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 ¹H and ¹³C 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 IC₅₀ values 16.10 µg/ml. LC₅₀ 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 IC₅₀ of ASE was measured and they are regarded as safe with LC₅₀ > 1,000 for ASE and LC₅₀ > 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).
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, iso-aaptamine, aaptoine, and dimethyilaaptamine (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 F254 (*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 1H, and 13C-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 1.5625 µg/ml, respectively, to determine the IC50 values.

Data recorded were calculated by as follows:

\[
\text{Percentage of scavenging radical} = \frac{\text{abs.of blank} - \text{abs.of sample}}{\text{abs.of blank}} \times 100
\]

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). IC50 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.

\[
\text{Percentage of lethality} = \frac{\text{total larvae - number of live larvae}}{\text{total larvae}} \times 100
\]

The various concentrations used were 2,000 ppm–15,625 ppm for the sample and positive control (K+) and 12.5%–0.03907626% 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.

LC50 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. Fraction 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 \(\delta 0–3\) ppm. The \(^1\)H NMR signal of compound AS1 was characterized with six distinctive peaks of five high-intensity methyl group at \(\delta 0–3\) ppm. The \(^1\)H NMR signal of compound AS1 had a similar proton signal of steroid, which was characterized by overlapping proton signals at \(\delta 0–3\) ppm.

The \(^{13}\)C NMR signal of compound AS1 was characterized with six distinctive peaks of five high-intensity methyl group at \(\delta 0–3\) ppm. The \(^{13}\)C NMR signal of compound AS1 was characterized with six distinctive peaks of five high-intensity methyl group at \(\delta 0–3\) ppm. The \(^{13}\)C NMR signal of compound AS1 was characterized with six distinctive peaks of five high-intensity methyl group at \(\delta 0–3\) ppm. The \(^{13}\)C NMR signal of compound AS1 was characterized with six distinctive peaks of five high-intensity methyl group at \(\delta 0–3\) ppm.

| C# | \(\delta_c\) | \(\delta_H(\Sigma H, m, J \text{ Hz})\) | C# | \(\delta_c\) | \(\delta_H(\Sigma H, m, J \text{ Hz})\) |
|----|-------------|---------------------------------|----|-------------|---------------------------------|
| 1  | 37.1        | –                               | 1  | 37.2        | –                               |
| 2  | 35.5        | –                               | 2  | 35.7        | –                               |
| 3  | 35.7        | 3,57 (1H, m, 4)                  | 3  | 35.6        | 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                              | 10 | 32.3        | –                               |
| 11 | 19.7        | 11                              | 11 | 19.8        | –                               |
| 12 | 40.1        | 12                              | 12 | 40.3        | –                               |
| 13 | 36.3        | 13                              | 13 | 36.4        | –                               |
| 14 | 42.7        | 14                              | 14 | 42.8        | –                               |
| 15 | 23.9        | 15                              | 15 | 24.1        | –                               |
| 16 | 28.4        | 16                              | 16 | 28.5        | –                               |
| 17 | 56.6        | 17                              | 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                              | 20 | 38.4        | –                               |
| 21 | 18.8        | 0.96(3H,d,12)                   | 21 | 18.9        | 0.9 d                           |
| 22 | 35.9        | 22                              | 22 | 36.0        | –                               |
| 23 | 28.1        | 23                              | 23 | 28.2        | –                               |
| 24 | 39.6        | 24                              | 24 | 39.7        | –                               |
| 25 | 28.8        | 25                              | 25 | 29.0        | –                               |
| 26 | 22.7        | 0.89(3H, 8)                     | 26 | 22.7        | 0.87 d                          |
| 27 | 22.9        | 0.84(3H, 8)                     | 27 | 23.0        | 0.86 d                          |

Table 1. Comparaison of \(^1\)H and \(^{13}\)C NMR of compound AS1 (ppm, measured under 100 MHz \((^{13}\text{C})\) and 400 MHz \((^{1}\text{H})\) in CDCl\(_3\), \(\delta_{\text{TMS}} = 0\).
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).

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

**Figure 2.** Compound AS1 (cholesterol).

**Table 2.** Signal $^1$H NMR of compound AS2 (in ppm, measured under 400 MHz ($^1$H) in CD$_3$OD, $\delta$ TMS = 0).

| No. | $\delta_1$ (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 $\pi$ bonds) |
| H-7 | 3,62(d, 4)           | Methylene/Methine (bond to electronegative atoms or have $\pi$ bonds) |
| H-8 | 3,93(s)              | Methoxy                                        |
| H-9 | 3,98(d, 8)           | Methylene/Methine (bond to electronegative atoms or have $\pi$ 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                                        |
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 LC₅₀ 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

### Table 3. signal of 'H NMR of compound AS3 (in ppm, measured at 400 MHz ('H) in CD₃OD, 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/methine         |
| 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      |

**Figure 3.** 'H NMR of compound AS2.
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. IC₅₀ values of sample.

| Sample                          | Concentration (µg/mL) | %Inhibition* | IC₅₀ (µg/ml) |
|---------------------------------|-----------------------|--------------|--------------|
| Ascorbic Acid (AA)              |                       |              |              |
|                                 | 200                   | 82,46 ± 0,66 |              |
|                                 | 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| 23,36        |
|                                 | 200                   | 60,61 ± 0,18 |              |
|                                 | 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  | 16,10        |
|                                 | 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 |              |
|                                 | 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* | IC₅₀ (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 were 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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