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Isolation and identification of secondary metabolite acetone extract *Aaptos* sp. and its antioxidant properties and acute toxicity

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ABSTRACT

Aaptos sp. can be developed and utilized as a new antioxidant source. This study aims to investigate the antioxidant activity and its acute toxicity of acetone ASE stands for *Aaptos sp. Extract* and its isolates. *Aaptos* sp. was extracted by acetone, followed by fractionating with vacuum liquid chromatography, liquid–liquid partition, and radial chromatography. Each step was intervened with thin-layer chromatography. Isolates identified by comparing their physical properties and ^{1}H and ^{13}C NMR stands for *Nuclear Magnetic Resonance* spectrum with literature data. Antioxidant activity assayed qualitatively and quantitatively, and the acute toxicity assayed with brine shrimp lethality test. Isolates of ASE (44 g) obtained were AS1 (50 mg), AS2 (23 mg), AS3 (8.3 mg), and AS4 (22 mg). AS1 is identified as cholestanol. Antioxidant activity assayed qualitatively showed that ASE, AS1, AS2, and AS5 were showing as antioxidant activity, only ASE had IC_{50} values 16.10 µg/ml. LC_{50} of ASE, AS1, AS2, and AS5 were 1,041.5 µg/ml; 1,488.33 µg/ml; 681,87 µg/ml; and 783,21 µg/ml, respectively. In conclusion, there are four isolates from the ASE although only cholestanol (AS1) successfully identified. ASE, AS1, AS2, and AS5 have antioxidant activity but only IC_{50} of ASE was measured and they are regarded as safe with $LC_{50} > 1,000$ for ASE and $LC_{50} > 200$ for its isolates.

INTRODUCTION

The free radicals are the presence of an unpaired electron that is unstable and highly reactive. The free radical formation is a side product of metabolism or from external sources. Pollution, UV stands for *ultraviolet* radiation, smoke, fast-food, high-fat meal, and addictive substance are external sources of the formation of free radicals in the body. Normally, the body will produce endogenous antioxidant, such as glutathione, alpha-lipoic acid, coenzyme Q, ferritin, uric acid, bilirubin, superoxide dismutase, catalase, and glutathione peroxidase as the homeostatic process of free radical formation, thereby they will be balanced. Exposures of these factors unwittingly hasten up the formation of free radical rate. The endogenous antioxidant defense will not be enough if the formations of free radicals are excessive (Lobo *et al*., 2010).

Stress oxidative must be avoided because it can damage the cell components, including proteins, DNA, and lipid membranes, thus resulting in necrosis. It leads to degenerative deterioration and development of diseases, such as cancer, cardiovascular disease and diabetes mellitus type II, cataract, arthritis, autoimmune disease, and neurodegenerative diseases (Kabel, 2014; Wojick *et al*., 2010). The human body does not synthesize excessive amounts of the endogenous antioxidant, thus exogenous antioxidants are needed (Dharini *et al*., 2010).

The discovery of exogenous antioxidants is a choice for the development and utilization of natural resources. Indonesia is one of the largest archipelago countries in the world that has abundant natural resources. They can be developed and utilized in the discovery of novel medicines. Marine sponges including Aaptos sp. are one of the biota that can be used for drug discovery because many bioactive substances can be found (Kudo *et al*., 2014; Uli *et al*., 2017).

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The previous study showed that sponge *Aaptos* sp. exhibits activity as an anticancer, antibacterial, and antidepressant (Shaari *et al*., 2009). Sponge *Aaptos* sp. contains secondary metabolites, such as aaptamine, isoaaptamine, aaptoline, and dimethylaaptamine (Aoki *et al*., 2006; Quiao and Uy, 2013; Tsukamoto *et al*., 2010; Kudo *et al*., 2014).

Despite the discovery of a novel drug is essential, knowledge of potential toxic effect is equally important. The toxicity test is important to conduct as well as an initial parameter of drug safety prior tested to human. This is because each substance has potential toxicity depending on the dose in the body (Andreanus *et al*., 2002). Therefore, this study aims to investigate secondary metabolites of Sponge *Aaptos* sp. by isolating the isolates to discovering its antioxidant property and proving its acute toxicity thus can support the treatment of medication accurately in the future.

MATERIALS AND METHODS

Sponge collection

Sponge sample (*Aaptos* sp.) was collected from Bintang Samudra Marine Edu-Park, Soropia Sub District, Konawe District, South East Sulawesi. Sponge sample was collected from the reef slope (70°) with depth 10 m above sea level by SCUBA (*Self Contained Underwater Breathing Apparatus*) diving. The sample was determined by staff of Faculty of Fisheries and Marine Science, Universitas Halu Oleo (NO. 537/un29.20/KAPUSLIT/2019). The sample collected then put in the icebox.

Extraction

Sponge sample (*Aaptos* sp.) was washed and cleaned from impurities, then chopped into pieces. The sample was macerated with acetone for 3 × 24 hours. Filtrate and reside were separated and replaced the solvent. Collected filtrated was then concentrated by using vacuum rotary evaporator (*Buchi*®) and yielded amount 44 g concentrated extract.

