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Sponge Biology Characteristics and Steroids Secondary Metabolite of *Haliclona* sp. from Lemukutan Island, West Kalimantan, Indonesia

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Present study reports the sponge biology characteristics and secondary metabolites of steroids of *Haliclona* subgenus *Haliclona* sp. Morphological characteristics of sampled sponge coded as LMK-2 from the Lemukutan Island waters were identified as *Haliclona* (*haliclona*) sp. Furthermore, isolates coded as $M_2R_{14}K_{4a}$ from secondary metabolites isolation of *n*-hexane fraction yield needle-shaped white crystals.

Keywords: Porifera, Halicloniid sponge, Steroid, Spermonde.

INTRODUCTION

Secondary metabolite compounds such as alkaloids, steroids, terpenoids, polyketides, polypeptides and others exhibit various biological activities such as cytotoxic, antibiotic, antifungal, anti-tumor, antiviral, antifouling and enzyme activity inhibitors [1]. One of the secondary metabolite sources are sponges that inhabit coral reef ecosystems. Several studies have demonstrated that sponges can produce toxin compounds as protection from predators [2] and more than 10% indicated cytotoxic activity [3].

Steroid compounds have been developed in the medical world through their activity as antioxidants and their high toxicity. Steroids are found widely in plants and animals. For example, steroid in red algae *Eucheuma cottonii* [4], in sponges such as *Phyllospongia foliascens* [5], *Axinella sinoxea* [6], *Xestospongia testudinaria* [7], *Theonella swinhoei* [8-10], *Crella incrustans* [11] and *Ircinia mutan* [12]. Other potential of steroid compounds is an antifungal, antitumor, anticancer drug [13], cytotoxic [12], antimalarial [14], antibacterial [15], *etc*. Sponges are also known to be a source of steroids [16] and some have been reported to exhibit pharmacological effects. Sterols such as Kaimanol and Saringosterol were isolated from *Xestospongia* sp. The sponge exhibited antiplasmodial activity [14], langcosterol A from *Xestospongia testudinaria* exhibited moderate cytotoxic activities [7], eryloside A as an antifungal and Eryloside K and L as an antitumor from *Erylus lendenfeldi* sponge [13]. Five sterols, namely theonellasterol K, acetyl-theonellasterol, acetyldehydroconicasterol, theonellasterol and theonellasterone have been isolated from *T. swinhoei* sponge from Pingtung Beach, Taiwan. Theonellasterol K showed significant cytotoxic activity against cancer cell lines HCT-116, K562 and lymphoblastic leukemia (Molt 4) [8]. Another steroid that contains sulfate and chlorine groups, namely chalinulasterol, is discovered and isolated from sponge *Chalinula molitba*. *T. swinhoei* is also known to have sulfate sterols content, namely solomonsterol A and B with a cholestant skeleton [17].

A new type of sterol with a stigmastan skeleton, namely (24E)-5α,6α-epoxystigmasta-7,24(28)-dien-3β-ol has also been isolated from the sponge *Phyllospongia foliascens* from the South China sea [5]. Two new sulfonated sterols in the form of dimers, namely manadosterols A and B, were isolated from the *Lissodendryx* fibrous sponge from Indonesia and have the potential to become anticancer agents [18]. Another sterol is β-sitosterol which is similar to cholesterol and is known to act as a nutrient for humans and has potential as an anticancer of the breast and colon [19]. This is consistent with studies reporting on the induction of apoptosis by β-sitosterol and on the inhibition of cellular proliferation and the induction of

apoptosis in U937 leukemia cells by β-sitosterol by down regulating the pro-apoptotic protein Bcl-2 and caspase 3 activation [20]. The presence of β-sitosterol in several sponge species has been widely reported, The presence of sitosterol in several sponge species has been widely reported, including information for the development of potential anticancer drugs. This study also provides important information about the presence of β-sitosterol and the biological characteristics of the *Haliclona (haliclona)* sp. sponge from Lemukutan Island, West Kalimantan, Indonesia.

