Optimizing antioxidant substances extraction produced by *Holothuria atra* using ultrasonic with response surface method

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Abstract. This study was to discover the effect of ultrasonic extraction based on the factors of extraction time and amplitude, to increase the yield recovery of *H. atra* extract and antioxidant activity (IC50). The process in optimizing ultrasonic extraction was performed using Response Surface Methodology (RSM) with Central Composite Design (CCD). *H. atra* extract produced by the 2 different extraction methods indicated a significant difference in yield (maceration 5.48%, ultrasonic 10.43%) and antioxidant activity (maceration 149.38 ppm, ultrasonic 84.85 ppm). The antibacterial activity against *B. subtilis, E. coli, and S. aureus* also the toxicity LC50 at 457.77: 443.63 ppm were not significantly difference. It was found that ultrasonic extraction method needs shorter time with higher yield and more antioxidant activity. The sea cucumber extract that was produced from the ultrasonic method contains alkaloid, triterpenoid, and saponin, while steroid undetected, and from the maceration method contains no alkaloid and steroid.

1. Introduction

Concern to geographically, Indonesia is located between Pacific Ocean and the Indian Ocean, which means that this country is the best habitat for marine organisms, including sea cucumbers [1]. Indonesia is the world’s highest imported of Holothuroidea [2]. The common species of Indonesian sea cucumber which are highly economic valued are golden sea cucumber (*Stichopus hermanii*), black teatfish (*Holothuria nobilis*), white teatfish (*Holothuria fuscogilva*) and sandfish (*Holothuria scabra*)[2]. The most highly valued of sea cucumbers caused increasing of exploitation was being proposed to be listed in the endangered marine biota species and will become restricted for any kind of international commercial trade [3]. Another species of sea cucumber (such as black sea cucumber or *Holothuria atra*), is one of marine biota whose population is still considerably abundance, and this species is not highly-priced. Komala [4] says that 55% of the sea cucumber population in Kepulauan Seribu, Jakarta, is classified as *Holothuria atra*.

Bioactive substances content in *H atra* extract has been reported some researcher. The extract of *H. atra* contains phenolic compounds (chlorogenic acid, pyrogallol, rutoside, coumaric acid, catechin, and ascorbic acid) [6].As well as alkaloid, triterpenoid, steroid, saponin, anthraquinone, and glycoside which are considered to be the potential antioxidant [7]. It has also found that sea cucumber extract tends to act as antibacterial, antifungal [8] anticoagulant, antihypertension, antimalaria, antivirus [9].
antithrombotic, overcome osteoarthritis [10] anti-inflammation, immune-stimulant [7], antitumor [11] and anticancer [12].

The common method used to extract sea cucumber was maceration. Extraction using maceration method generates the yield of about 6% [13] or 0.11% [14] in 3 x 24 hours. Using the maceration method will result in the low of extract yield in longer time processes. Thus, there should be any new method that could be increasing the extraction yield and cutting the time process. The ultrasonic method that has the cavitation phenomenon now being used as the alternative for reducing extraction time and multiplying the yield [15].

The main targets to be optimized were yield and the antioxidant activity, which is performed using Response Surface Methodology and Central Composite Design. This method is known as an effective statistic method for finding optimal condition [16]. As the independent variables of this experiment were the extraction time and the ultrasonic amplitude, which would be compared with the ‘control’ extraction yield generated from the maceration method. The other parameter such as chemical group screening and antibacterial activity would also be performed.

2. Experimental
2.1 Materials
The dried sea cucumber (Holothuria atra) were obtained from the Lampung in 2018. The fresh body wall of sea cucumber was separated with the viscera. The body wall was boiled for 1 hour and dried directly under the sun (above bamboo/para-para) for several days. The dried sea cucumber was continued for drying to reduce water content using the oven at 60 C during 24 hours. The sea cucumbers were powdered using a blender (Waring Commercial) and sifted using the mesh with 20 strainers. Powdering the sea cucumbers were easier to extract than slices once because the smaller size of materials it will have a broader surface. A broad material surface may enhance the efficiency of contact between the materials with the solvent [17], and the extraction of bioactive components can be optimized [18, 19, 20, 21].

