Increasing anthraquinone compounds on callus leaf *Morinda citrifolia* (L.) by elicitation method using chitosan shell of shrimps (*Penaeus monodon*)

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**Abstract.** Elicitation is one method to increase the content of secondary metabolite compounds in plant tissues. Anthraquinone is one of the secondary metabolite compounds that have potential of being a medicinal agent and produced by plants. The purpose of this research is to increase anthraquinone content in *Morinda citrifolia* (L.) leaf callus using elicitation method with chitosan from shrimp shell extract (*Penaeus monodon*) as elicitors. *M.citrifolia* (L.) callus was obtained from young leaf explants grown on Murashige and Skoog (MS) medium with 2.4- D addition of 1.75 mg / L and Kinetin 1.5 mg / L aseptically. The second subculture of callus is elicited by elicitor of chitosan each of 0.5 ml. The concentration of elicitor used was 0, 0.5, 1.5, 2 and 2.5 mg / ml. Harvesting of elicitation result done on day 0, 2, 4 and day 8 after elicitation. The results showed that callus containing anthraquinone could be induced in MS medium with addition of 2,4-D 1.75 and Kinetin 1.5 mg / L and addition of chitosan at some concentrations may increase anthraquinone. The highest anthraquinone content was produced at concentration 2 mg / ml and 4 days harvest time.

1. **Introduction**

Natural plant products have become an important part of medicine throughout human history [1]. The main source of large amounts of bioactive compounds commonly used as medicinal ingredients comes from plants [2]. Plant bioactive is usually a secondary metabolite product and plays an important role in world health [3]. Secondary metabolites have biological and pharmacological properties that are useful in treatment [4].

One of the medicinal plants that are widely used as medicinal plants is *Morinda citrifolia* (L.) or often known as noni. Noni identified as having more than 70 beneficial bioactive compounds and reported to have various therapeutic effects, including antibacterial, antiviral, anti-fungal, anti-tumor, anti-HIV, anti-helmin, analgesic, anti-inflammatory, and immune enhancing effects [5]. From several studies that have been done, it can be seen that almost all parts of noni plants contain chemicals and nutrients that can be useful for health [6].

Bioactive compounds in noni plants are spread in various organs such as roots, leaves and fruit [5]. There are some important including anthraquinone (quinone class), scopoletin (alkaloids), ascorbic acid (vitamins), β-carotene, arginine, proseronine and proseroninase which are precursors of seronine (class alkaloids) [7]. Although the content of bioactive compounds is very diverse, there are compounds that are most commonly found in the genus Morinda, is Anthraquinon [8]. Anthraquinone is secondary
metabolite compound that can be used as an anti-cancer, anti-tumor, and able to overcome inflammation and others.

Anthraquinone is also important group of secondary metabolites in bacteria, fungi, mosses and plants [9,10]. Anthraquinone is widely used in industry and medicine for various purposes. Anthraquinone component is a molecule that clinically important, especially rubiadin, damnacanthal, alizarin and purpurin, which are used in the formula for chemotherapy drugs [11].

Secondary metabolites can be obtained by extracting directly from adult plant organs [5]. However, besides the expensive extraction and isolation process, this method requires large-scale plant cultivation. In addition, the use of plants in the production of desired compounds can continuously affect the availability of these plant species. Meanwhile, synthetic metabolites are also expensive because active structures are very complex [12]. Therefore, it is necessary to develop alternative methods in the extraction of plants for the production of bioactive compounds [13]. Therefore, it is necessary to develop alternative methods in the extraction of plants for the production of bioactive compounds [13].

Tissue culture techniques are one method that can be used in producing secondary metabolites. Tissue culture has several advantages: it can continuous and reliable production of natural compounds [14], not dependent on environmental factors, the production system can be regulated, does not require a lot of land and the purification process is easier because the culture cells does not contain pigment [15].