Isolation, purification, and identification of isolates

Fractionation and Isolation of phytochemicals of acetone extract of *Aaptos* sp. were using vacuum liquid chromatography (VLC), liquid–liquid partition, and radial chromatography (RC). Every step of fractionation and isolation was intervened by thin-layer chromatography (TLC) Si-Gel F_{254} (*Merck*®) and observed under UV light (UVG-58) 245 and 366 nm and sprayed with cerium sulfate (CeSO4) (*Merck*®).

Isolates obtained was identified by its physical properties, TLC profile, and ^{1}H, and ^{13}C-NMR spectrum (*Agilent*®). Isolates were then elucidated and compared with literature data.

Measurement of antioxidant activity of isolates

Antioxidant activity was assayed qualitatively and quantitatively. Qualitatively, antioxidant activity was assayed with the dot blot assay method. The various concentrations of extract and isolates, as follows:

Positive Control (Ascorbic Acid (AA)):5,000 µg/ml; 2,500 µg/ml; 1,250 µg/ml; and 625 µg/ml

Acetone extract of *Aaptos* sp. (ASE) : 25 µg; 12.5 µg; 6.25 µg; and 3.125 µg
Isolate AS1 : 25 µg; 12.5 µg; 6.25 µg; and 3.125 µg
Isolate AS2 : 25 µg; 12.5 µg; 6.25 µg; and 3.125 µg
Isolate AS5 : 25 µg; 12.5 µg; 6.25 µg; and 3.125 µg

The sample then spotted and run on the TLC plate and dipped into DPPH stands for *2,2-diphenyl-1-picrylhydrazil*. The plate observed on visible light and UV 366 nm.

Quantitatively, antioxidant activity was measured under spectrophotometer (Genesys-20) with modified DPPH assay. The various concentrations of extract and isolates were 200; 100; 50: 25: 12.5: 6.25: 3.125; and 1.5,625 µg/ml, respectively, to determine the IC_{50} values.

Data recorded were calculated by as follows:

$$\text{Percentage of scavenging radical} = \left(\text{abs. of blank} - \frac{\text{abs.of sample}}{\text{abs.of blank}} \right) \times 100$$

Statistical analysis was done by using SPSS® (Statistical Product and Service Solution) 21.0 for determining the significant difference between isolates and positive control ($p < 0.05$). IC_{50} values were determined by GraphPad Prism 5 (GraphPad Software®, La Jolla, California, USA).

Acute toxicity test

Acute toxicity test was done by brine shrimp lethality test.

$$\text{Percentage of lethality} = \frac{(\text{total larvae} - \text{number of live larvae})}{\text{total larvae}} \times 100$$

The various concentrations used were 2,000 ppm–15.625 ppm for the sample and positive control (K+) and 12.5%–0.03902626% for the negative control (K-). Samples used were acetone extract of *Aaptos* sp. (ASE), compound AS1, compound AS2, and compound AS5. The positive control used was potassium dichromate and the negative control used was DMSO.

LC_{50} of isolates was obtained from the Probit analysis among the variation dose of extract and isolates and the number of death larvae of *Artemia salina* by using Minitab® ver 17.1.2.

RESULTS AND DISCUSSION

Isolation and purification of isolates

Acetone extract of *Aaptos* sp. (44 g, 10%) was fractionated by VLC with n-hexane:ethyl acetate (10:0, 9:1, 8:2, 7:3, 5:5, and 3:7), ethyl acetate:methanol (10:0), and methanol 100% to increase the polarity. Fractions 1–5 and fraction washed-methanol were obtained from eluates that showed similar TLC profiles. Faction 2 was purified with RC and obtains the compound **AS1 (50 mg)** with eluent n-hexane: ethyl acetate (9:1). Fraction washed-methanol was partitioned with n:hexane:ethyl acetate (1:1) and thereafter, the partition was combined after TLC profile that showed similar eluates and continued with RC with eluent n-hexane:dichloromethane:ethyl acetate (1:8:1) obtained Fraction A-E. Purification of fraction B with RC with eluent n-hexane:dichloromethane:ethyl acetate (1:8:1) obtained three different fractions (fraction A1, A2, and A3), followed purification fraction A2 with RC with eluent n-hexane:ethyl acetate:dichloromethane (1:7:2) provided compound **AS2 (23 mg)** and AS3 **(8.3 mg)**. Fraction D was purified with RC with eluent ethyl acetate:dichloromethane:methanol (4:5:1) provided compound **AS4 (22 mg)**.

Identification with ^{1}H, and ^{13}C-NMR spectrum

Compound AS1

Compound AS1 was white crystal. AS1 was not showing spot under UV 254 nm and 366 nm although showed spot after sprayed with cerium sulfate ($CeCl_3$) reagent. Thus, the steroid compound suspected. The ^{1}H NMR AS1 compound had a similar proton signal of steroid, which was characterized by overlapping proton signals at δ 0–3 ppm. The ^{1}H NMR signal of compound AS1 was characterized with six distinctive peaks of five high-intensity methyl group at δ 0.63 (H-18), 0.78 (H-19), 0.084 (H-26), 0.89 (H-27), and 0.96 (H-21) ppm and proton signal at δ 3.57 ppm (H-3) showed proton signal hydroxylated-methine, hence hydroxide bond/ hydroxyl group (-OH) suspected in structure (Table 1).