2.2. Methods
2.2.1. Sea cucumber (Holothuria atra) extraction using ultrasonic and maceration method.
About 10 g of sea cucumbers powder was macerated with 96 % ethanol in 500 mL beaker glass, the ratio between sea cucumber powder and the solvent was 1:10. The samples were extracted using two methods, the first was maceration as a control method, processed for 3 x 24 hours in room temperature. The second was the ultrasonic method (treatment method) to perform comparison (Cole Palmer/cpx 130, 220 V, 130 Watt). Experiment design of ultrasonic method was performed using Response Surface Methodology with the model of Central Composite Design (Table 1 and 2) through statistic software so-called Design Expert. The filtrates generated from an extraction process using either maceration or ultrasonic methods were strained using Whatman no. 4 paper strainer and evaporated using rotary vacuum evaporator at the temperature of 40 degrees Celsius with the speed of 90 rpm to coagulate and turn it into coarse sea cucumber extract. The coarse extract would be analyzed and compared.

2.2.2. Experiment design using RSM and statistical analysis.
This experiment was performed using RSM with the model of Central Composite Design (CCD) on two independent factors, which were extraction time (XI) and extraction amplitude (X2) (Table 1).
Table 1. Independent variables of CCD experiment design.

| Code | Factor            | Variable Code |
|------|-------------------|---------------|
|      | Time (minute)     | -α 0 -1 +1   |
| X₁   |                   | 23.78 30 45 60 66.21 |
|      | Amplitude (decibel)| 47.92 50 55 60 62.07 |

Each factor range was stipulated based on the preliminary research findings which the yield and antioxidant activity (IC₅₀) as the dependent variable. The completed design comprised 13 experimental points, including 5 center points, 4 factorial points, and 4 axial points. The CCD data were analyzed using multiple regressions to adjust it with the second-order polynomial model, as stated in the following equation:

\[
Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1, j=2}^{k-1, k} \beta_{ij} X_i X_j + \epsilon
\]  

(1)

\( \beta_0; \beta_i; \beta_{ii}, \) and where the terms for regression coefficients in the intercept, linear, quadratic, and interaction; \( X_i; X_{ii}; X_{ij} \) were the independent variables; \( Y \) was the response and was the random error component.

Design Expert was previously used for obtaining the quadratic model of the polynomial coefficient. This model was more effective since easy to define \( R^2 \) value, Adjusted \( R^2 \) value, and adjusted with the significant statistic result with the F test.

Table 2. \( H. \ atra \) ultrasonic extraction with Central Composite Design (CCD).

| Run | Time (minute) | Amplitude (decibel) | Time (minute) | Amplitude (decibel) | Yield (%) | IC₅₀ (ppm) |
|-----|---------------|---------------------|---------------|---------------------|-----------|------------|
| 1   | -1            | -1                  | 30            | 50                  | 4.92      | 96         |
| 2   | 1             | -1                  | 60            | 50                  | 8.8       | 125        |
| 3   | -1            | 1                   | 30            | 60                  | 5.2       | 108        |
| 4   | 1             | 1                   | 60            | 60                  | 9.01      | 134        |
| 5   | -1.41421      | 0                   | 23.7868       | 55                  | 4.5       | 88         |
| 6   | 1.414214      | 0                   | 66.2132       | 55                  | 10.4      | 129        |
| 7   | 0             | -1.41421            | 45            | 47.92893            | 6.06      | 118        |
| 8   | 0             | 1.414214            | 45            | 62.07107            | 8.23      | 129        |
| 9   | 0             | 0                   | 45            | 55                  | 10.82     | 85         |
| 10  | 0             | 0                   | 45            | 55                  | 10.53     | 83         |
| 11  | 0             | 0                   | 45            | 55                  | 10.91     | 84         |
| 12  | 0             | 0                   | 45            | 55                  | 10.25     | 85         |
| 13  | 0             | 0                   | 45            | 55                  | 10.9      | 83         |
| Prediction | 45.52 | 54.92 | 10.7417 | 84.4356 |
| Verification | 45.52 | 54.92 | 10.43 | 84.85 |