EXPERIMENTAL

Sponge sample was taken from Lemukutan Island, West Kalimantan, Indonesia using SCUBA diving at a depth of 5 m. The samples coded as LMK-2 were cleaned from other adhering biotas, weighed, put in plastic bags, documented. Furthermore, samples were divided in half for taxonomic identification and extraction processes. The chemicals used were *n*-hexane, ethyl acetate, dichloromethane, chloroform, methanol, Lieberman-Burchard reagent and silica gel 60 230-400 mesh (Merck).

Sponges identification: Identification comprises of histological preparations that are spicule preparation, to determine the diversity of spicules in the skeleton and a thick section through the sponge tissue, to determine the structure of skeleton, the structure of water-canal system and other aspects of histology. Bleach digestion was used for this spicule preparation. Small fragments of 'tissue' were placed in sample bottles, and a small quantity of sodium hypochlorite was later added. After a short period of time, the organic components will dissolve leaving only the mineral skeletons. This was followed by three times rinsed with distilled water followed by 70% ethanol for spicule preservation. Thick tangential and perpendicular hand-cut sections of 1.0-1.5 mm were procured from a preserved fragment to examine their skeletal arrangement. Sections were placed onto a glass slide and left to dry, while stacked with weight. Clean spicule suspensions were then pipetted onto a glass slide and topped with a cover slip, likewise, to dried skeleton sections that were completely dried. Both spicules and skeleton were being mounted with Entellan® 107960 Merck Millipore. After the microslides were completely dried, observations were conducted with compound microscope, Olympus® CX31 and Optilab "Filter Upgrade Edition" with a magnification of 40X and 100X. The taxonomic identification of sponges was carried out based on the sponge identification manual from Hooper and van Soest 2002 along with the World Porifera Database (WPD) website [21].

Extraction and partition: The extraction process is carried out by the maceration method. In brief, by immersing about 300 g of sample in an organic solvent for several days at room temperature until the extract becomes clear to remove the organic compounds contained in sample [22], then extract was filtered and evaporated until dryness (crude methanol extract). The crude methanol extract was suspended in 5% water in methanol. The methanol extract suspension was partitioned several times with *n*-hexane until part of *n*-hexane to be clear. The *n*-hexane fraction was evaporated by a rotary evaporator until dryness. The methanol crude extract was identified, while the steroid presence was identified using Liberman-Burchard reagent. Then, the methanol fraction was partitioned using dichloromethane and ethyl acetate as solvent.

Fractionation and purification: Fractionation was carried out on *n*-hexane fraction from crude methanol extract of LMK-2 using thin layer chromatography (TLC), vacuum liquid chromatography (VLC) and flash column chromatography (FCC). In VLS and FCC, *n*-hexane fraction is put into a column containing silica gel then eluted with several solvents in a gradient manner. TLC was used to determine the eluent composition to be used on VLC and FCC and to monitor the results of VLC and FCC.

Separation was carried out using silica gel G60 F_{254} in VLC and FCC using silica gel G60 (0.04-0.63 mm). Column chromatography results were tested by TLC then checked using UV lamp and exposed vanillin reagents. The fractions showing the same spots were combined and then evaporated to obtain dry isolates. Purification was carried out by recrystallization and checking on two-dimensional TLC using three solvents combinations (*n*-hexane, dichloromethane and ethyl acetate). The obtained needle crystals of isolate was coded as $M_2R_{14}K_{4a}$. The purity test of $M_2R_{14}K_{4a}$ isolate was carried out using two-dimensional TLC. The eluent used was *n*-hexane:ethyl acetate (90:10) and chloroform:ethyl acetate (85:15).

Molecular structure identification: Pure isolates were analyzed using several instruments such as FTIR, ^{1}H NMR, and ^{13}C NMR to identify the types of secondary metabolites. The output data in the form of spectrum are interpreted according to the characteristics and function of each instrument with FTIR, ^{1}H NMR and ^{13}C NMR.