The ^{13}C NMR signal was not presented above 90 ppm describing the steroid-AS1 compound, were not having a carbon-carbon double bond. The ^{13}C NMR signal compound AS1 presented 6 methyl carbon (CH_3) signal observed at δ 12.2 (C-18), 12.4 (C-18), 18.8 (C-21), 22.7 (C-26), and 22.9 (C-27) ppm in AS1 structure. Total 27 carbons of the steroid-AS1 compound with 12 carbon methylene (CH_2); 8 carbon methine (CH); and 12 carbon quartern (Cq). Carbon methine signal presented at δ 71.5ppm (C-3) which supposed to present at 35–50 ppm. Thus, concluded that the carbon methine is hydroxylated (Pavia *et al*., 2009). The hydroxylated-carbon methine was correlated with methine proton signal at ^{1}H NMR as well as bonded at methine at ^{1}H NMR (Figure 1). Based on the signal presented at ^{1}H NMR and ^{13}C NMR compared with literature (Mohamad *et al*., 2009), compound AS1 predicted as cholestanol (Figure 2).

Table 1. Comparasion of ^{1}H and ^{13}C NMR of compound AS1 (ppm, measured under 100 MHz (^{13}C) and 400 MHz (^{1}H) in $CDCl_3$, δ TMS = 0.

Compound AS1			Pembanding (Mohamad, dkk., 2009)		
C#	δ_C	δ_H(ΣH, *m*, J Hz)	C#	δ_C	δ_H
1	37,1	–	1	37,2	–
2	35,5	–	2	35,7	–
3	71,5	3,57 (1H, m, 4)	3	71,6	3,59 m
4	44,9	–	4	45,1	–
5	54,4	–	5	54,6	–
6	21,3	–	6	21,5	–
7	24,3	–	7	24,4	–
8	31,6	–	8	31,8	–
9	56,4	–	9	56,5	–
10	32,2	–	10	32,3	–
11	19,7	–	11	19,8	–
12	40,1	–	12	40,3	–
13	36,3	–	13	36,4	–
14	42,7	–	14	42,8	–
15	23,9	–	15	24,1	–
16	28,4	–	16	28,5	–
17	56,6	–	17	56,7	–
18	12,2	0,63(3H,s)	18	12,3	0,65 s
19	12,4	0,78(3H,s)	19	12,5	0,81 s
20	38,3	–	20	38,4	–
21	18,8	0,96(3H,d,12)	21	18,9	0,9 d
22	35,9	–	22	36,0	–
23	28,1	–	23	28,2	–
24	39,6	–	24	39,7	–
25	28,8	–	25	29,0	–
26	22,7	0,89(3H,t, 8)	26	22,7	0,87 d
27	22,9	0,84(3H,d,8)	27	23,0	0,86 d

Compound AS2

Compound AS2 was yellowish mass. AS2 was showing as a yellowish spot only visible under UV 366 nm. Compound AS2 is still under further identification hence the structure cannot be displayed in this paper. The initial identification of compound AS2 was possibly indicating proton signals of methyl, methylene, methine, methoxy, C-C double bond, and aromatic group. Signal proton of compound AS2 presented in Table 2 and Figure 3.

Compound AS3

Compound AS3 was yellowish mass. The spot was only visible under UV 254 nm as a yellowish spot. The compound AS3 is still under further identification, thus, cannot be displayed in this paper. The initial identification process was indicating of proton signal of methyl, methylene, methine, methoxy, C-C double bond, and aromatic group. Signal proton of compound AS3 presented in Table 3 and Figure 4.

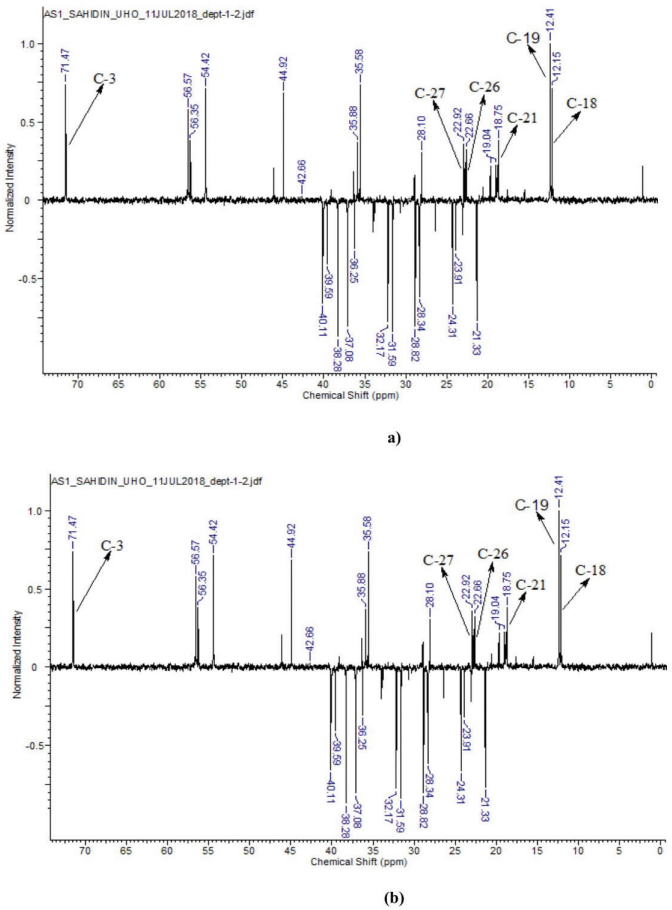

Figure 1. (a) ^{1}H NMR of compound AS1; (b) ^{13}C NMR of compound AS1.