Yield

The percentage of extraction yield was generated to the following formula:

\[
\% \text{ of yield} = \frac{\text{extraction yield}}{\text{mass of material}}
\]

(2)
Antioxidant Activity (IC\textsubscript{50})
Analysis of antioxidant activity (IC\textsubscript{50}) was performed using 1,1-diphenyl-2-pyrihidrazil (DPPH) (concentration: 0.1 Mm or 39.4 mg DPHH in 1 L of ethanol). Approximately 100 µl of the sample was mixed with 3.9 mL DPPH. Incubation was performed in a dark with room temperature for 2 hours. The absorption was measured (λ = 517 nm) using UV-Vis spectrophotometer. Solvent control was prepared using 100 µl ethanol and 3.9 ml DPPH. The activity of free radical scavenger was counted with this formula:

\[
\% \text{ of DPPH inhibition} = \frac{A^\circ - A'}{A^\circ} \times 100
\]  

(3)

Note: \(A^\circ\) = ‘Control’ Absorption; \(A'\) = Sample Absorption.

DPPH capability to prevent radicals was indicated by absorption reduction in the IC\textsubscript{50} value measurement from the measurement in the inhibition percentage (%) as much as 50% [22, 23].

2.2.3. Characterization of H. atra extract.
The extract resulted from maceration and ultrasonic methods will be compared for secondary metabolite content (alkaloid, steroid, triterpenoid, and saponin), the yield, antioxidant activity, toxicity, and antibacterial. The chemical group content was done using Harbone method, (Brine Shrimp Lethality Test) using Mayer [24] method and antibacterial against Bacillus subtilis, Escherichia coli, Vibrio eltor and Staphylococcus aureus using agar diffusion method. The analysis was done in Oceanography LIPI laboratory, while the Chondroitin sulfate analysis was performed in PT. Saraswati Indo Genetech.

3. Results and Discussion
The value obtained from each response variable observed from each experiment is shown in Table 2. Dependent variable affected by independent factor was analyzed statistically, including fix summary model, analysis of variance, normal plots of residual, and graphic model of response.

3.1. Effect of extraction time and amplitude to the yield
Base to the yield response, which informed at Table 2, the highest yield of sea cucumber extract could be achieved with an extraction time of 45 minutes and amplitude of 55 decibels, and the yield was approximately 10.25-10.91%. The lowest yield of H. atra extracts achieved with extraction time 23 minutes and amplitude reach 55 decibels. These conditions were gained the extraction yield about 4.5%. The data were used in the statistical analysis through Design Expert for stipulating analysis model for various kinds of H. atra extraction yield responses.

The Lack of Fit Test generates model compatibility if the p-value is > 0.05 [25, 26]. If the R\textsuperscript{1} is getting closer to 1, it indicates compatibility between observation value and prediction value [27, 28]. Table 3 showed that the model can be counted on the experimental value, it can be used as the theoretical base for the yield response in the H. atra ultrasonic extraction process.

Table 3 indicated that there was a significant difference in yield response generates during H. atra ultrasonic extraction. The distinction between the percentages of the yield was a response to various treatments, including extraction time and amplitude. Based on the partial test for every variable, it can be seen that the actual distinction was in the extraction time (X\textsubscript{1}), amplitude of ultrasonic extraction (X\textsubscript{2}) and the quadrat of the two factors (X\textsubscript{1}\textsuperscript{2} and X\textsubscript{2}\textsuperscript{2}), meanwhile, the interaction between both factors (X\textsubscript{1}X\textsubscript{2}) was not actually different because the p-value was > α (0.05).
Table 3. Regression coefficients and significance of the predicted second-order polynomial models.

| Coefficient | Yield (%) | Antioxidant (IC$_{50}$) |
|-------------|-----------|------------------------|
| Intercept $\beta_0$ | 10.68** | 84.00** |
| Linear $\beta_1$ | 2.00** | 14.12** |
| $\beta_2$ | 0.44* | 4.57** |
| Interaction $\beta_1 \beta_2$ | -0.018 | -0.75 |
| Quadratic $\beta_1^2$ | -1.69** | 12.19** |
| $\beta_2^2$ | -1.85** | 19.69** |
| Mean | 8.50 | 103.61 |
| Standard deviation | 0.44 | 1.13 |
| $R^2$ | 0.98 | 0.99 |
| Adjusted $R^2$ | 0.96 | 0.99 |
| F-value (model) | $< 0.0001$ | $< 0.0001$ |
| F-value (lack of fit) | 0.0932 | 0.3135 |