RESULTS AND DISCUSSION

Identification of sponge specimens: Macroscopic examination of LMK-2 specimens displayed that sponges possess a branched shape with a slightly brittle consistency, dark brown-black colour, slightly dry moss colour and has a very clear and large osculum at each end (Fig. 1). Furthermore, when preserved with ethanol, the colour of sponge turns pale light brown.

Fig. 1. Macroscopic picture of LMK-2 specimens

Microscopic examination of sponges displayed an isotropic spicules pattern in the ectosome layer with regular reticulation, whereas a rhombus reticulation was displayed at the choanosome layer supported by regular spongin fibers (Fig. 2b-c). Spicules possess a curved oxeas shape with dimensions of 104.1–**161.06**–173.4 µm × 8.7–**13.42**–21.5 µm and straight oxeas with dimensions of 142.5–**165.3**–174 µm × 8.8–**12.32**–18.8 µm (Fig. 2d).

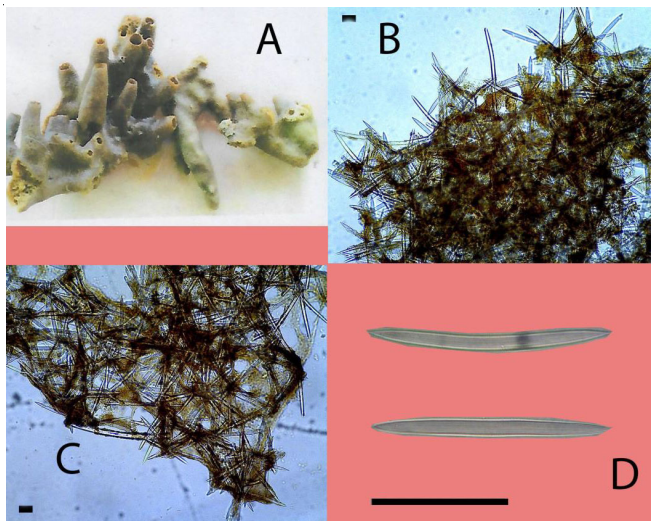

Fig. 2. A, "Lifeform": samples of species A, B, longitudinal cutting of tissue with a magnification of 40x C, cross-cutting of tissue at 40x magnification, D, oxeas spicules with 400x magnification Scale B, C and D 1=100 µm

Extraction and partition: The colour change from dark brown to clear for extraction and partition process indicates that secondary metabolites have dissolved in methanol and *n*-hexane, respectively. The result extraction by maceration was 16.6814 g. The partition process with *n*-hexane solvent was carried out several times until a clear *n*-hexane extract was obtained, and after the evaporation process, the weight of *n*-hexane fraction was 0.2971 g. Then, the LMK-2 methanol extract was tested with Lieberman-Burchard reagent and indicated that the steroid was positive, which changed colour to light green.