Compound AS4

Compound AS4 was solid reddish oil which fluorescents into orange under light UV 254 nm and turned out into orange when dissolving with the organic solvent. The signal proton of compound AS4 is cannot be interpreted with ^{1}H NMR thus the structure cannot be determined (Figure 5).

Antioxidant activity

The antioxidant activity test was carried out on the acetone extract of *Aaptos* sp. (ASE), AS1, AS2, and AS4 isolates. The test was not conducted on AS3 due to the limited amount of isolate. Qualitatively (Figure 6), showed that ASE, AS2, and AS5 had antioxidant constituent which was visualized on the visible light. The result is characterized by spots with faded yellow on the plate and visualized on UV light with 366 nm showed spot with light blue although they have low diameter and intensity than ascorbic acid (Badarinath *et al*., 2010). Discoloration of DPPH occurs due to the effect of the reduction of DPPH radicals compound by mechanism electron donation/hydrogen donation, thus the production of DPPH in non-radical form and reduces the intensity of purplish DPPH color (Molineux, 2004). Visualization under light UV 366 nm is proving the antioxidant component characterized by the light blue spot on the plate (Gu *et al*., 2009).

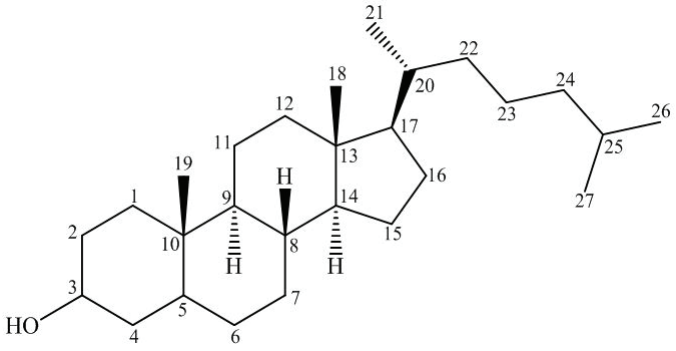

Figure 2. Compund AS1 (cholestanol).

Acetone extract and isolates were measured quantitatively from the antioxidant activity against DPPH free radical (Table 4; Figure 7). acetone extract of *Aaptos* sp. (ASE) is the only extract that has IC_{50} value above 50% namely, 16.10 µg/ml. For control, ascorbic acid (AA) provide IC_{50} values of 23.36 µg/ml. The IC_{50} value of ASE was lower than the IC_{50} value of AA possibly due to ASE consisted of a mixture of active compounds and synergically reduces DPPH. *Aaptamine* is one of predominant compounds that can be found in *Aaptos* sp. and has acted as an antioxidant (Larghi *et al*., 2009; Shaari *et al*., 2009). According to the structure of a compound with one or several OH molecules can inhibit oxidation and capture reactive free radicals from compounds that can destroy cells. The antioxidant is a reducing agent that is easily oxidized by free radical due to the double bond and OH

Table 2. Signal ^{1}H NMR of compound AS2 (in ppm, measured under 400 MHz (^{1}H) in CD_3OD, δ TMS = 0).

No.	δ_H(m, J Hz)	Estimated functional group
H-1	0,87(s)	Metal
H-2	1,26(s)	Metal
H-3	2,02(d, 8)	Methylene
H-4	2,24(t, 4)	Methylene/Methine
H-5	2,64(s)	Methine
H-6	3,44(d, 8)	Methylene/Methine (bond to electronegative atoms or have π bonds)
H-7	3,62(d, 4)	Methylene/Methine (bond to electronegative atoms or have π bonds)
H-8	3,93(s)	Methoxy
H-9	3,98(d, 8)	Methylene/Methine (bond to electronegative atoms or have π bonds)
H-10	4,02(s)	Methoxy
H-11	4,10(s)	Methoxy
H-12	4,19(d, 8)	Double bond
H-13	4,23(s)	Double bond
H-14	4,32(q, 8)	Double bond
H-15	6,68(s)	Double bond/aromatic
H-16	7,05(d, 8)	Double bond/aromatic
H-17	7,14(d, 4)	Double bond/aromatic
H-18	7,19(dd, (8, 4))	Double bond/aromatic
H-19	7,43(t, 4)	Double bond/aromatic
H-20	7,60(t, 4)	Double bond/aromatic
H-21	7,69(t,4)	Double bond/aromatic
H-22	8,32(s)	Aromatic