*p < 0.05; **p < 0.01

The length of time in the ultrasonic extraction process affected the yield. When the extraction time gradually increased, the yield continuously increased. When the time was passed to the optimum limit, the yield was reducing. The longer the extraction time, abundance yield will be reached. It can be explain because the chance of the solvent to contact with the material is higher due to the cavitation process [29, 30]. The extracted yield will optimum when the material is highly blended in the solvent until the solvent was completely saturated. Yield will be reduced when the extraction time is extended due to the degradation extraction compound [28]. Garcia [29] informed that the time needed by ultrasonic extraction process is shorter than the non-ultrasonic extraction process. The study performed by Zhao [28], regarding polysaccharides extraction, achieves the best time in the minute of 25th when the yield was abundantly generated.

The amplitude being observed by the researcher positively affected the extract yield. The yield was increased along with the enhancement of extraction time and amplitude. In the early extraction time with the amplitude of 50 decibels, the yield was continuously increased and the reduced when reaching a certain point of amplitude. Vinatoru [31] reported that the higher of amplitude, the higher chance for the cavitation bubbles (which are located in the membrane of the sample) to burst. This will generate a bigger micro facture. There is a relation between amplitude and energy (joule). The energy was directly proportional to the amplitude squared, it can be stated that the higher the amplitude, the higher the energy consumed [32]. The high amplitude which goes beyond the medium will generate pressure and shear force by the solvent molecule. This condition will generate local change in the density and modulus elasticity and increasing the mass movement interphase. It would be increasing the yield in less time [33].

3.2. Effect of extraction time and amplitude on the antioxidant activity

Antioxidant activity shown in the Table 2 implied that the highest antioxidant activity could be achieved when the extraction time was 45 minutes and the amplitude was 55 decibels, with the IC$_{50}$ of 83 ppm, and the lowest activity could be achieved when the extraction time was 60 minutes and the amplitude was 62.07 decibel, with the IC$_{50}$ of 134 ppm. The data were used in the statistical analysis through Design Expert for stipulating analysis model for various kinds of $H. atra$ extraction yield response.
Analysis of antioxidant (IC_{50}), based on the F (p-value) and the lack of fit in the Table 3 described that ultrasonic extraction time and amplitude in the quadratic model significantly affected the antioxidant activity (IC_{50}). It was indicated by the F value of quadratic model < 5% (p < 0.05) as much as < 0.0001 which means the error chance was < 0.01%. F value of the lack of fit was in the amount of 0.3135 which indicates p > 5% (p > 0.05), so the model incompatibility is not significant. It can be concluded that the quadratic model was compatible. P-value and the lack of fit which was over to the limiting threshold indicated that the model was significantly implicated [25].

The antioxidant (IC_{50}) generated by the two methods was significantly different. This distinction happened as the response of treatment variation, including time and amplitude. It can be seen that the actual difference was in the extraction time (X_1), amplitude of ultrasonic extraction (X_2) and the quartet of the two factors (X_1^2 and X_2^2), meanwhile, the interaction between both factors (X_1X_2) was not significantly different because the p-value was > α (0.05).

Time length of the ultrasonic extraction process significantly affected antioxidant activity (IC_{50}). In the early time of the process, the antioxidant was continuously increased because of the bioactive compounds. However, the time extension will reduce antioxidant activity. The ultrasonic method is suitable for to be applied to the extraction of a bioactive compound which cannot stand high heat, like the process of pomegranate extraction with the most optimal extraction time of 53 minutes [34]. Jiang [36] also stated that the early times of ultrasonic extraction generate a positive effect, but, if extended, then the quality of the composition will be reduced.

