhibition with an IC₅₀ value of 1.05 µg/mL. The methanolic extract of *Petrosia* sp. exhibited 61.21% inhibition against 5α-reductase. Further study is needed to study the chemistry behind the active samples.

ACKNOWLEDGEMENT

This research was funded by the Ministry of Research, Technology, and Higher Education of the Republic of Indonesia under grant number 01/E/KPT/2018.

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