Fractionation and purification: The number of fractions obtained from the VLC fractionation was 17 fractions eluted using a gradient solvent, namely 100% *n*-hexane, *n*-hexane: ethyl acetate (95:5, 90:10, 85:15, 80:20, 75:25, 70 :30, 65:35, 60:40, 55:45), 100% ethyl acetate, ethyl acetate:methanol (80:20, 60:40, 40:60, 20:80) and 100% methanol. The results of VLC were monitored at wavelength of 254 and 366 nm and sprayed using vanillin reagent (Fig. 3). The VLC results were grouped into three combined fractions, namely M_1 (fraction 1-2), M_2 (fraction 5-11), and M_3 (fraction 12-17). The mass of the M_2 fraction was 0.2193 g and selected for the first fractionation step using FCC and eluted gradient with 100% *n*-hexane, *n*-hexane:ethyl acetate (95:5, 90:10, 85:15, 80:20, 60:40), 100% ethyl acetate and 100% methanol) and obtained 320 fractions grouped into 20 combined fractions with codes M_2R_1 to M_2R_{20}. All fractions were monitored using an ultraviolet (UV) lamp at a wavelength of 254, 366 nm and sprayed using vanillin reagent (Fig. 4). TLC results for the fraction with the code M_2R_{14}, namely vials 155-188, showed a major spot that still showed a tail with an isolated mass of 63.6 mg. Therefore, it is necessary to do a second fractionation phase using a smaller column of FCC to separate the isolates from impurities. The M_2R_{14} fraction was then eluted gradient using 100% *n*-hexane, *n*-hexane:ethyl acetate (95:5 and 70:30), 100% ethyl acetate and 100% methanol and obtained 90 fractions then grouped into five combined fractions ($M_2R_{14}K_1$ till $M_2R_{14}K_5$) (Fig 5a). Spot on vials 28, 36, and 42 still show tailings, so vials 28, 36 and 42 were combined into $M_2R_{14}K_4$ and then separated using preparative TLC with *n*-hexane:ethyl acetate as eluent (95:5). The results of the preparative TLC showed that there were two separate bands with codes $M_2R_{14}K_{4a}$ and $M_2R_{14}K_{4b}$ (Fig. 5b), then crushed and each isolate dissolved in chloroform then filtered, evaporated slowly till form crystals with a mass of 16.5 mg for $M_2R_{14}K_{4a}$ isolated. The purity test on isolate $M_2R_{14}K_{4a}$ was carried out using two-dimensional TLC (Fig. 6). The eluents used were *n*-hexane:ethyl acetate (90:10) and chloroform:ethyl acetate (85:15).

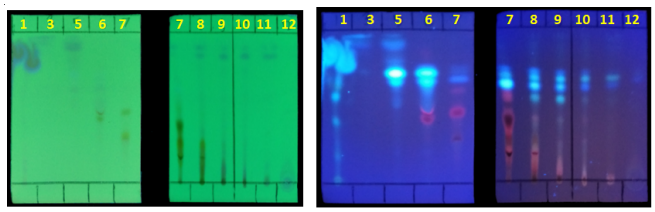

Fig. 3. Monitoring results of VLC using TLC under UV lamp at λ 254 nm and 366 nm

FTIR spectrum analysis: In the FTIR spectrum of LMK-2 methanol extract (Fig. 7), there is absorption at 3743.83 cm^{-1}, 3419.79 cm^{-1} (OH *str.* or NH *str.*), 2956.87 cm^{-1}, 2927.94 cm^{-1}, 2873.94 cm^{-1} (CH_3 *str.* and CH_2 *str.*), 1739.79 cm^{-1} (C=0 *str.*), 1645.28 cm^{-1} (C=C *str.*), 1564.27 cm^{-1}, 1456.26 cm^{-1}, 1413.82 cm^{-1}, 1346.31 cm^{-1} (CH_3 *bend.* and CH_2 *bend.*) , 1257.59 cm^{-1} (C-N *str*), 1112.93 cm^{-1} (C-O *str.*), 653.87 cm^{-1} (CH_2 *rock.*). It appears that the FTIR spectrum of methanol extract shows the complexity of the compound represented by the strain and bending absorption data of various functional groups. The complexity of the compound causes the presence of =C-H absorption not to appear because of the overlapping with -OH absorption, but presence of C=C absorption represents the indication for the presence of the =C-H group. The LMK-2 FTIR spectrum data also correlates with the phytochemical test results of the LMK-2 methanol extract, which showed positive results for alkaloids and steroids.

In contrast to FTIR spectrum of $M_2R_{14}K_{4a}$ isolate (Fig. 8), which do not have carbonyl groups (C=O). The wide absorption at 3442.94 cm^{-1} indicates the presence of O-H strecthing, while the absorption at 3028.34 cm^{-1} indicates the presence of =C-H absorption due to the olefin group. The presence of olefin group was supported by C=C stretching absorption at 1645.28 cm^{-1}. The strong stretching absorption of the C-H bond of CH_2 and CH_3 appear at 2866.22 cm^{-1} and 2935.66 cm^{-1}, respectively. These absorptions are supported by the presence of bending

