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RESEARCH ARTICLE

# MITIGATION OF *ALEXANDRIUM TAMIYAVANICHII* USING ACTIVE FRACTIONS FROM ETHANOL EXTRACT OF ORNAMENTAL PLANT, *SANSEVIERIA TRIFASCIATA*

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**ABSTRACT**

Blooms of toxic *Alexandrium tamiyavnichii* have been recorded in several parts of the world including Malaysia. This Harmful algal bloom (HAB) has led to human illness and loss to fishery industries. In order to control the bloom and minimize the effects, the growth of the species needs to be inhibited using a mitigation agent, preferably environment friendly agent. In this study, an ornamental plant, *Sansevieria trifasciata* will be used to inhibit the growth of *A. tamiyavanichii*. The plants were fractionated to obtain fractions (dichloromethane (DCM) and methanol) from ethanol fresh and dried plants extracts. Eight concentrations (0.001, 0.01, 0.1, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/mL) of these fractions were tested on the algae for 24 hours and removal efficiencies (RE) were determined. Toxicity test was conducted on Artemia using 10, 50, 100 and 500 mg/mL concentrations of active fractions for 24 hours. Phytochemical compounds were detected using standard procedures. Results obtained showed that the growth of *A. tamiyavanichii* was inhibited by all concentration tested. Active fractions from DCM using both fresh and dried plants showed good results with more that 80% RE values at 5 mg/mL within 2.5 to 5 hours. During the experiments, DCM used did not change the pH of the culture medium compared to methanol fraction. For phytochemical screening tests, compounds detected in all fractions were alkaloids, tannins, glycosides, reducing sugars and terpenoids. These compounds might cause the inhibition of targeted algae, however further study is needed to determine the bioactivity and its specific effect on HAB species. To conclude, fractions of DCM from fresh and dried *S. trifasciata* have the potential in the mitigation of *A. tamiyavanichii.* This could help to minimize the impact of this species on human health and reduce the loss to fishery industries.

**KEYWORDS**

Mitigation, Harmful Algal Bloom, environmentally friendly, active fractions.

## 1. INTRODUCTION

Harmful Algal Bloom (HAB) is a recurrent problem in Malaysia, particularly in Sabah coastal waters. The occurrences of HAB are due to the availability of optimum environmental conditions needed for the microalgae to grow reaching millions of cells per litre. A lot of negative effects have been recorded caused by HAB such as human illnesses, loss to fishery and tourism industries. In Malaysia, there were many HAB cases caused by different species including *Alexandrium tamiyanichii, A. minutum*, *Pyrodinium bahamense* var. *compressum* and *Margelifidinium polykrikoides* have been reported (Mohammad-Noor et al., 2018a).

There were many study conducted elsewhere to find the best method for mitigation of HABs. One of the established methods was applying clay sprayed to affected areas whereby the clay will remove HAB cells through flocculation and sedimentation. This method has been adapted by several countries for examples Japan, South Korea, and USA (Imai et al., 2006; Kim et al., 2010; Sengco and Anderson, 2004). Besides, physical approach, other studies on HAB inhibition focused on applying biological and chemical approaches. This includes using extract of Chinese fir to inhibit the growth of *A.tamarense* (Yang et al., 2009). All methods that conducted mostly produced positive results by mitigating the specific target algae. However, some of the methods conducted affected the environment by causing pollution and cause harm to other marine creatures. Therefore, studies on allelopathy become an alternative for mitigation of HABs due to its low cost and environmental friendly (Wang et al., 2007).