Ultrasonic extraction amplitude (X_2) was significantly affected the IC_{50}, similar to square factors (X_1^2 and X_2^2) which significantly affect IC_{50} of H. atra extract. It was probably due to the cavitation phenomenon. Liu [15] stated that ultrasonic cavitation may break the cell membrane mechanically and increasing material transfer. This cavitation phenomenon brings physical effect and chemical effect on the extraction process if extraction time and amplitude increased. Jiang [35] stated that the higher of ultrasonic power or the higher length time of extraction process will increase the number of hydroxyl radicals, which could damage the chemical compound due to the oxidation brought by the ultrasonic wave. Balachandran [36] observed that antioxidant such as cyanidin-3-glucoside may be decreased to 20% in the 4 hours sonication. On the other hand, this radical can be used as food material function enhancer. The study performed by Balachandran [36] indicated that the sonication process on the phenol compound changes hydroxylation into ortho-, meta- and para-. So, it can be said that the establishment of OH•, due to the sonication process, may enhance the antioxidant in an herbal compound such as flavonoid.

3.3. Optimization of time and amplitude

Based on the quadratic equation and the two or three-dimension contour graphic model (Figure 1, 2 and 3) were reflecting the relation between both independent variables. Figure 1 represents time and amplitude to yield response. Contour plot as shown in Figure 1, showed that extraction time affects the yield. Different color in contour plot graphic showed the yield value. Yellow to red color showed constantly increased yield, whereas blue color showed low yield. This contour described that the yield was increased when the time increases from 30-52.50 minutes, and decreased when the extraction time above 52.50 minutes. The difference in surface-level indicated different yield response value in each combination of formula components. The low area showed low response yield value, while a high area showed high yield response value. In the three-dimension graphic, it can be seen that response yield to the sonication time and amplitude started to stabilize in the range of 10.00% yield number up to 11%.

Figure 2 represents time and amplitude effect on antioxidant activity response (IC_{50}). It showed the extraction time which affects the antioxidant activity (IC_{50}). Different color in contour plot graphic showed different antioxidant activity. Green to blue color of high antioxidant activity (decreased IC_{50} number), otherwise, the red color showed low antioxidant activity (high IC_{50} value). Based on the graphic 3 dimensions, high antioxidant activity response starting from 30 minutes to 52.50 minutes and amplitude of 57.50 decibels.
Figure 3. represents extraction optimization result with time and amplitude treatment to the yield response and antioxidant. The optimum point in the extraction process (Table 2) wanted is in the form of goal, which is % yield maximally chosen and minimum antioxidant IC\textsubscript{50}. The chosen formula solution is the formula with the period combination extraction of 45.52 minutes and 54.92-decibel amplitude. This formula was predicted to have 10.74% yield and 84.44% antioxidant. Desirability value of 0.972 means that the formula will produce a product having the characteristics corresponding to the optimization target of 97.2%. Desirability value reaching 1 showed good optimization function, in which the program can fulfill the purpose based on the criteria desired in the end product [25, 37]. The blue color in contour showed low desirability value while red color showed high desirability value. This contour showed the colors emerged are blue to yellow with maximum desirability value was 0.972.

Verification stage of optimization result was aimed to ensure that the optimum point solution obtained and Design Expert software can produce response value following the optimum response condition solution given by the software. Verification will be conducted by implementing the optimum solution in \textit{H. atra} extraction process with ultrasonic during 45.52 minutes at 54.92 decibels, with three times repetition, and the resulted yield and antioxidant activity (IC\textsubscript{50}) observation were performed. Verification and software prediction result will be analyzed with paired T-test using Minitab 17. P-value. The paired T-test above 5% showed that the prediction value and verification were not significant, it's mean that the resolution or prediction supports verification. Verification result of \textit{H. atra} extraction with the ultrasonic method can be seen in Table 2.