*M. citrifolia* (L.) secondary metabolite compounds can be propagated in vitro, by callus culture and cell suspension culture. This is in accordance with [16] that callus production can be used directly to regenerate plantlets, extract or to manipulate some primary metabolites and secondary metabolites. [16] callus culture and plant cell culture in vitro are potential sources for secondary metabolite production [17]. Callus are amorphous (non-shaped or undifferentiated) cells formed from cells that divide continuously in vitro or in a tube [18]. Callus culture can produce secondary metabolites that are more numerous than those produced by the parent plant. But unfortunately the secondary metabolite content in some callus cultures and cell cultures is still relatively low, so a method is needed in tissue culture that can increase secondary metabolite content.

One technique in tissue culture that can be used to increase secondary metabolites is elicitation method. Elicitation is a method for simultaneously inducing phytoalexin formation, constitutive secondary metabolites or other secondary metabolites that normally do not accumulate [5]. Elicitation can be done by adding an elicitor. Elicitor is a biological and non-biological compound that can cause an increase in phytoalexin production when added to plants or plant cell cultures [5]. One of the elicitors that can be used is chitin found in shrimp shell.

Chitin is the second largest natural polymer (biopolymer) found in nature after cellulose. Chitin compounds are found in the cell walls of low-level plants such as mushrooms and are also found on the outer skin of low-level animals such as insects, shrimp and crabs. The highest chitin content is found in crabs and shrimp. Chitosan is a derivative of chitin. Chitosan can be produced from chitin by deacetylation. Chitosan has been found to induce a variety of plant defense responses [19]. However, the effect of chitosan on the induction of secondary metabolites has not been widely studied [20]. Chitosan from shrimp shells can be used as an elicitor which can increase anthraquinone bioactive compounds in callus culture of *Morinda citrifolia* (L.) leaves in MS (Murashige and Skoog) medium. Elicitation process occurs due to inhibition of cell viability and disruption of ion transport. This is a response to stress [21]. As a result there was an increase in secondary metabolite content.

The use of chitin or chitosan as an elicitor can also be an effort to utilize shrimp shell waste. Because if this waste is not managed properly, it can affect the environmental balance, especially the issue of odors and poor environmental aesthetics [22].

Based on the background described above, a research was conducted to improve the anthraquinone secondary metabolites found in noni leaf callus with the help of chitosan elicitor from shrimp shell.
2. Method
The callus was used from the second and third leaves of *Morinda citrifolia* (L.) planted in Murashige & Skoog (MS) medium with addition of 2,4-D 1.75 mg / L and Kinetin 1.5 mg / L [23]. Callus that has been sub-cultured on the same medium are then used as materials for elicitation and measurement of the results of the analysis of secondary metabolites. Callus which is used which has a weak and brown callus characteristic indicates a secondary metabolite has been formed.

Before elicitation is carried out, determination of the growth curve is carried out, until the best elicitation time is found, which is at the exponential end phase. From the results of the measurement of the growth curve obtained the best time is on the 20th day.

Provision of elicitor material in the form of chitosan, is briefly described as follows: wet shrimp shells → washed and dried iling milled and sifted until they pass the sieve (-35 + 48 mesh) or average diameter of 0.356 mm protein protein refining (deproteinization) → washed with water → demineralization → washed with water warna color removal → washed with water and dried (chitin formed) ilangan removal of acetyl group (deacetylation) → washed with water and dried osan chitosan biopolymer formed → FTIR test to determine the degree of deacetylation per cent (%) purity of chitosan [24].

The chitosan produced is then dissolved by: Stock solution made with 10 mg / ml concentration of chitosan elicitor dissolved in acetic acid solvent and stirred until homogeneous at 40°C, then concentration concentration is made according to the research design by using the retail formula $V1M1 = V2M2$ according to the amount needed, then sterilized at 120°C for 20 minutes and checked pH 5.7-5.8. In this study, the concentration of chitosan used was 0 mg / ml, 0.5 mg / ml, 1 mg / ml, 1.5 mg / ml, 2 mg / ml and 2.5 mg / ml (modification [21]).