Although HAB cases in Malaysia have been reported almost every year, study on identifying suitable material for the mitigation of HAB is still limited. Study has been conducted on the suitability of using local soils to inhibit *A. minutum* and *A. andersonii* (Roziawati et al., 2016). Their study indicated promising results using two types of soil viz. paddy field and pottery clay. In this study, an ornamental plant, *S. trifasciata* will be used to inhibit the growth of *A. tamiyavanichii*. Previously, water extract of dried and fresh *S. trifasciata* were tested on *A. tamiyavanichii* and showed a positive inhibition of the algae, however, the extract caused the decrease

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of pH on the cultured medium (Mohammad-Noor et al., 2018b). Therefore, this study will investigate the potential of DCM and methanol fractions to inhibit the growth of *A. tamiyavanichii*. The outcomes of this study are expected to complement previous study and increase our understanding on the potential of *S. trifasciata* to mitigate HAB species, particularly *A. tamiyavanichii*.

## 2. Materials and Methods

### 2.1 *A. tamiyavanichii* culture

Culture of *A. tamiyavanichii* used in this study came from the same source as reported by Mohammad-Noor et al., (2018b). The cultures were maintained in ESDK medium enriched with f/2 vitamin, temperature of 26ºC, light cycle of 12:12 L:D (light and dark) and light intensity of 2000 lux.

### 2.2 Extraction of *S. trifasciata*

The plant was processed (washed, weighted and cut) and dried in an oven at 40ºC until constant weight were obtained. Then, both fresh and dried plant samples were soaked in 95% ethanol and left at room temperature for 9 days. Every three days, extracts were collected and replaced with fresh ethanol. The collected crude extracts were filtered through 0.45 µm Whattman filter using vacuum pump and concentrated with rotary evaporator to remove the extracting solvent.

### 2.3 Fractionation process

Fractionation was conducted to all the extracts to obtain several fractions (dichloromethane (DCM) and methanol). Twenty gram of ethanol plant extract was dissolved in 95% ethanol and was put in the VLC column packed with 150 g of silica gel. Then, eluted using solvent with increasing polarity (dichloromethane and methanol) and were poured through the VLC column to obtain the fractions.

### 2.4 Phytochemical screening

Phytochemical screening test was conducted to detect phytochemical compounds produced by *S. trifasciata* that might have the potential to inhibit the growth of *A. tamiyavanichii.*

#### *2.4.1 Alkaloids*

Twenty milligram of plant extract was mixed with 10 mL ethanol, heated in water bath and filtered before adding 1% hydrochloric acid. Then, few drops of Mayer's reagents were added. The appearance of cream precipitate indicates the presence of alkaloids.

#### *2.4.2 Tannin*

A small amount of plant extract was mixed with water, heated in water bath and added with ferric chloride. The appearance of dark green colour indicates the presence of tannin.

#### *2.4.3 Anthraquinones*

Half gram of plant extract was boiled with 10% HCl for a few minutes in water bath. Then it was allowed to cold to room temperature. The appearance of pink colour showed the presence of anthraquinones.

#### *2.4.4 Glycoside*

Half gram of plant extract was dissolved in 2 mL of glacial acetic acid containing a few drops ferric chloride solution before mixed with 1 mL of concentrated sulphuric acid. The appearance of brown ring at interface indicates the presence of glycoside.

#### *2.4.5 Reducing sugar*

Plant extract was dissolved in water and filtered. Then a few drops of Fehling solutions A and B were added before boiled a few minutes. The formation of orange precipitate indicates the presence of reducing sugar.

#### *2.4.6 Saponins*

Plant extract of 0.2 g was shaken with 5 mL of distilled water and heated. The appearance of creamy miss of small bubble indicates the presence of saponins.

#### *2.4.7 Flavonoids*

Plant extract was mixed with a few drops of hydrochloric acid. The formation of red colour indicates the presence of flavonoids.

#### *2.4.8 Phlobatanins*

Half gram of plant extract was dissolved in distilled water before filtered. The filtered solution was then boiled with 2% HCl. The appearance of red precipitate indicates the presence of phlobatanins

#### *2.4.9 Steroids*

Plant extract was mixed with 2 mL of chloroform before sulphuric acid was added. The appearance of red colour at the lower layer of chloroform indicates the presence of steroids.