The optimum extraction condition predicted by the program was 45.52 minutes at 54.92 decibels. The optimum response obtained by this prediction reached the yield 10.74717% and antioxidant activity (IC\textsubscript{50}) value was 84.4356 ppm. The verification result performed in the laboratory was 10.43% of yield and 83.85 ppm of antioxidant (IC\textsubscript{50}). Verification result with Minitab 17, showed that T-test was not significantly different with the prediction resulted from Design Expert software. P-value resulted for yield was 0.30 and for antioxidant (IC\textsubscript{50}) was 0.25. P-value > 0.05 can be interpreted that there is no significant difference between software prediction and the actual condition. It can be concluded that the model is accurate form extraction optimization with maximum yield and high antioxidant activity with low IC\textsubscript{50} value. Wangtueai [38] said that if the differentiation between prediction value and research value is not more than 5% indicated that the model is correct.

Figure 1. Graphic 3-dimensions of relation between sonication time and amplitude with yield response.

Figure 2. Graphic 3-dimensions of relation between sonication time and amplitude with antioxidant activity (IC\textsubscript{50}).

Figure 3. Graphic 3-dimension of relation between sonication time and amplitude with desirability value.
3.4. Characterization of H. atra Extract of Maceration and Ultrasonic Method

The filtrate obtained from extraction process with maceration or ultrasonic after filtering with Whatman no. 4, showed the difference color, filtrate result of ultrasonic method extraction is more dense, indicating that the antioxidant content is higher.

| Phytochemical screening | The positive result according to literature | Result |
|-------------------------|--------------------------------------------|--------|
| Alkaloid                | Orange-red sediment is formed (Dragendorff reactor). White sediment is formed (Mayer reactor) brownish-red sediment is formed (Wagner reactor) | Maceration: - | Ultrasonic: + |
| Steroid                | The greenish-blue color is formed | Maceration: - | Ultrasonic: - |
| Triterpenoid            | Brownish or violet color is formed | Maceration: + | Ultrasonic: + |
| Saponin                 | Stable foam is formed | Maceration: + | Ultrasonic: + |

Preliminary identification of secondary metabolite group with phytochemical analysis is an initial important step in research to know the chemical structural framework [39]. This analysis includes alkaloid, triterpenoid, steroid, and saponin test. Secondary metabolite compound analysis result of sea cucumber extract extracted with maceration and ultrasonic methods can be seen in Table 4. Secondary metabolite content of sea cucumber extract with 96% ethanol solvent extracted with maceration and ultrasonic methods showed that on both methods there was no indication of steroid, but triterpenoid and saponin content were identified. While alkaloid in the extract using the ultrasonic method can be identified, and not detected in the maceration method. This result is in line with the research conducted by Putram [40] H. atra sea cucumber ethanol extract is positive contains triterpenoid, flavonoid, alkaloid, and saponin. Whereas there was a difference in the research result conducted by Rasyid [14], that Holothuria sp. extract didn’t contain triterpenoid and alkaloid, but steroid was identified. It was possible because the difference in sea cucumber variety, also difference in solvent extraction will produce the difference in secondary metabolite.

3.5. The measurement result of yield (%)

The yield obtained from different extraction process can produce different yield. It can be seen in Figure 4[a]. The Yield generated from ultrasonic extraction method was 10.43% while from maceration was 5.48%. This data was similar to the T-test, maceration and ultrasonic methods that generated significantly different yield. By using ultrasonic would gain higher yield than maceration method. The benefit when the applied ultrasonic method in the extraction process was the higher energy transfer. It will increase the ability and reduce the time of response production level on the extraction process.[41].

3.6. The result of the antioxidant test

Antioxidant activity of holothurian extract using of maceration and ultrasonic method can be seen in Figure 4[b]. The result of the antioxidant activity test from the maceration method has IC50: 149.38 ppm, it is categorized into medium activity. Extraction using Ultrasonic method I generated higher antioxidant activity IC50:84.45 ppm and categorized into strong activity. Based on T-test of maceration and ultrasonic method have different antioxidant activity value and better that maceration, indicated by 0.003 p-values (PV < 0.05). It can be said that the ultrasonic extraction method can increase antioxidant activity compared to the maceration method.

