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Research Article

UNPRECEDENTED ISOLATION OF β-SITOSTEROL ACETATE FROM DICHLOROMETHANE FRACTION OF HYDROID, *Aglaophenia cupressina* LAMOUROUX, AND ITS ANTIBACTERIAL ACTIVITIES

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ABSTRACT

Hydroids, one type of marine invertebrates, known to produce numbers of interesting metabolites with a various practical application, such as antimicrobial, anticancer, and anti-inflammation. Aims of this investigation are to isolate, purify, and determine the structure of molecules from dichloromethane fraction of hydroid, *Aglaophenia cupressina* Lamouroux, and further test its antibacterial activities against *Staphylococcus aureus* and *Escherichia coli*. Unexpectedly, β-sitosterol acetate was isolated with an amount of 9 mg and m.p 134-135 ° C. A further testing against *Staphylococcus aureus* and *Escherichia coli* also gave an inhibition zone diameter of 11.5 and 8.15 respectively which considerably good for antibacterial agent

Keywords: β-sitosterol acetate, hydroid, *Aglaophenia cupressina* Lamouroux, isolation, purification, spectroscopy, antibacterial.

INTRODUCTION

Marine environment currently becomes one of the most important areas, not only because of the roles as sources of human food consumption, but also as a supplier for huge varieties of chemical entities with different practical usage,e.g., biological, medicinal, pharmacological, and others[1]. One of the major problems in medication at present is the high demands of new antibiotic because of bacterial resistant. In order to resolve this problem, marine inhabitants become the highly recommends solution because until recently only very limited exploration have been done in term of its active chemical entities. Hydroids, one type of marine invertebrates, found to be fascinate and unique inhabitant which grown independently or in a symbiotic ways with other marine organisms like sponges, corals, molluscs, micro and macro algae, and even micro-organisms to produce some interesting compounds[2]. As a lower rank animal, without physical defence system, hydroid using the chemical system of protection by directly synthesized chemical toxins, or in a symbiotic way with other marine inhabitants, like bacteria, who can synthesise those chemical toxins and will be co-jointly used in order to protect both against predators or competitors[3].

As reported from the literature, numbers of chemical entities with varieties of activity have been isolated from hydroids. Aiello and co-workers[4] have isolated from *Aglaophenia pluma* three β-carboline type of molecules. Further, anticancer Gymnangiamide were isolated from *Gymnangium (Aglaophenia) regae* as well as an antioxidant Tridentatol from *Tridentata marginata*[5,6]. More recently Johannes and co-workers[7] and Afriansyah and co-workers[8] isolated hexadecanoid acid and β-sitosterol from a non-polar fraction of *A Cupressina* Lamouroux which found considerably potent as an antibacterial against *E Coli* and anti mitotic for the sterols. This investigation are aims to primarily isolate and determine the structure of dichloromethane fraction of *A Cupressina* Lamouroux and further examine its antibacterial potency against *S Aureus* and *E Coli*

MATERIALS AND METHODS

Samples collection and treatment

Samples of hydroid, *A Cuppresina* Lamouroux, were collected from Samalona island, nearby Spermonde archipelago, South Sulawesi, Indonesia. Fresh sample was then washed several times time seawater continued with fresh water and air-dried to obtain a dried sample.

Extraction, fractionation, and purification

Approximately 750 grams of dried samples of hydroid was macerated with 750 mL MeOH p.a for 24 hours and filtered with filter paper of Whatman No.42. This process was then repeated for three times and all MeOH filtrates were combine and evaporated under reduced pressure to obtain approximately 16.57 gram dark colour crudes methanol extracts. These crude extracts were fractionated undergoes Kupchan's fractionation and its modified methods[9]. Roughly 0.736 grams of the obtained dichloromethane fraction were then subjected to purification under several chromatography methods starting with finding a proper eluate which gave better separation in TLC plate. Afterwards, the purification continued with a vacuum and flash column chromatography to obtain the pure compound of dichloromethane fraction. The pure compound of DCM fraction was then put into an assessment for spectroscopy test, including FTIR, 1H-NMR, 13C-NMR and melting point, to determine the possible structure of the compounds. Prior to the spectroscopy test, a phytochemical test was carried out using the standard test[10,11]

Table 1: FTIR Spectrum of Compound 1

Wave length (in cm-1)	Functional group
2918.30 and 2848.86 (str)	stretching of -CH aliphatic
1739.79(str)	Strain of C=O
1625.99(w)	Stretching of aliphatic C=C
1465.9 (str)	stretching and bending of -CH2 aliphatic
1381.03 (m)	stretching and bending of -CH3 aliphatic
1014.56 (w)	bending of C-O