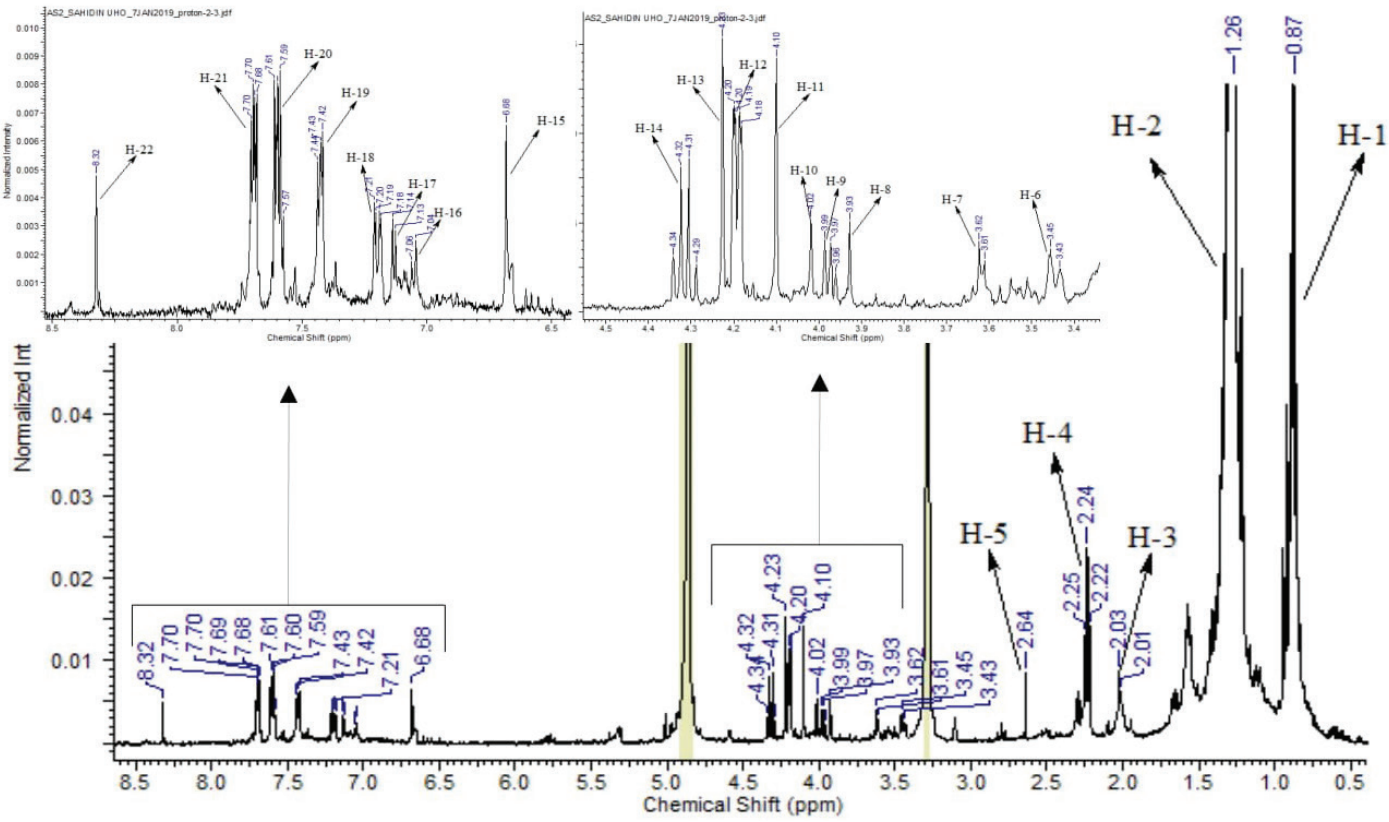

Figure 3. [1]H NMR of compound AS2.

Table 3. signal of [1]H NMR of compound AS3 (in ppm, measured at 400 MHz ([1]H) in CD_3OD, δ TMS = 0).

No.	δ_H(m, J Hz)	Estimated functional group
H-1	0,87(s)	Metal
H-2	1,26(s)	Metal
H-3	1,82(s)	Methylene
H-4	2,25(t, 8)	Methylene/methinee
H-5	3,62(s)	Methoxy
H-6	3,98(d, 8)	Methylene/Methine (bond to electronegative atoms or have π bonds)
H-7	4,19(d, 8)	Double bond
H-8	4,31(q, 4)	Double bond
H-9	6,59(d, 8)	Double bond/aromatic
H-10	6,68(s)	Double bond/aromatic
H-11	6,83(q, 4)	Double bond/aromatic
H-12	7,19(s)	Double bond/aromatic
H-13	7,38(t, 8)	Double bond/aromatic
H-14	7,43(t, 4)	Double bond/aromatic
H-15	7,59(q, 4)	Double bond/aromatic
H-16	7,69(q, 4)	Double bond/aromatic
H-17	7,82(d, 8)	Double bond/aromatic

molecular bonds become double bond, thereby the free radicals will accept hydrogen atom resulting in the formation of oxygen radicals. Thereafter, oxygen radical is delocalized by resonance, thus produces more stable radicals (Afrianty *et al*., 2010).