#### *2.4.10 Terpenoids (Salkowski test)*

Plant extract (0.2 g) was mixed with 2 mL of chloroform before 3 mL of concentrated sulphuric acid was added. The appearance of a reddish-brown layer indicates the presence of terpenoids.

### 2.5 The effect of fractions on the growth of *A. tamiyavanichii*

Test on *A. tamiyavanichii* was done at different concentrations of fractions (0.001, 0.01, 0.1, 0.5, 1, 1.5, 2 and 2.5 mg/mL) in three replicates. The tests were conducted in 60×10 mm petri dish with approximately 1000 to 2000 cells/mL cell density of *A. tamiyavanichii*. Control was set up for each concentration whereby no fractions were introduced to the cells. The effects of each concentration on cells were determined at time interval of 0, 2.5, 5, 10 and 24 hours. Number of cells were counted twice using Sedgewick rafter chamber, under light microscopy at total magnification of 100X. pH was recorded during experiments. Finally, the effects of the fractions on the growth of *A. tamiyavanichii* were determined based on removal efficiency (RE). The higher removal efficiency indicated the higher effect of the fraction in removing the cells.

$$Re = \frac{\text{initial cell concentration} - \text{sample cell concentration}}{\text{Initial cell concentration}} \times 100\%$$

### 2.6 Toxicity test

The test was done on Artemia (brine shrimp) at different concentrations (10, 50 and 100 mg/mL). Artemias were hatched and experiments were conducted following method by Mohammad-Noor et al. (2018b). $LC_{50}$ at the 95% confidence limits were obtained by plotted survivors percentages with concentrations tested.

### 2.7 Statistical analysis

One-way ANOVA was run using SPSS 2.0 to analyse significant different between concentration tested for both active fractions. Correlation analysis was done to investigate the relationship between RE and time of exposure, and between RE and concentration tested.

## 3. Results and Discussions

### 3.1 Phytochemical screening

Based on phytochemical screening, several important compounds were detected from both fractions i.e. alkaloids, tannins, glycosides, reducing sugars and terpenoids (Table 1). Previous study showed that *S. trifasciata* produced carbohydrates, saponins, glycosides and steroids (Yoshihrio et al., 1996). These compounds have been reported to have antibacterial or anti-microbial properties which includes tannins, glycosides and terpenoids (Borokini and Ayodele, 2012; Kar et al., 2013; Samejo et al., 2013). Determination of phytochemical compounds in *S. trifasciata* is considered as a basic step in identifying potential chemical compounds that can be used in mitigating HAB species. However, further study is needed to confirm the specific compound.

**Table 1:** Phytochemical screening on active fractions of *S. trifasciata*

| | MeOH fraction (Fresh) | MeOH fraction (Dried) | DCM fraction (fresh) | DCM fraction (Dried) |
|---|---|---|---|---|
| **Alkaloid** | + | + | + | + |
| **Tannins** | + | + | + | + |
| **Anthraquinones** | - | - | - | - |
| **Glycoside** | + | + | + | + |
| **Reducing sugars** | + | + | + | + |
| **Saponins** | - | - | - | - |
| **Flavonoids** | - | - | - | - |
| **Phlobatanins** | - | - | - | - |
| **Steroids** | - | - | - | - |
| **Terpenoids** | + | + | + | + |

+ = present; - = absent

### 3.2 Test of extracts on *A. tamiyavanichii*

Generally, each concentration tested resulted in positive indication to inhibit the growth of A. *tamiyavanichii* although each concentration from different fractions has different strength.

#### *3.2.1 DCM fractions*

From results obtained, the highest RE for DCM fraction from ethanol fresh plant was at 1.5 mg/mL with 80.9% at 5 hours while the lowest RE was at 0.5 mg/mL with 38.78% at 5 hours (Figure 1). There were significant differences were found between times of exposure ($p<0.05$) for RE of all concentrations of this fraction. However, negative correlation was obtained between RE and time (r=-0.7) but positive correlation was recorded between RE and concentration (r=0.7). The range of pH recorded during the experiment was 7.81 to 8.23.

