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PHYTOCHEMICAL PROFILE, FREE RADICAL SCAVENGING ACTIVITY, AND α-GLUCOSIDASE INHIBITORY ACTIVITY OF FRESHWATER SPONGES *Oncosclera asiatica* AND *Eunapius carteri* FROM EAST JAVA, INDONESIA

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ABSTRACT

Sponges from freshwater environments are poorly known producers of phytochemicals with pharmacological potency. *Oncosclera asiatica* and *Eunapius carteri* are freshwater sponges from the Porong River, East Java province. They can be considered promising new sources of bioactive compounds. This study aims to investigate the pharmacological potency of *O. asiatica* and *E. carteri* extracts. Qualitative phytochemical and GC-MS analyses were performed on the *O. asiatica* and *E. carteri* extracts. Antioxidant capacity was determined using DPPH and ABTS free radical scavenging activities, whereas in vitro anti-diabetic activity was evaluated using α-glucosidase inhibition. The phytochemicals were identified by GC-MS in *O. asiatica* and *E. carteri* extracts, such as fatty acid esters including hexadecanoic acid, methyl ester; 9-hexadecenoic acid, methyl ester, (Z); 9,12-Octadecadienoyl chloride (Z, Z); 9-Octadecenamide (Z); and decanedioic acid, dibutyl ester, and others. In addition, the *n*-hexane and ethyl acetate fractions from *E. carteri* exhibited good α-glucosidase inhibitory activity. This study revealed that *O. asiatica* and *E. carteri* extracts are potential sources of pharmaceutically useful bioactive compounds of natural origin.

Keywords: Antioxidant, α-glucosidase, Diabetes, Freshwater Sponges, Phytochemicals

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INTRODUCTION

Bioprospection has developed into a vibrant scientific discipline that investigates fresh applications for the use of natural goods. Most of the work in chemical research over the past 60 years or more has been concentrated on metabolites from the sea.[1] Evolutionary pressure from a wide range of species and environments that are very different from those in terrestrial ecosystems has led to the creation of novel

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compounds that may be employed for a variety of pharmaceutical applications, including the treatment of cancers and Alzheimer's disease.[2] The river region is home to a variety of invertebrate animals, including sponges, which contain natural chemicals that are crucial for the discovery and development of new drugs.[3] Freshwater sponges (Porifera) are a rare kind of sponge and their surroundings set them apart from their marine counterparts. Sessile suspension feeders and freshwater sponges can be found adhering to submerged surfaces in the majority of inland water habitats. Although roughly 150 species of sponge's dwell in freshwater areas such as lakes and rivers, the great majority of the estimated 15,000 sponges exist in marine ecosystems.[4] In contrast to marine natural products, there are comparatively few chemical compounds from any freshwater animal or plant species that have been isolated and described. Alkaloids, steroids, terpenoids, polyketides, polypeptides, and other phytochemicals have a variety of biological effects, including cytotoxicity, antibiotic activity, antifungal activity, antiviral activity, antitumor activity, and enzyme activity inhibitory effects.[5] Sponges that live in freshwater environments are some of the sources of secondary metabolites. Studies have shown that sponges may manufacture toxins as a defense against predators and more than 10% of those substances showed cytotoxic actions. These animals' chemical composition is made up of a wide range of fatty acids, sterols, and uncommon specialized metabolites that are exclusive to freshwater sponges.[6-8] These characteristics are highly valuable in pharmacology and biotechnology, which raises the value of these organisms as a source of novel bioproducts. The bioactivity of these animals against a number of therapeutic targets, including acetylcholinesterase and malaria, has lately drawn attention to their unique metabolism.[9] Therefore, this study was conducted to investigate the presence of phytochemicals in less studied species of Oncosclera asiatica (Manconi & Ruengsawang, 2012) and Eunapius carteri (Bowerbank, 1863), which were discovered in Porong River[10], East Java province, Indonesia, and to assess their pharmacological potential in free radical scavenging activity and α-glucosidase inhibitory activity. This study is also the first attempt to do so.