Acute toxicity

The LC_{50} of acetone extract of *Aaptos* sp. (ASE), compound AS1, compound AS2, compound AS5 were 1041.5, 1488.33, 681.87, and 783.21 µg/ml, respectively (Figure 8). According to

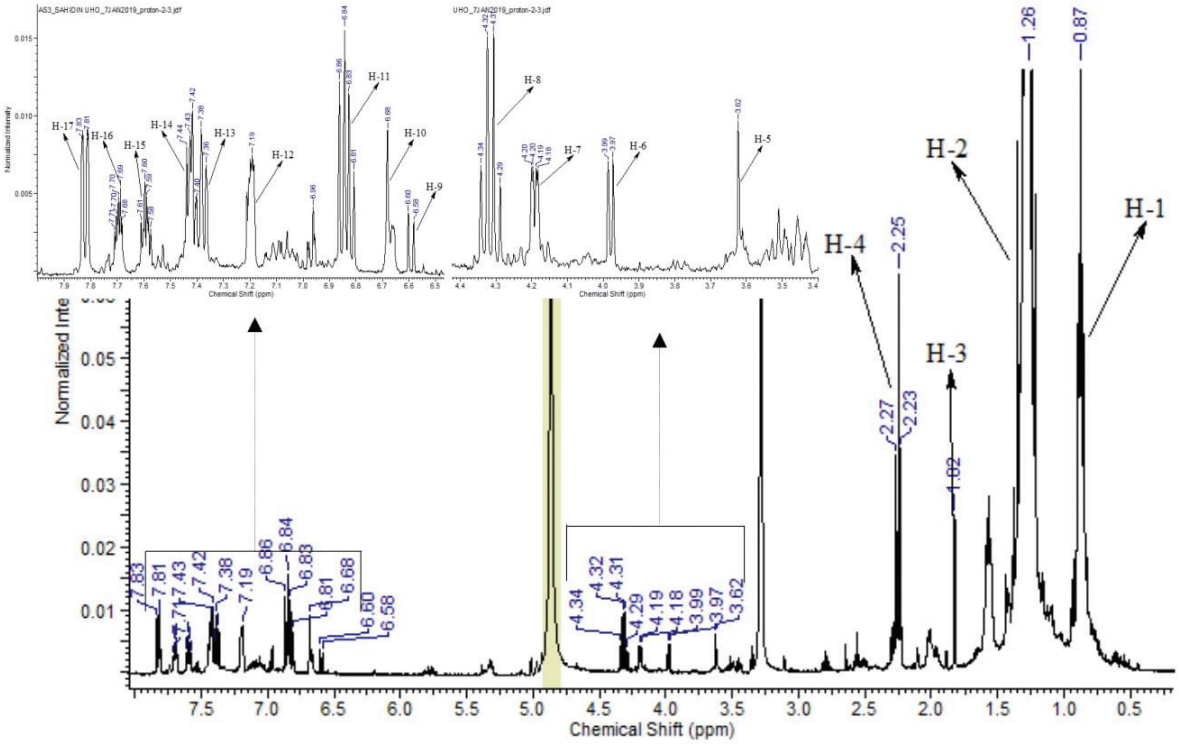

Figure 4. [1]H NMR of compound AS3.