Elicitation is done by adding 0.5 ml of elicitor on callus with a concentration of 0 mg / ml, 0.5 mg / ml, 1 mg / ml, 1.5 mg / ml, 2 mg / ml and 2.5 mg / ml. In the control callus added sterile aquadest. Elicitation harvesting is carried out on 0, 2, 4 and 8 days [21]. Then the elicitation callus was analyzed for its anthraquinone content by means of: Callus dried in an oven at 50°C until the weight was constant, then crushed using mortar and pestle until smooth (modification [21]). Dry callus is then weighed and separated 0.02-0.2g FW added with 5ml 80% ethanol solvent p.a then evaporated for 10 minutes at 60oC. After that it was centrifuged at 1500 g / 4700 rpm for 5 minutes. The supernatant is separated by a pellet, then put together and made to a volume of 10 ml using the same procedure. The above procedure is repeated until the extractant reaches a volume of 10 ml (modification [25]). The absorbance value and anthraquinone content were measured by spectrophotometer at a wavelength of 434 nm using alizarin as a standard [26].

3. Result and Discussion
Callus was successfully grown in Murashige and Skoog (MS) media with the addition of ZPT 2.4 D 1.75 mg / L and Kinetin 1.5 mg / L, as shown in Figure 3.1. Many studies have been carried out, as reported [27] that with the addition of 2,4-D growth regulators can stimulate callus growth and cell enlargement in explants so as to trigger callus formation. This is also reinforced by [28] that auxin can induce callus formation which begins with the process of dedifferentiation and cytokines can induce callus by cell division and cell differentiation.
Figure 1. The initial morphology of callus formation (a)
White explants swell and also appear white spots from leaf
explants (b) Callus almost covers all leaf explants

Callus is formed, then subculture on the same medium and after 2 weeks of callus when is ready to be elicitate. Along with preparing callus for elicitation, also prepared the elicitor form of chitosan. Callus before and after elicitation can be seen in Figure 3.2.

Based on the callus growth curve it is known that the logarithmic phase occurs on the 20th day and the stationary phase is reached on the 24th day. Therefore, elicitation treatment is carried out on 20th days. The effect of chitosan elicitor on the growth of M.citrifolia (L.) can be known through morphological observations based on the texture and color of callus before elicitate and after it has been elicitate. The results showed that until the end of the treatment, the callus texture remained compact. On the top surface of the explant, there are watery tissue conditions. This condition is caused by contacting with the elicitor its acts as an absorption area of nutrients for explants.

The callus color of M. citrifolia (L.) without elicitor was not changed, while the callus that was quenched experienced a slight change. Callus undergoes browning along with increased elicitor concentration and elicitation. According [29], elicitated callus was darker (brown / yellowish) (Figure 3.2). This is thought to be a hypersensitive response shown by the tissue due to stress. According [30], hypersensitivity response is a good mechanism to limit the spread of pathogens. This hypersensitive response is a mechanism to kill cells infected with pathogens (in this case the elicitor) so as not to spread to other cells.

Explants that tend to be brownish may be caused by explant conditions that internally have high phenol content so that the influence of light will cause phenol oxidation to phenolic quinone [31]. Antrakuinone is one of the secondary metabolic products produced by explants and belongs to the phenolic quinone class which is derived from phenol derivatives in biosynthesis. Therefore, the explant selection process that will be cultured on the treatment media is usually based on the color of the explant to be cultured.

After experiencing elicitation, the callus is then harvested according to the harvesting time determined, then the anthraquinone content is analysed. To measure the concentration of anthraquinone
According to [26] the standard of alizarin was used, this was because alizarin was one of the groups of anthraquinone. Alizarin is an anthraquinone derivative with the same molecular formula. In this study used yellow GG Alizarin (Merck). Alizarin standard curve is obtained by including the absorbance value of alizarin solution in various concentration ranges from 1 ppm to 10 ppm. Alizarin is dissolved in 80% ethanol. Then each concentration measured in absorbance using a spectrophotometer (Genesys 10v) at a wavelength of 434 nm. The results of the measurement of anthraquinone content in elicitation callus can be seen in Table 3.1. and Figure 3.3.

Based on these tables and figures, it can be seen that there is an effect of elicitor addition on anthraquinone content and even this effect has occurred on day 0. This is possible because the elicitor initiates activity in plant cells through receptor interactions on the plant cell plasma membrane, and this means that receptor interactions have occurred on the plasma membrane after several hours of elicitation.