EXPERIMENTAL

Material and Methods

Two freshwater sponges, *Oncosclera asiatica* (OA) and *Eunapius carteri* (EC), were freshly collected from Porong River at 7°28'17.4 "S, 112°31'07.3 "E on July 25, 2020 in the city of Mojokerto using a sharp knife for removing them from other encrusting. Identification of the sponges was based on their morphological characteristics, DNA barcoding, and phylogenetic analysis by mitochondrial DNA (mtDNA) and Internal transcribed spacer (ITS), which was carried out at the Zoology and Animal Engineering Laboratory of the Faculty of Science and Data Analytics, Institut Teknologi Sepuluh Nopember.[10] Pro analysis of *n*-hexane, dichloromethane, ethyl acetate, and methanol were purchased from Merck (Darmstadt, Germany). The Baker's yeast α-glucosidase, p-NPG (4-Nitrophenyl-α-D-glucopyranoside), butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA) were obtained from Sigma Aldrich. Meanwhile, DPPH (2,2 diphenyl-2-picrylhydrazyl) and ABTS ((2,2-Azinobis 3-ethyl benzothiazoline 6-sulfonic acid) were obtained from TCI (Tokyo Chemical Industry).

Extraction Process

In ascending order of their polarity, *n*-hexane, dichloromethane, ethyl acetate, and MeOH were used to extract the sponge materials. Using a vacuum rotary evaporator (BUCHI R-100) at 40°C to get the crude extracts, the extracts were filtered and concentrated before being stored at 4°C until use. The extracts were tested for antioxidant activity, α-glucosidase inhibitory activity, GC/MS analyses, and phytochemical screenings.

Phytochemical Screenings

Qualitative phytochemical analysis was conducted on the four extracts (*n*-hexane, dichloromethane, ethyl acetate, and MeOH) of sponge materials of *O. asiatica* and *E. carteri*. It was carried out in previous studies to determine phytochemical components, such as alkaloids, flavonoids, saponin, phenolic, and triterpenes.[11,12]

GS/MS Qualitative Analysis

The identification of bioactive compounds from n-hexane, dichloromethane, and ethyl acetate extract of two freshwater sponges (*O. asiatica* and *E. carteri*) was characterized by Agilent 7890 GC series equipped

with an HP-5 silica capillary column (5% diphenyl-and 95% dimethyl-polysiloxane, 30 m x 0.32 mm ID, 250 µm film thickness) coupled to mass spectrometry (MS) 5977B MS selective detector with slight modification.[13] The oven temperature was maintained at 40ºC, then it was increased by 2oC/min to 230ºC. The identification was carried out using the NIST 2017 MS Library.

DPPH Free Radical Scavenging Activity

The free radical scavenging activity of four extracts (*n*-hexane, dichloromethane, ethyl acetate, and MeOH) from sponge materials of *O. asiatica* and *E. carteri* was measured by DPPH free radical with some modifications.[14] Briefly, the reaction mixture containing 100 µL of DPPH solution and 20 µL of samples with various concentrations was shaken vigorously and incubated for 30 minutes at room temperature. Afterward, absorbance was read at 517 nm. The lower absorbance of the reaction mixture is indicated by higher free radical scavenging activity. BHT and BHA were used as the antioxidant standard.

ABTS Free Radical Scavenging Activity

The ABTS free radical scavenging activity was examined using the previous method with some modifications.[15] Briefly, 20 µL of various concentrations of the samples were added to 100 µL of ABTS solution. The reaction mixture was incubated at room temperature for 30 minutes, then the absorbance was measured at 750 nm using a ThermoScientific Multiscan Sky High microplate reader. BHT and BHA were used as the antioxidant standards.

α-Glucosidase Inhibitory Activity

The α-glucosidase inhibitory activity was slightly modified according to standard procedures following the hydrolysis of nitrophenyl glycosides.[16] Briefly, the reaction mixture contained 40 µL of enzyme solution (Baker's yeast α-glucosidase), 50 µL of *p*-NPG in buffer phosphate pH 6.9, and various concentrations of test samples. The reaction mixture was incubated at 37ºC for 20 minutes and the reaction was terminated by adding Na_2CO_3 1M. After incubation, the absorbance was measured spectrophotometrically at 405 nm. Acarbose was used as the positive standard.