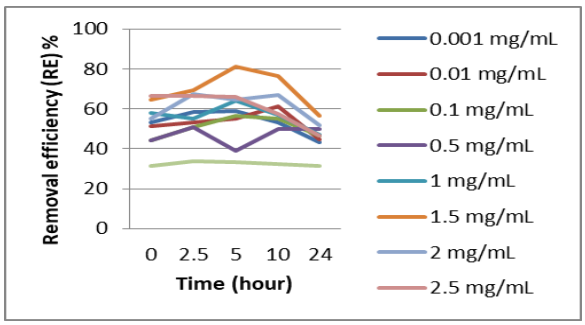


**Figure 1:** Removal efficiency of DCM fraction from ethanol fresh plant extract on *A. tamiyavanichii*

The highest RE showed by DCM from ethanol dried plant extract was 83.91% at 1.5 mg/mL at 2.5 hour whereas the lowest RE recorded was at 0.01 mg/mL which was 46.98% at 10 hour (Figure 2). RE showed significant differences between time ($p<0.05$) for all concentration tested. Negative correlations were obtained between RE and time (r=-0.94) but positive correlations were found between RE and concentration (r=0.84). The range of pH recorded during the experiment was 7.81 to 8.23.

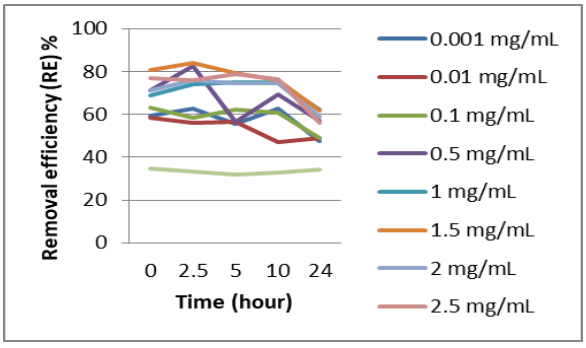


**Figure 2:** Removal efficiency of DCM fraction from ethanol dried plant extract on *A. tamiyavanichii*

#### *3.2.2 Methanol fractions*

For methanol fraction from ethanol fresh plant, the highest RE was recorded at 0.1 mg/mL which was 93.86% at 2.5 hour while the lowest RE was recorded at 0.01 mg/mL with 25.16% at 0 hour (Figure 3). Significant differences were found between time ($p<0.05$) for all concentration tested. However, negative correlations between both time (r=-0.37) and concentration with RE (r=-0.05) were found. The range of pH recorded during experiment was 7.46 to 8.20 for 0.001 to 0.1 mg/mL and 5.22 to 6.47 for 0.5 to 2.5 mg/mL.

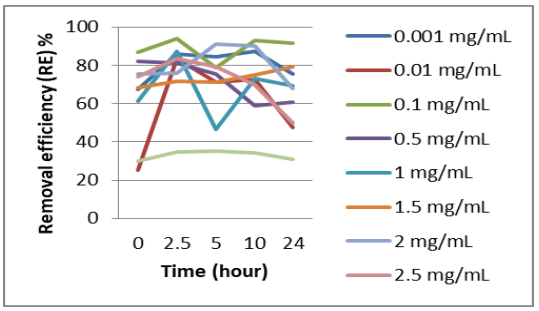


**Figure 3:** Removal efficiency of methanol fraction from ethanol fresh plant extract on *A. tamiyavanichii*

For methanol fractions from ethanol dried plant extract showed the highest RE result was at 0.1 mg/mL which was 91.35% at 2.5 hours whereas the lowest RE was at 1 mg/mL which was 47.81% at 2.5 hour (Figure 4). There were significant differences ($p<0.05$) of RE between time for all concentration tested. Negative correlations were found between RE and time (r=-0.56) and, RE and concentration (r=-0.30). The ranges of pH recorded for methanol fraction of ethanol dried plant was similar to methanol fraction of ethanol fresh plant which were 7.46 to 8.20 for 0.001 to 0.1 mg/mL and 5.22 to 6.47 for 0.5 to 2.5 mg/mL.