Table 2: 1H(500 MHz) and 13C(125 MHz) NMR Assignment for Compound 1

Carbon No.	Compound 1		Pierre *et al*., 2015		Prakash *et al*., 2012		Chem NMR	
	δ13C (ppm)	δ1H(ppm)	δ13C (ppm)	δ1H(ppm)	δ13C (ppm)	δ1H(ppm)	δ13C (ppm)	δ1H(ppm)
1	37.08	-	36.72	-	37.5	-	37.2	1.05
2	31.93	-	29.71	-	31.9	-	31.7	1.52
3	73.76	4.60 (dt,1H)	71.97	3.53 (m,1H)	72.0	3.53 (tdd,1H)	71.6	4.60
4	42.38	-	42.35	-	42.5	-	41.8	2.21
5	139.80	-	140.94	-	140.9	-	140.8	-
6	122.67	5.36(s,1H)	121.32	5.38 (s,1H)	121.9	5.36(t,1H)	121.8	5.27
7	32.02	-	31.71	-	32.1	-	32	1.94
8	31.93	-	29.24	-	32.1	-	31.8	1.27
9	50.08	-	50.03	-	50.3	-	50.8	1.17
10	36.68	-	36.16	-	36.7	-	37.7	
11	21.11	-	24.32	-	21.3	-	21.1	1.38
12	39.79	-	39.82	-	39.9	-	39.9	1.31
13	42.38	-	42.1	-	42.6	-	43	
14	56.76	-	56.9	-	57.1	-	56.9	1.04
15	25.16	-	24.32	-	26.3	-	26.4	1.9
16	28.32	-	28.9	-	28.5	-	28.6	1.9
17	56.18	-	56.03	-	56.3	-	56.4	1.24
18	11.94	0.86(s,3H)	12.06	1.29 (d,3H)	12.0	-	12.0	0.84
19	19.42	0.66(s,3H)	19.06	0.74 (d,3H)	19.0	0.68 (s,3H)	19.3	1.01
20	36.26	-	36.82	-	36.3	-	36.1	2.08
21	19.42	0.99(d,3H)	19.06	1.20 (d,3H)	19.0	0.93 (d,3H)	19.3	0.88
22	34.8	-	34.0	-	34.2	-	33.9	1.19
23	25.15	-	26.3	-	26.3	-	26.4	5.27
24	50.01	-	46.21	-	46.1	-	46.1	1.88
25	29.80	-	30.01	-	29.04	-	30.04	1.63
26	21.11	0.91(d,3H)	21.12	0.84 (d,3H)	20.1	0.83 (s,3H)	21.1	0.83
27	21.11	-	21.12	-	20.1	-	21.0	0.83
28	23.91	-	23.32	-	23.3	-	23.2	1.44
29	11.94	1.00(t,3H)	12.06	1.04(t,3H)	12.2	1.01(t,3H)	12.2	0.83
30	21.11	-	-	-	-	-	21.0	0
31	173	2.01(s,3H)	-	-	-	-	171	2.02

Table 3: Measurement of Inhibition of Compound 1 Against *E Coli* and *S Aureaus*

Type of Bacteria	Incubation time	Inhibition Zone Diameter		
		Compound 1	Neg.Control (DMSO	Pos.Control (Ampicillin)
E Coli	24 hour	9.45	5.15	18.75
	48 hour	10.25	5.15	19.45
	72 hour	11.50	5.15	20.25
S Aureaus	24 hour	6.25	5.10	18.25
	48 hour	7.40	5.10	19.31
	72 hour	8.15	5.10	20.10

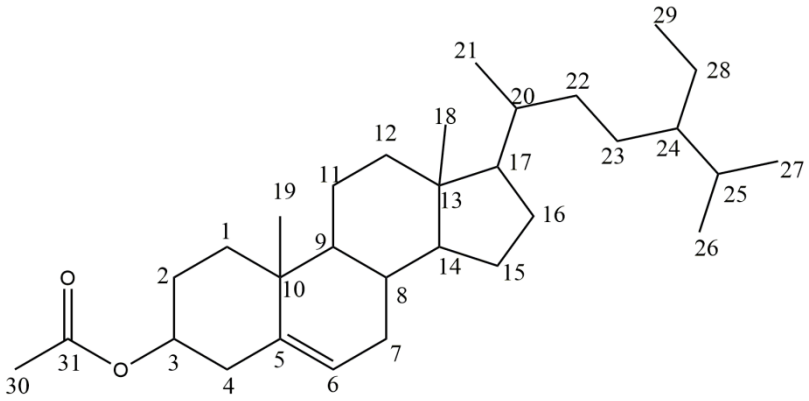

Figure 1: Structure of β-Sitosterol Acetate

Antibacterial test

Pure compounds from DCM fraction were tested for its antibacterial properties against *S Aureus* and *E Coli* undergoes Agar Diffusion Methods[10]. This method used a Nutrient Agar (NA) medium of growth and the bacteria were scratched aseptically to the Petri-disc contain of Nutrient Agar (NA) media. A disc-paper with a diameter of 5 mm was dipped into the sample solution and fluttered until no samples drop. Ampicillin and Dimethyl Sulphoxide (DMSO) as a positive and negative control were treated same as the samples. Small paper-disc that contains samples, positive control, and negative control, was put at the surface of media in Petri disc and incubated at a temperature of 37 ^{o}C for 3 days. Measurement of the transparent zone which form at surrounding paper-disc were measured by a vernier calipers.