RESULTS AND DISCUSSION

Table-1 shows the findings of the phytochemical screening test performed on the four different freshwater sponge extracts. In short, it was observed that the process of partitioning from non-polar (*n*-hexane) to polar solvent (methanol) demonstrated reasonably effective separation. All extracts of freshwater sponges (*O. asiatica* and *E. carteri)* resulted in positive for the alkaloid test, except for the polar fraction (MeOH). Furthermore, the *n*-hexane and dichloromethane extract of *O. asiatica* showed the presence of triterpenoids, while the ethyl acetate extract of *O. asiatica* showed the presence of saponins. Meanwhile, the *n*-hexane and dichloromethane extract of *E. carteri* showed the presence of alkaloids and triterpenoids. Unfortunately, other phytochemicals such as flavonoids and phenolic were absent in all freshwater sponge extracts. However, this finding is in line with previous studies that reported that sterols and long chains (fatty acids) compounds were present in the genus of *Metaniam* and *Drulia* (Metaniidae), and *Ochridaspongia rotunda* Arndt (Malawispongiidae) freshwater sponges.[4,8,9]

Table-1: Phytochemical Screening of Freshwaters Sponges (*O. asiatica* and *E. carteri*)

No	Samples	Phytochemicals				
		Alkaloids	Flavonoids	Saponins	Phenolic	Triterpenoids
1	*Oncosclera asiatica*					
	n-Hexane fraction	+	-	-	-	+
	Dichloromethane fraction	+	-	-	-	+
	Ethyl acetate fraction	+	-	+	-	-
	MeOH fraction	-	-	-	-	-
2	*Eunapius carteri*					
	n-Hexane fraction	+	-	-	-	+
	Dichloromethane fraction	+	-	-	-	+
	Ethyl acetate fraction	+	-	-	-	-
	MeOH fraction	-	-	-	-	-

(+) presence; (-) absence

To the best of the authors' knowledge, no records of phytochemicals of *O. asiatica* and *E. carteri* freshwater sponges inhabiting the Porong River area were found in any previous study.

Table-2 displays the results of a qualitative GC-MS analysis of the phytochemicals found in the *n*-hexane, dichloromethane, and ethyl acetate extracts of the two freshwater sponges. 43 chemical components of *O. asiatica* and *E. carteri* were found in the *n*-hexane, dichloromethane, and ethyl acetate extracts together with their retention times. By comparing the peak retention time and mass spectrum fragmentations of both freshwater sponge extracts to their analog published in the NIST library, the GC-MS chromatogram of *n*-hexane, dichloromethane, and ethyl acetate extracts was identified (data not shown). Figure-1 shows that four fractions of freshwater sponges *O. asiatica* and *E. carteri* contain a variety of secondary metabolites. In this observation, the presence of fatty acid esters such as hexadecanoic acid, methyl ester; 9-hexadecenoic acid, methyl ester, (Z); 9,12-Octadecadienoyl chloride (Z, Z); 9-Octadecenamide (Z); and decanedioic acid, dibutyl ester demonstrated various biological activities, including analgesic, ulcerogenic, anti-inflammatory, anti-bacterial, and hypolipidemic effect.[17,18] Hexadecanoic acid, also known as methyl ester of α-linoleic acid, was found in a prior study that showed its biological activity as an anticancer, antibacterial, and anti-inflammatory agent.[16]