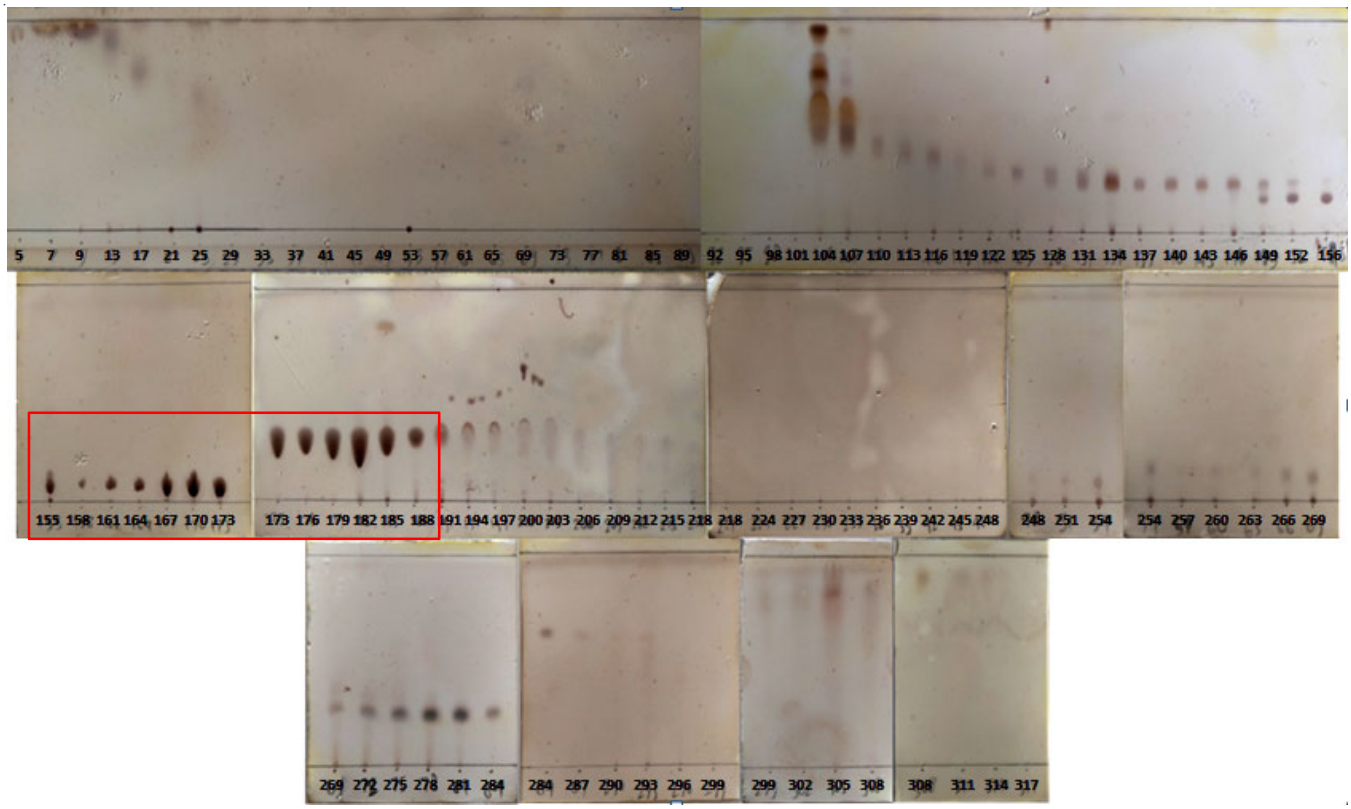

Fig. 4. Monitoring to all fractions of FCC using TLC and vanillin reagen

Fig. 5. TLC profile: (a) Five combined fractions of 90 fractions, (b) Preparative TLC of $M_2R_{14}K_4$ fraction