The antioxidant content in H. atra extracts observed by Esmat [6] with analysis using high-performance liquid chromatography (HPLC), displaying phenolic components, such as chlorogenic, routine, coumaric acid, catechin and ascorbate acid. HPLC analysis is supported by GC-MS result.
showing the existence of 59 compounds belong to the flavonoid, phenolic, triterpenoid, saponin, and alkaloid category [7]. Murniasih research [42] exposed that *H. atra* extract separated with column chromatography on silica gel and elution with gradient system increases polarity. The active fraction of *H. atra* extract showed significantly restricted radical scavenging activity (35.3487%) in 1 mg/ml concentration. The result of Gas Chromatography-Mass Spectrophotometer analysis of *H. atra* produces 7 compounds, which is in fraction 2. Besides, fraction active 7 showed some compounds, which are cycloicosane (17), pentadecyl trichloroacetate (18), 14β-pregnane (21) and cis-4-siano-2-(2-hydroxycyclohexyl) pyridin.

### 3.7. Result of antibacterial activity and toxicity test

The comparison of the antibacterial activity of *H. atra* extract between maceration and ultrasonic method can be seen in Table 5. The data above shows that the sea cucumber capsule has antibacterial activity. It indicates that *H. atra* extract can inhibit the growth of *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, and *Vibrio El Tor*. Based on T-test, between maceration and ultrasonic methods didn’t prove that there was a significant antibacterial activity, indicated with p-value > 0.05. Sea cucumber is known contained some bioactive antibacterial [43]. Abraham [8] reported the antimicrobial effect of *H. scabra* extract, *H. scabra* extract was reported active against *Aeromonas hidrofila*, *Escherichia coli*, *Enterococcus sp.*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus*, *Vibrio harveyi*, and fungi coming from fish, *Aspergillus*.

The toxicity test result was displayed in Figure 4[c]. there is no significant difference between maceration and ultrasonic method. The IC50 value of extract by using the maceration method was 457.7 ppm, while ultrasonic was 443.6 ppm. Concern to this result both of them categorized moderately toxic. IC50 value will be more effective in preventing cancer cell growth, Dhinakaran [7] research stating that *H. atra* extract shows strong prevention activity to tumor growth in human’s cervix cancer cell (HeLa) also as human’s breast cancer cell (MCF-7). In Janakiram et al. [11] research, sea cucumber (*Curcumaria frondosa*) extract was stated has potential as anti-cancer for colon cancer patient since it contains monosulfated triterpenoid glycoside Frondoside A, the disulfated glycoside Frondoside B, the trisulfated glycoside Frondoside C, 12-methyltetradecanoic acid, eicosapentaenoic, and fucosylated chondroitin sulfate.

### Table 5. Antibacterial activity assays of *H. atra* extract with maceration & ultrasonic method.

| Sample                  | *B. subtilis* (cm) | *E. coli* (cm) | *S. aureus* (cm) | *V. El Tor*     |
|-------------------------|--------------------|----------------|------------------|-----------------|
| Ampicillin (10 µg = 0.01 mg) | 3.941± 0.17<sup>a</sup> | 2.303± 0.04<sup>a</sup> | 2.5925± 0.05<sup>a</sup> | 3.122± 0.12<sup>a</sup> |
| *H. atra* maceration     | 1.39± 0.08<sup>b</sup>  | 1.241± 0.08<sup>b</sup>  | 1.211± 0.14<sup>b</sup>  | 1.1123 ± 0.06<sup>b</sup> |
| *H. atra* ultrasonic     | 1.48± 0.14<sup>b</sup>  | 1.247± 0.08<sup>b</sup>  | 1.383± 0.14<sup>b</sup>  | 1.2823 ± 0.025<sup>b</sup> |

<sup>a</sup>Description: The value is in average ±deviation from 3 times repetition, Sample: 10 mg/ml
4. Conclusion
Response Surface Method (RSM) could be used to optimize H. atra extraction using ultrasonic method. Two important parameter such as yield and antioxidant activity were affected by the extraction time and amplitude. The predicted and validated values are not significantly different on 95% trust level. The model in this research can be used to optimize the yield and antioxidant activity by time and amplitude variety. The optimum extraction process can be performed within 45.52 minutes of extraction time and 54.92-decibel amplitude. Extraction method using ultrasonic compared to maceration method showed differences in phytochemical contain and yield. The antioxidant activity were known to be higher in the ultrasonic method than maceration. While, it was not significantly different in bioactivity & toxicity (LC50).

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