Table-2: Chemical Components of *O. asiatica* and *E. carteri*

Samples*	Chemical Compounds	RT**
Oncosclera asiatica		
n-hexane	1-Hexadecanol	17.55
	Tributyl acetylcitrate	21.24
Ethyl acetate	2-butyltetrahydrofuran	14.53
	1-Hexadecanol	17.55
	Hexadecanoic acid, methyl ester	18.00
	1-Propene-1,2,3-tricarboxylic acid, tributyl ester	20.29
	Tributyl acetylcitrate	21.29
	9-Octadecenamide, (Z)-	22.14
Dichloromethane	benzeneacetonitrile, alpha-[(4-cyanophenyl) methylene]-4-nitro	115.84
	Morphinan-6-ol, 4,5-epoxy-N-methyl -, (5alpha., 6beta)	117.25
	6-Bromo-1-methyl-2-oxoquinoline-4- carboxylic acid	117.85
Eunapius carteri		
n-hexane	1,3,5,7-Cyclooctatetraene	4.32
	2-butyltetrahydrofuran	14.51
	Hexadecamethyl cyclooctasiloxane	15.23
	Methyl tetradecanoate	15.87
	1-Tetradecanol	16.61
	Octadecamethyl cyclononasiloxane	16.99
	9-Hexadecenoic acid, methyl ester, (Z)	17.80
	Hexadecanoic acid, methyl ester	18.00
	Didecyl phthalate	18.43
	Cycloheptasiloxane, tetradecamethyl-	18.57
	9,12-Octadecadienoyl chloride, (Z,Z)	19.75
	Methyl tetradecanoate	19.94
	13-Docosenamide, (Z)-	20.54
	Tributyl acetylcitrate	21.23
	9-Octadecenamide, (Z)-	22.15
	Propanoic acid, 3-ethoxy-, ethyl ester	22.15
Ethyl acetate	1-Dodecanol	12.96
	2-butyltetrahydrofuran	14.62
	Cyclooctasiloxane, hexadecamethyl-	15.24
	1-Hexadecanol	17.57
	Hexadecanoic acid, methyl ester	18.00
	1-Propene-1,2,3-tricarboxylic acid, tributyl ester	20.32

	Tributyl acetylcitrate	21.29
	9-Octadecenamide, (Z)	22.20
	1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	25.20
Dichloromethane	Decanedioic acid, dibutyl ester	82.60
	Butyl citrate	86.78
	4,5-Difluoro-2-methyl-phenyl) -(4-methyl-piperidin-1-yl)-methanone	103.90
	1-Naphthalenecarboxamide, N-(3-chlrophenyl)	104.70
	9-Octadecenamide, (Z)-	105.73
	6-Bromo-1-methyl-2-oxoquinoline-4-carboxylic acid	114.58
	Benzamide, N-(3-methylphenyl)-2,3, 4-trifluoro	117.50

* GC/MS analysis was not performed on the methanol extract
** RT (retention time) in minutes

Tributyl acetyl citrate was also shown to offer the potential to be a flavoring component in pharmaceutical medication production.[19] Oleamide, also known as 9-octadecenamide (Z), is a fatty acid amide. It may bind to cannabinoid receptors and cause a wide range of biological effects, including the induction of sleep, immunosuppression, and the activation of the serotonin and GABA receptors.[20] These findings collectively showed that the two freshwater sponges, *O. asiatica* and *E. carteri*, had a variety of secondary metabolites with potential biological activities. The chemical makeup of *O. asiatica* and *E. carteri* has not yet been the subject of any investigations that have been described in the literature.