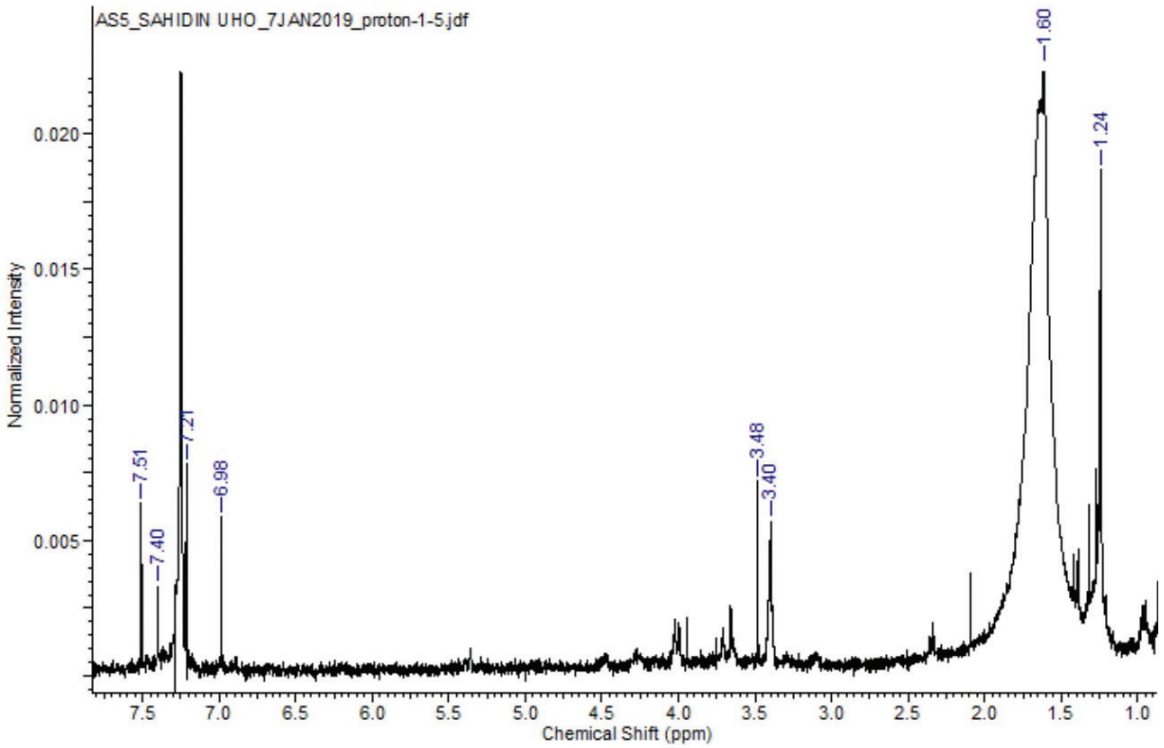

Figure 5. [1]H NMR of compound AS4.

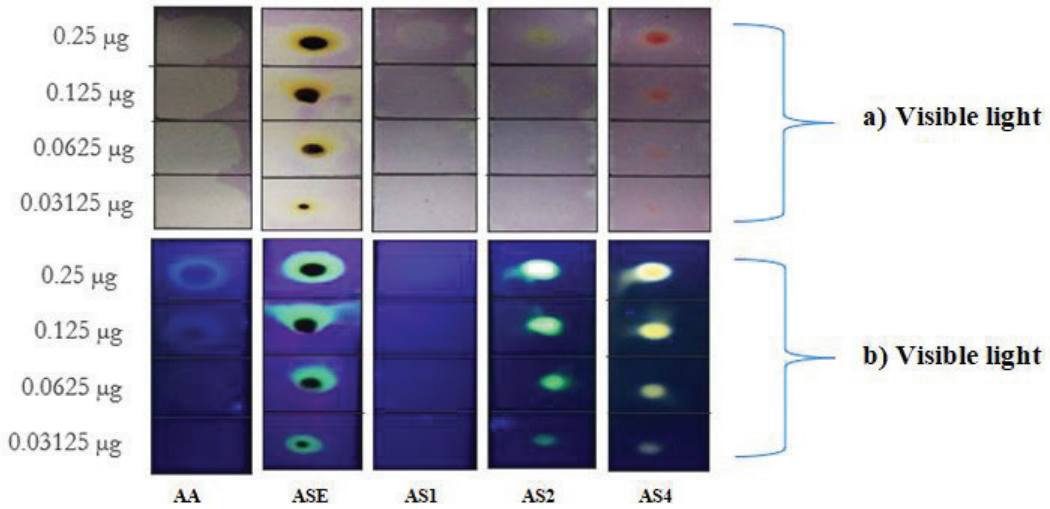

Figure 6. Qualitative test of antioxidant activity (a) Visible light; (b) UV light 366 nm.

Table 4. IC_{50} values of sample.

Sample	Concentration (µg/mL)	%Inhibition*	IC_{50} (µg/ml)
Ascorbic Acid (AA)	200	82,46 ± 0,66	23,36
	100	82,24 ± 3,32	
	50	83,05 ± 2,40	
	25	85,30 ± 2,61	
	12,5	65,43 ± 9,15	
	6,25	26,19 ± 15,55	
	3,125	13,24 ± 11,59	
	1,5625	15,78 ± 16,21	
Acetone ectract of *Aaptos* sp. (ASE)	200	60,61 ± 4,39	16,10
	100	60,47 ± 0,18	
	50	61,43 ± 0,29	
	25	44,70 ± 1,09	
	12,5	29,15 ± 2,83	
	6,25	13,33 ± 5,15	
	3,125	10,88 ± 3,31	
	1,5625	7,43 ± 7,07	
AS1	200	16,89 ± 2,56	–
	100	19,53 ± 7,51	
	50	13,32 ± 3,41	
	25	14,67 ± 5,87	
	12,5	16,04 ± 7,19	
	6,25	14,99 ± 5,77	
	3,125	14,03 ± 5,38	
	1,5625	13,73 ± 1,29	
AS2	200	21,55 ± 6,40	–
	100	11,87 ± 0,58	
	50	12,88 ± 0,02	
	25	14,27 ± 1,96	
	12,5	14,11 ± 2,99	
	6,25	10,42 ± 4,31	
	3,125	14,37 ± 5,69	
	1,5625	13,05 ± 2,43	

(Continued)

Sample	Concentration (µg/mL)	%Inhibition*	IC_{50} (µg/ml)
AS4	200	22,08 ± 1,61	–
	100	23,41 ± 3,94	
	50	16,15 ± 1,69	
	25	18,78 ± 0,24	
	12,5	19,03 ± 4,01	
	6,25	17,60 ± 3,49	
	3,125	17,78 ± 4,01	
	1,5625	14,03 ± 0,04	

*: n + SD = 6.