RESULTS AND DISCUSSION

Resulted darkest brown colour of dichloromethane fraction approximately 0.736 grams were purified undergoes series of chromatography works which initiated by a thin layer chromatography using silica gel plate and combination of n-hexane, chloroform, and ethyl acetate as an eluent starting with a non-polar solvent and gradually increasing to the more polar as well as the combining of both solvent. After finding the suitable solvent system the Vacuum column chromatography were then applied to the sample in order to re-grouping the group of the more simple molecules. This process resulted in 17 sub-fractions and among these sub-fraction of 5-9 gave similarity in Rf spot were then combined to resulted a yellow amorf with weight of 69.7 mg. Resulted sub-fraction was the continue re-purification with a flash column chromatography using a gradient mixture of n-hexane/chloroform from 100 % n-hexane to 100% chloroform and resulting a much more pure combining sub-fraction of 6-9 approximately 35.6 mg. The final preparative TLC were then applying to this sub-fraction with an eluent of n-hexane/chloroform 1:1 ratio to result a crystals which after recrystallization give a pure compound about 9 mg, single spot appeared in TLC with UV-longwave and with an Rf of 0.3. These pure compounds were then subjected to a phytochemical test[10,11] and gave a dark bluish colour after adding with acetic acid anhydride and sulphuric acid which indicated the presence of steroids class of molecules. A melting point check also was carried out and gave the m.p of the pure Compound 1 about 134-135 ^{o}C which also clarify the purity of the compound.

In order to determine the structure of this pure compound, a series of spectroscopy analysis were done including FTIR, proton and carbon NMR. Table 1 and 2 shown the FTIR, proton and carbon NMR spectrum of Compound 1.

FTIR spectrogram of Compound 1 **(**Table 1.**)** clearly showed strong absorption band at wave length of 2918.30 and 2848.86 cm^{-1} which indicated the presence of C-H aliphatic which also corresponded with and absorption at 1465.9 and 1381.03 cm-1 for a -CH2 and -CH3. Moreover, a strong absorption at wave length of 1739.79 cm^{-1} is due to the presence of C=O carbonyl functional group and these also confirmed by the weak absorption band at 1014.56 cm^{-1} which shows clear sign of the presence of C-O bending. Another important functional group which is C=C olefin were signed by the weak absorption at 1625.99 cm^{-1}.

Proton NMR spectrum of Compound 1 (Table 2.) shows a unique signal at chemical shift of δ 5.35 ppm with a singlet type of multiplicity (H6,1H,s) indicated the presence of an olefin (C=C) functional group which binds to a quaternary carbon. Much higher chemical shift at δ 4.60 ppm (H3,1H, m) belong to the proton attached to carbon that binds with a heteroatom (-OCH). Beside that the signal at δ 2.01 ppm (H31,3H,s) with a singlet multiplicity corresponding with the proton attached to the carbon that directly bind with the carbonyl group.

Carbon NMR signal of Compound 1 shows 31 signals with various chemical shift as stated in Table 2. Major signal at chemical shift of 73.76 ppm belong to the oxycarbon (C-O), and further sp^{2} carbon signal at 122.68 ppm and 139.81 ppm corresponding with the presence of two olefin carbon. Another important chemical shift at δ 173.47 ppm refer to the signal of carbonyl (C=O). Both proton and carbon NMR data were reconfirmed with the NMR data that obtain from literatures [12, 13,14]. The results were seems to match accordingly.

After comprehended all data from phytochemical assessment, melting point, and spectroscopy (FTIR, 1HNMR, and 13CNMR), with further compared to the data from literatures, it was summarised that Compound 1, which was isolated and purified from DCM fraction of Hydroid *A Cupressina* Lamoureaoux is β-Sitosterol Acetate, a derivative of β-Sitosterol. The structure of Compound 1 was described in Figure 1

Compound 1 were then subjected to a bioactivity test against *E coli* and *S aureus*. The results, as stated in Table 3, showed that highest inhibition activity of Compound 1 is after incubation length of 3 x 24 hours with the diameter inhibition zone of 11.50 mm for *E coli* and 8.15 mm for *S Aureus*. This result is lower than Ampicillin antibiotic as the positive control but still relatively moderate as antibacterial agents.

CONCLUSION

This research has successfully isolated and purified β-sitosterol acetate (Compound **1**), a typical steroids, from dichloromethane fraction of hydroid *A Cuppressina* Lamouroux approximately 9 mg, yellow amorf crystals with m.p of 134-135 ^{o}C. Antimicrobial test against *E coli* and *S aureus* of the pure compound shown a quite promising inhibiting capacity of 8.50 mm and 5.15 mm inhibiting zone diameter.

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