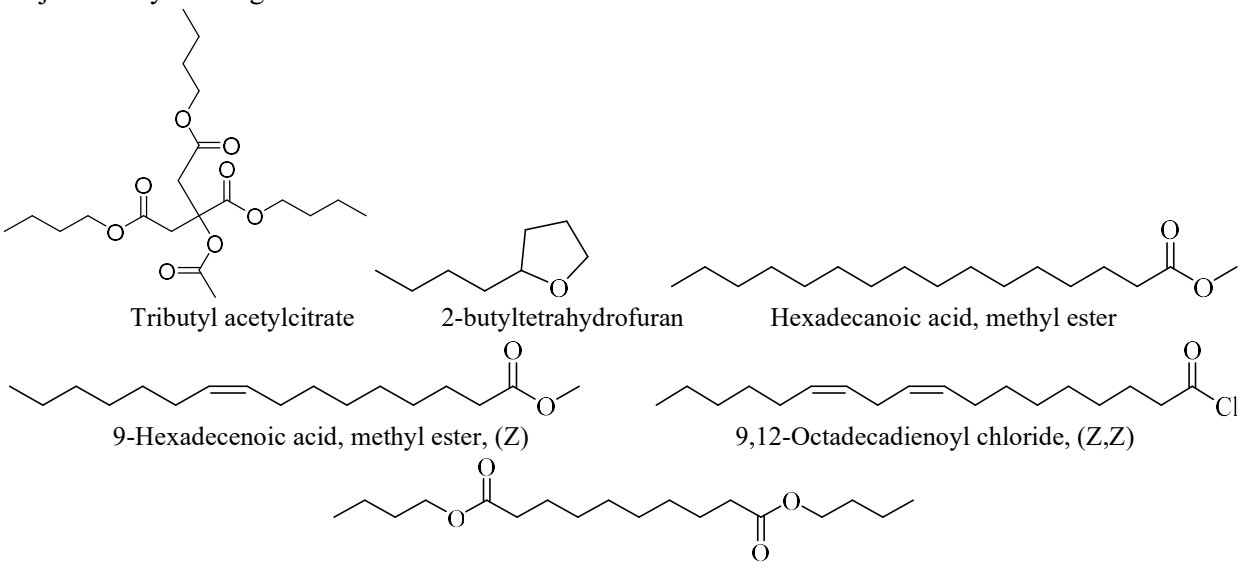

Fig.-1: GC-MS Detection of Possible Bioactive Compounds (selected) from two Freshwater Sponges (*O. asiatica* and *E. carteri*)

The investigated biological activities of two freshwater sponges (*O. asiatica* and *E. carteri*) were further determined using α-glucosidase inhibitory activity and free radical scavenging activity. In this study, two different approaches were successfully employed to examine the antioxidant capacity of the two freshwater sponges, namely DPPH and ABTS free radical scavenging activities presented in Fig.-2 and Fig.-3. These results were compared with butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) as positive standards.

The antioxidant activity of four fractions of the two freshwater sponges was determined on the basis of DPPH free radical scavenging activity (Fig.-2). Among the samples, the *n*-hexane fractions of both *O. asiatica* and *E. carteri* displayed the best percentage of inhibition values of 39.90% and 47.78% respectively. Meanwhile, the other fractions exhibited the lowest percentage of inhibition at 10 mg/mL. Different solvent extracts of the two freshwater sponges showed the potency scavenging activity in descending order of *n*-hexane > dichloromethane > ethyl acetate > methanol.

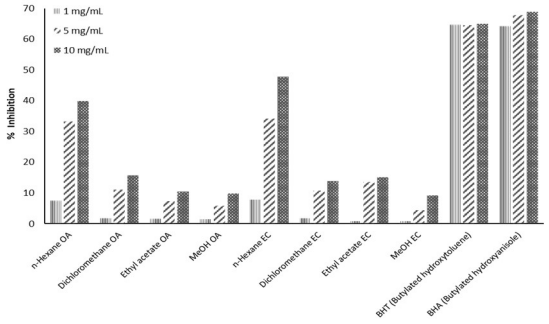

Fig.-2: DPPH Free Radical Scavenging Activity of *O. asiatica* and *E. carteri*

These results are quite similar to a previous study that reported that freshwater sponges from Lake Baikal had very low antioxidant activity.[21] Furthermore, the free radical scavenging activity of the two freshwater sponges was also assessed using ABTS free radicals. The inhibition percentage (%) values of the two freshwater sponge extracts against ABTS free radical are shown in Fig.-3. Unfortunately, all examined extracts showed the lowest inhibition against ABTS free radicals.