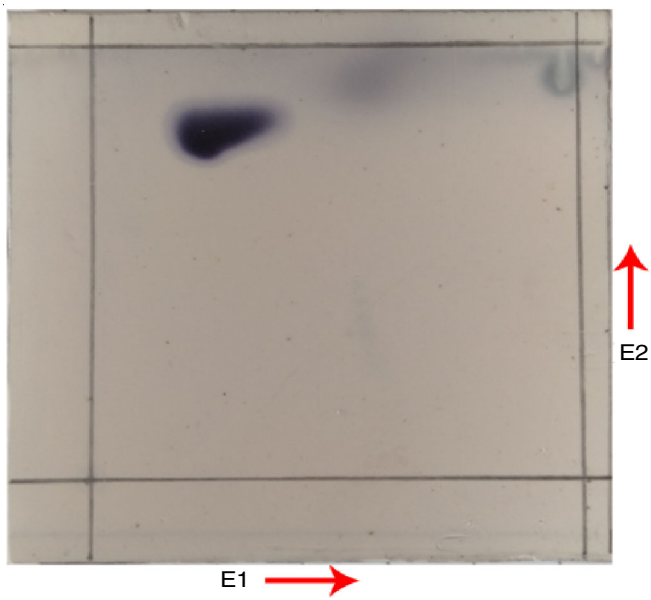

Fig. 6. Profile of $M_2R_{14}K_{4a}$ isolate on two-dimensional TLC

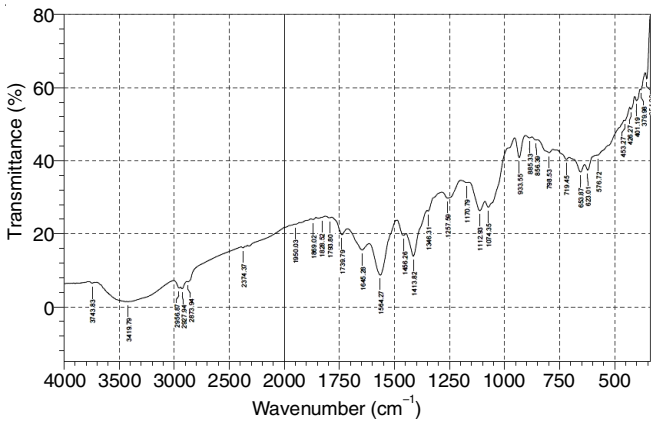

Fig. 7. FTIR spectrum of methanol extract LMK-2

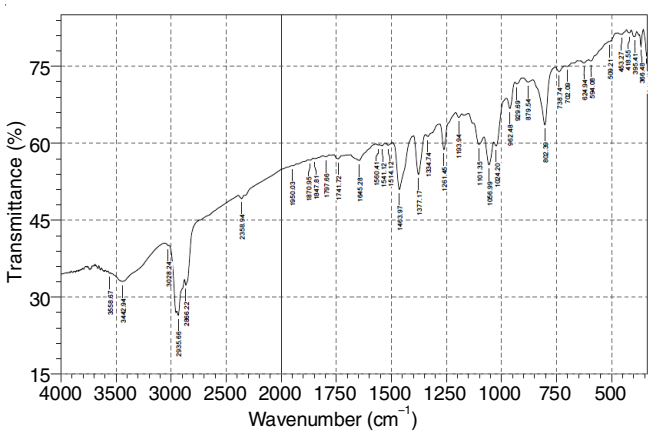

Fig. 8. FTIR spectrum of isolate $M_2R_{14}K_{4a}$

absorption for CH_3 and CH_2 at 1463.97 cm^{-1} and 1377.17 cm^{-1}, respectively.

NMR analysis: Based on the interpretation results of ^1H NMR on $M_2R_{14}K_{4a}$ isolate (Fig. 9), it can be seen that there is signal above 5 ppm, and overlapping signal group in areas below 2 ppm. Multiplet signal overlapping CH_3, CH_2, and CH at δ 0.4-2.6 ppm, signal doublet at δ 5.33 ppm for =CH olefin supported by the ^{13}C NMR signal, namely C=C at δ 139.71 and 120.71 ppm. Signal doublet at δ 5.33 ppm similar with signal at 5.34 (1H) [23] and δ 5.35 (1H) of a double bond between the carbons 5 and 6 of the four sterols with the same skeleton as β-sitosterol [24]. Multiplet proton signal at δ 3.50 ppm for CH from C3 and supported by ^{13}C NMR signal from CH-O at δ 70.79 ppm. Signal multiplet at δ 3.50 ppm is also similar to the signal at δ 3.56-3.51 (m, 1H) [25] and 3.51 (m, 1H) [23]. All these signals indicated that the $M_2R_{14}K_{4a}$ isolate is a sterol.