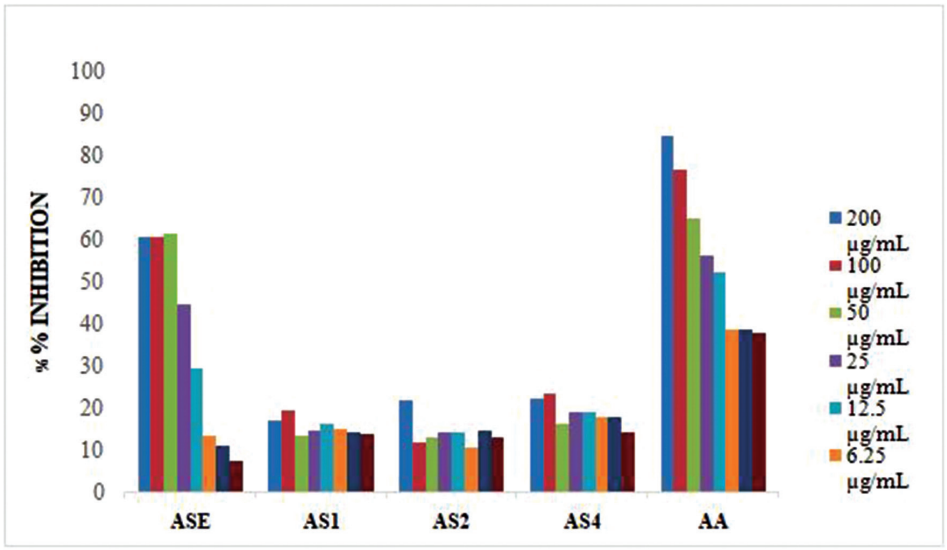

Figure 7. Graphic bar of %inhibition of extract, isolates, and control (ASE = Acetone extract of *Aaptos* sp.; AS1 = Compound AS1; AS2 = Compound AS2; AS4 = Compound AS4; A4 = Ascorbic acid).

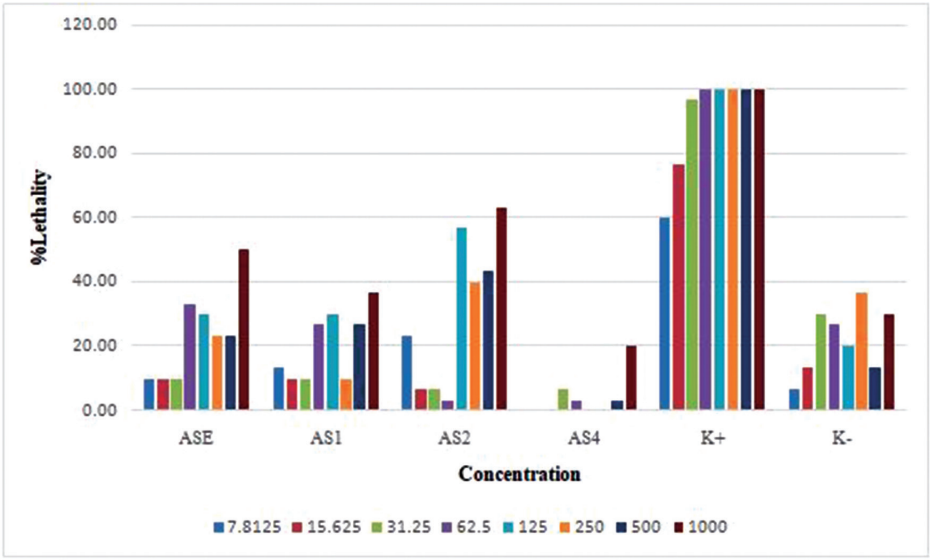

Figure 8. Graphic bar of lethality percentage of *A.salina* leach (ASE = Acetone extract of Aaptos sp.; AS1 = compound AS1; AS2 = Compound AS2; AS4 = Compound AS4; K+ = potassium dichromate; K- = DMSO).

results concluded that LC_{50} of ASE is not toxic (IC_{50}>1,000 µg/ml) and as well the compound AS1, compound AS2, and compound AS5 are not toxic (LC_{50} > 200 µg/ml) (Meyer *et al*., 1982).

CONCLUSION

Compounds isolated from acetone extract of *Aaptos* sp. were four isolates although only cholestanol (AS1) successfully identified. Compound AS2, AS3, and AS4 were not identified yet. Acetone extract of *Aaptos* sp. has antioxidant activity according to results with IC_{50} value is 16.10 µg/ml (Ascorbic acid as the positive control, IC_{50} value is 23.36 µg/ml). Besides that, the compound AS1, compound AS2, and compound AS5 were not measured due to the percentage of inhibition inadequate to 50%. Acetone extract of *Aaptos* sp. (ASE), AS1, AS2, and AS5 are not toxic with LC_{50} values weres 1,041.57, 1,488, 681.87, and 783.21 µg/ml, respectively.Further study needed to investigate secondary metabolites of *Aaptos* sp. and its activities in many aspects, thus broaden information about a pharmacological aspect of *Aaptos* sp. in treatment. MS and IR spectrum data are needed for determining the further chemical structure

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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AUTHORSHIP

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