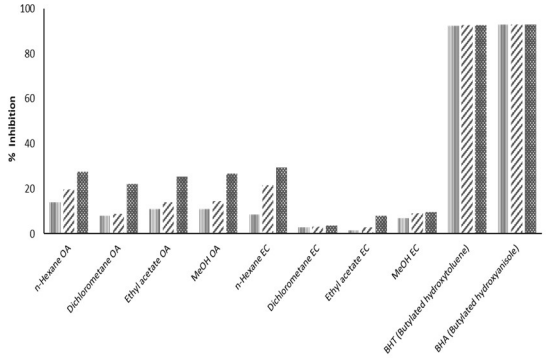

Fig.-3: ABTS Free Radical Scavenging Activity of *O. asiatica* and *E. carteri*

To continue our investigation, the antidiabetic activity of *O. asiatica* and *E. carteri* fractions was also evaluated against α-glucosidase. As presented in Table-3, among four *O. asiatica* fractions at concentrations of 1, 5, and 10 mg/mL, n-hexane fraction demonstrated a concentration-dependent inhibitory activity against α-glucosidase with percent inhibition of 10.46%, 11.64%, and 23.43% respectively. Unfortunately, other fractions of *O. asiatica* showed no inhibitory activity against α-glucosidase.

Table-3: α-Glucosidase Inhibitory Activity of *O. asiatica* and *E. carteri*

No	Samples	Percent inhibition (%)[a]		
		1 mg/mL	5 mg/mL	10 mg/mL
1	*Oncosclera asiatica*			
	n-Hexane fraction	10.46 ± 3.13	11.64 ± 0.55	23.43 ± 0.40
	Dichloromethane fraction	NI[b]	NI	NI
	Ethyl acetate fraction	NI	NI	NI
	MeOH fraction	NI	NI	NI
2	*Eunapius carteri*			
	n-Hexane fraction	38.96 ± 1.78	48.59 ± 1.63	55.86 ± 1.28
	Dichloromethane fraction	NI	NI	NI
	Ethyl acetate fraction	60.07 ± 1.47	70.24 ± 1.20	79.96 ± 1.15
	MeOH fraction	NI	NI	NI
3	Quercetin 0.1 mg/mL	60.03 ± 1.79		
	Quercetin 1 mg/mL	87.56 ± 3.11		

[a]Data is shown as the mean of triplicate experiments ± SD.
[b]No inhibition, inhibitory activities less than 10% at 10 mg/Ml

Furthermore, the inhibitory effects of α-glucosidase were measured also in four *E. carteri* fractions. The *n*-hexane and ethyl acetate fractions demonstrated good inhibitory activity against α-glucosidase among the

four fractions assayed. Surprisingly, as shown in Table-3, the ethyl acetate fraction of *E. carteri* exhibited good inhibitory activity with a percent inhibition of 79.96% at a concentration of 10 mg/mL, whereas the *n*-hexane fraction exhibited with percent inhibition of 55.86%. Quercetin, a positive standard α-glucosidase inhibitor, also demonstrated a concentration-dependent inhibitory effect at 0.1 mg/mL and 1 mg/mL. These results indicated that freshwater sponges *O. asiatica* and *E. carteri* can become a potential source with an inhibitory effect against α-glucosidase. As far as the authors know, there has been no report of α-glucosidase inhibitory activity in freshwater sponges *O. asiatica* and *E. carteri*. Taken all together, further research will be directed toward a detailed chemical components analysis of these two freshwater sponges including isolation and identification of phytochemicals that are responsible for the biological activities observed.

CONCLUSION

Unknown freshwater sponges from the Porong River in East Java province, such as *Oncosclera asiatica* and *Eunapius carteri*, may contain a variety of pharmacological phytoconstituents. It has demonstrated numerous pharmacological activities in the current investigation, including antioxidant and α-glucosidase inhibitory action. This study's analysis of many phytochemicals, including fatty acids and sterols, suggested that freshwater sponges from the Porong River may be a new supply of those substances for the pharmaceutical sector. Consequently, more research is required to examine specific components of extracts of freshwater sponges from the Porong River that could be responsible for other biological processes.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID IDs, given below:

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