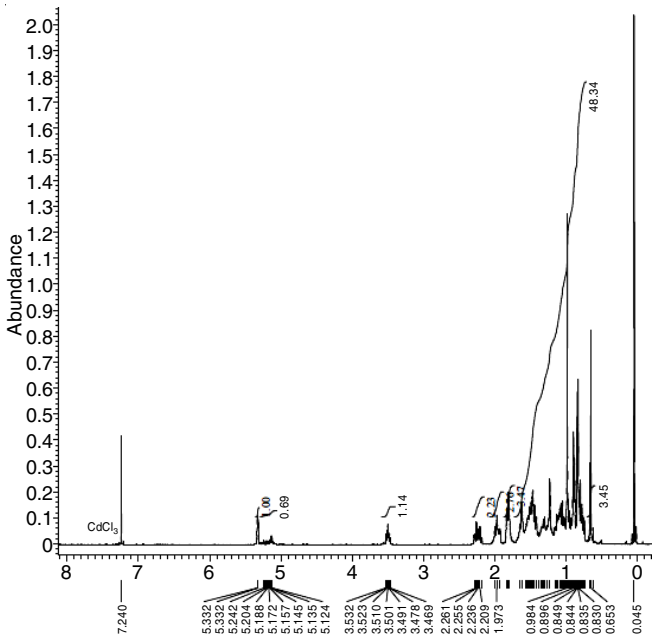

Fig. 9. ^1H NMR spectrum of isolate $M_2R_{14}K_{4a}$

The ^{13}C NMR spectrum shows $M_2R_{14}K_{4a}$ isolate (Fig. 10), which supports the data analysis that has been done previously. So, it can be concluded that $M_2R_{14}K_{4a}$ isolate a steroid of stigmast-5-en-3β-ol or β-sitosterol . The ^{13}C-NMR spectrum analysis of isolate $M_2R_{14}K_{4a}$ showed that it contained 29 carbons consis-ting of 11 methylene groups (CH_2), six methyl groups (CH_3), ten methine groups (CH), three C quaternary atoms and one OH functional group. Details of ^1H & ^{13}C NMR data of the $M_2R_{14}K_{4a}$ isolate and reference of sitosterol are given in Table-1. The interpretation results of data spectroscopy could be predicted that the molecule structure of isolate $M_2R_{14}K_{4a}$ was of β-sitosterol (Fig. 11).

Conclusion

In this study, one species of sponge were obtained and identified, namely *Haliclona* sp. The results of an isolated secondary metabolites from *n*-hexane fraction from *Haliclona* sp. (LMK-2) obtained isolates $M_2R_{14}K_{4a}$. The spectroscopic characterization data indicated that the isolate is a typical steroids and strongly suspected as a β-sitosterol or stigmast-5-en-3β-ol compound.

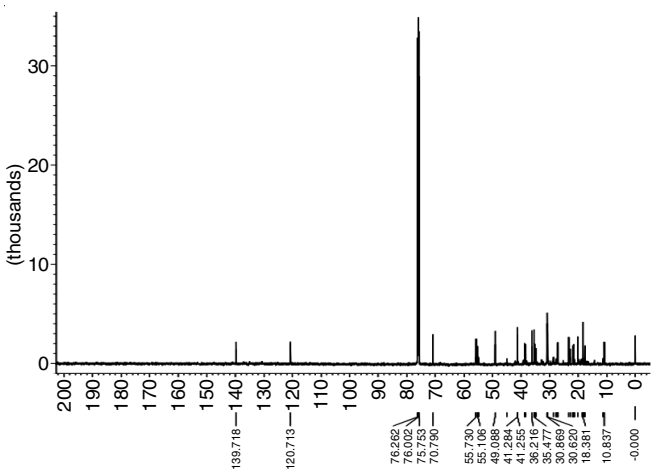

Fig. 10. ^{13}C NMR spectrum of isolate $M_2R_{14}K_{4a}$

TABLE-1
SPECTRUM DATA OF ^1H NMR AND ^{13}C NMR FOR $M_2R_{14}K_{4a}$ ISOLATE (SOLVENT, $CDCl_3$, 500 MHz)