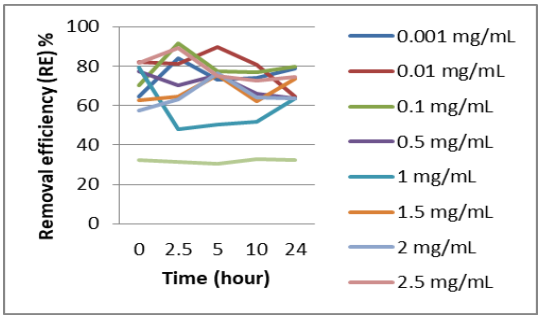


**Figure 4:** Removal efficiency of methanol fraction from ethanol dried plant extract on *A. tamiyavanichii*

### 3.3 The effect of fractions on the growth of *A. tamiyavanichii*

One of the important factors to determine the effectiveness of mitigation agent is time needed to inhibit the growth of HAB species besides concentration. The duration of time taken by different mitigation agent differ depending on its strength. For example, extract of medicinal plants at concentration of 800 mg/L required 7 days to mitigate 91.3% of *Microcystis aeruginosa* (Yi et al., 2012). For both DCM and methanol fractions of *S. trifasciata*, negative correlations were obtained between RE and time of exposure. Although an increase of RE was observed during the first 10 hours, the inhibition started to decrease slightly after that. Further study is needed to investigate why the active fractions loss it effectiveness to inhibit the growth of *A. tamiyavanichii* after exposed to a certain duration of time.

The application of mitigation agent on cells might cause the pH of the medium to change and this can inhibit the growth of algae. Depending on species, the tolerable range of pH for algal growth range from 7 to 9. The addition of mitigation agent (chitosan-silica composite) has caused the culture media to become acidic (Iqbal et al., 2019). This can lead to false positive result whereby the algae died due to the acidic medium but not due to the mitigation agent itself. In this study, DCM fractions for both ethanol fresh and dried plant extracts did not caused the pH to change much at all concentration tested compared to methanol fractions of fresh and dried plant extracts. Higher concentration of methanol fractions (0.5 to 2.5 mg/mL) caused the medium to become acidic and unsuitable to be used to mitigate *A. tamiyavanichii*.

Based on morphological observation, cells that exposed to all active fractions did not showed any morphological changes after immediate contact. However, after 5 to 10 hours, the cells tend to swim weakly and died. Some cells tend to rupture. Similar condition has been reported whereby algicidal actinomycete inhibited the growth of *A. tamarense* by causing the cell to lyse and therefore, released the cellular component as exposure time increased (Zhou et al., 2008).

### 3.4 Toxicity test

To investigate the possible effect of these active fractions on other marine organisms, toxicity study using Artemia was conducted. Figures 5 to 8 showed the results of $LC_{50}$ of active fractions on Artemia. For both DCM fractions and methanol fraction from dried plant the $LC_{50}$ of 95% confidence limits was at 300 mg/mL, respectively. However for methanol fraction for fresh plant, the $LC_{50}$ was at 150 mg/mL. The high $LC_{50}$ of both active fractions, except for methanol fraction of fresh plant indicates that they are safe to be used in marine environment, at least at this level. Furthermore, the concentration needed to inhibit the growth of *A. tamiyavanichii* is far lower than concentration needed to kill Artemia. Compared to extract of *S. trifasciata* using distilled water of fresh and dried plants, the $LC_{50}$ of the fractions to Artemia recorded were much lower i.e. 30 mg/mL and 70 mg/mL, respectively (Mohammad-Noor et al., 2018b). This further support that the DCM fractions are more suitable to be use to inhibit *A. tamiyavanichii* due to higher $LC_{50}$ recorded.