Table 1. The anthraquinone content of callus leaves of *M. citrifolia* (L.) after elicitation with chitosan from shrimp shells

| Concentration of elicitor (mg/ml) | Average anthraquinone concentration (mg / g FW callus) on harvesting day |
|----------------------------------|----------------------------------------------------------------------------------|
|                                  | 0       | 2       | 4       | 8       |
| 0                                | 3.491 ± 0.12a | 3.790 ± 0.34a | 3.716 ± 0.20a | 5.681 ± 0.90a |
| 0.5                              | 6.353 ± 0.32b | 7.158 ± 0.38b | 8.204 ± 0.15b | 8.330 ± 0.11c |
| 1                                | 7.158 ± 0.55c | 7.945 ± 0.36c | 8.371 ± 0.09b | 8.336 ± 0.18c |
| 1.5                              | 7.216 ± 0.69c | 8.147 ± 0.13cd | 9.796 ± 0.95c | 10.095 ± 0.61d |
| 2                                | 7.330 ± 0.19c | 8.239 ± 0.09cd | 11.302 ± 0.07e | 10.917 ± 0.59d |
| 2.5                              | 7.417 ± 0.21c | 8.339 ± 0.20d | 8.032 ± 0.51b | 7.543 ± 0.42b |

The harvesting time of the 2nd, 4th and 8th days shows that the elicitor given can be responded to by an increase in the anthraquinone content which is higher than the 0th day. However, at a concentration of 2.5 mg / ml, both for the harvest time for the 4th day and the 8th day of the anthraquinone content began to decrease compared to the concentration of the elicitor below. The occurrence of a decrease in anthraquinone levels at a concentration of 2.5 mg / ml, probably due to the concentration of all receptors that recognize the elicitor component has been saturated with the elicitor so that the addition of the elicitor given can no longer affect the increase in anthraquinone content. Anthraquinone content at day 4 was higher than day 8, especially at a concentration of 2 mg/ml. From this data it appears that harvesting time affects anthraquinone content. According to Sitinjak et al., (2000) the length of elicitation affects the anthraquinone content in the presence of post binding effects [29]. The post binding effect is an effect that occurs after the effector is received by the receptor. Post binding effects can be observed in the extracellular signal delivery mechanism.
According [32]), regulation of secondary metabolite biosynthesis pathways is carried out by specific transcription factors. This transcription factor is a DNA-binding protein sequence that regulates the initiation of mRNA and is related to the promoter region of the target gene. These proteins regulate gene transcription based on tissue type and / or in response to internal signals (plant hormones), and external signals (biotic elicitor or UV light).

Chitosan elicitor can affect gene activity. Gene activity begins with transcription of DNA into mRNA, followed by translation of mRNA. Next, the mRNA leaves the nucleus leading to the cytosol. In the cytosol, mRNA is translated in the ribosome. This mRNA translation causes the formation of new enzymes and activation of certain enzymes that lead to the process of growth and development and the synthesis of secondary metabolites directly through the regulation of enzymes [33].

In this study it was suspected that the added chitosan injected into the callus joined with a number of recipient proteins then the complex of growth regulating substances - the recipient protein moves into the nucleus affecting gene activity in the mRNA translation process which causes new enzymes and activates certain enzymes that lead to the process of forming and developing secondary metabolites through the regulation of enzymes. Elicitors play a role in the binding of protein membranes that have the potential for enzyme activity. According [34], elicitors are bound to the membrane of the recipient protein in the cell plasma membrane.

4. Conclusion

Based on the results of the study it can be concluded that the administration of chitosan elicitor with concentration of 0; 0.5; 1; 1.5; ; 2 and 2.5 mg/ml with elicitation time or harvesting 0, 2, 4 and 8 days significantly affected the anthraquinone content of callus from *Morinda citrifolia* (L.) leaves. The results above show that elicitor concentration and harvesting time affect the anthraquinone content in callus *Morinda citrifolia* (L.) culture. The highest anthraquinone content is obtained by the addition of 2 mg / ml elicitor and 4 days elicitation time, ie 11,302 mg / g callus or increased by 323.75% or 3 times the control.

5. References

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