No. C	Isolate $M_2R_{14}K_{4a}$		[Ref. 23]	
	^1H NMR (δ_H, ppm, *J* = Hz), Σ H	^{13}C NMR (δ, ppm)	^1H NMR (δ_H, ppm, *J* = Hz, Σ H	^{13}C NMR (δ, ppm)
1		35.47 (CH_2)		37.29 (CH_2)
2		30.62 (CH_2)		31.95 (CH_2)
3	3.50 (m, *J* = 4.50,1H)	70.79 (CH)	3.51 (m, 1H)	71.84 ((CH)
4		41.25 (CH_2)		42.36 (CH_2)
5		139.71 (Cq)		140.80 (Cq)
6	5.33 (d, *J* = 5, 1H)	120.71 (CH)	5.34 (d, *J* = 5.2, 1H)	121.73(CH)
7		70.79 (CH_2)		31.71 (CH_2)
8		38.49 (CH)		31.95 (CH)
9		45.00 (CH)		50.19 (CH)
10		38.64 (Cq)		36.08 (Cq)
11		21.54 (CH_2)		21.12 (CH_2)
12		38.64 (CH_2)		39.82 (CH_2)
13		41.28 (Cq)		42.36 (Cq)
14		49.08 (CH)		56.81 (CH)
15		23.27 (CH_2)		24.33 (CH_2)
16		27.22 (CH_2)		28.26 (CH_2)
17		55.73 (CH)		56.11 (CH)
18	0.65 (s, 3H)	11.30 (CH_3)	.,67 (s, 3H)	11.88 (CH_3)
19	1.23 (s, 3H)	17.69 (CH_3)	1.00 (s, 3H)	19.41 (CH_3)
20		36.21 (CH)		36.54 (CH)
21	0.96 (d, *J* = 8, 3H)	18.38 (CH_3)	0.92 (d, *J* = 6.0,3H)	19.07 (CH_3)
22		34.76 (CH_2)		34.00 (CH_3)
23		30.62 (CH_2)		26.16 (CH_2)
24		41.25 (CH)		45.39 (CH)
25		30.86 (CH)		29.23 (CH)
26	0.84 (d, *J* = 7.0, 3H)	20.05 (CH_3)	0.82 (d, *J* = 7.2,3H)	19.83 (CH)
27	0.83(d, *J* = 7.0, 3H)	17.94 (CH_3)	0.79 (d, *J* = 7.2,3H)	18.81 (CH_3)
28		17.69 (CH_2)		23.12 (CH_2)
29	0.89 (d, *J* = 6.5, 3H)	10.83 (CH_3)	0.85 (d, *J* = 7.8,3H)	12.01 (CH_3)

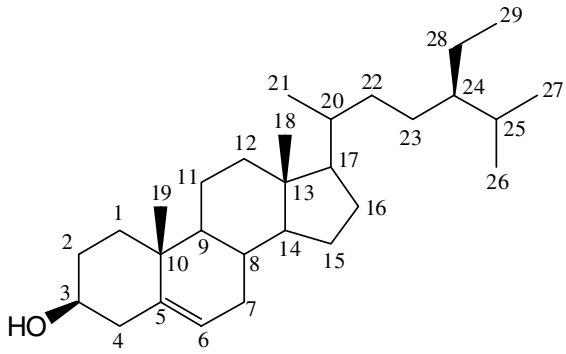

Fig. 11. Molecular structure of β-sitosterol

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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