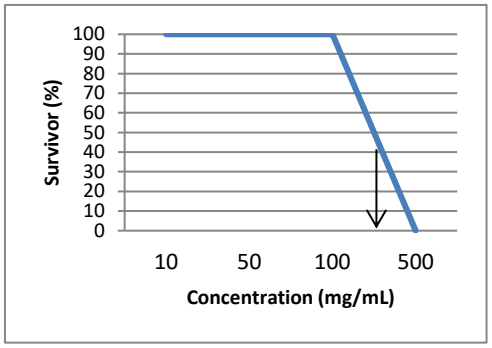


**Figure 5:** $LC_{50}$ of DCM fraction from ethanol fresh plant extract on Artemia

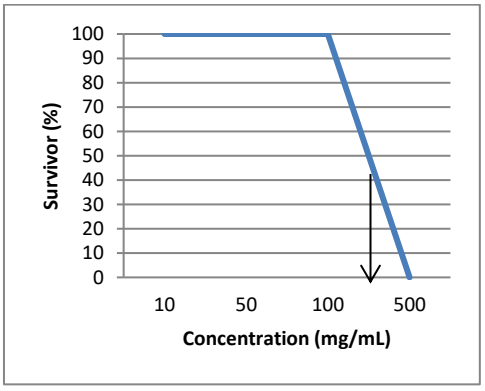


**Figure 6:** $LC_{50}$ of DCM fraction from ethanol dried plant extract on Artemia

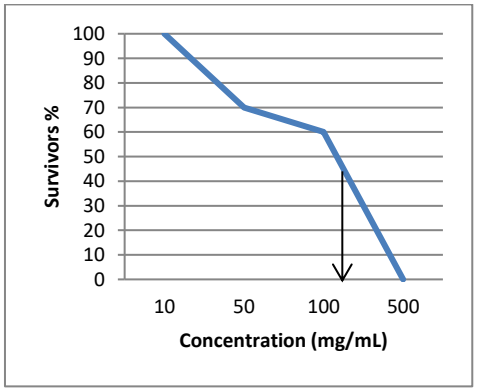


**Figure 7:** $LC_{50}$ of methanol fraction from ethanol fresh plant extract on Artemia

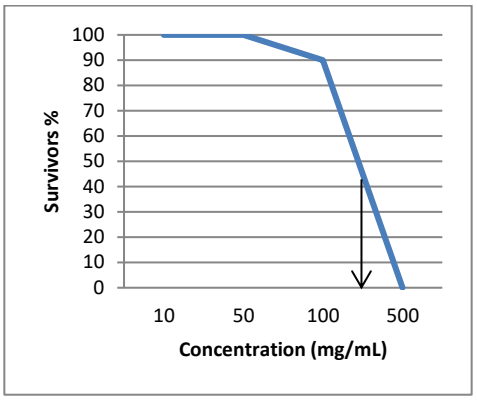


**Figure 8:** $LC_{50}$ of methanol fraction from ethanol dried plant extract on Artemia.

## 4. Conclusion

This study showed that ornamental plant, *S. trifasciata* can be used to inhibit the growth of *A. tamiyavanichii*. The recorded REs indicate that both DCM fractions are more suitable to be used as mitigation agent for *A. tamiyavanichii* compared to both methanol fractions. Both DCM fractions recorded REs more than 80% on *A. tamiyavanichii* at concentration of 1.5 mg/mL at duration of 2.5 to 5 hours. Toxicity test based on Artemia test also support the suitability of DCM fractions compared to methanol fractions whereby high concentration is needed to kill 50% of Artemia. These findings will add to available knowledge on biological and environmental friendly ways to mitigate HAB species and at the same time reduce possible effect on the environment.

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