การเลี้ยงฟองน้ำทะเลสีน้ำเงิน *Xestospongia* sp. ของไทยในสภาวะธรรมชาติ เพื่อผลิตสารต้านมะเร็ง Renieramycin M

Cultivation of a Blue Thai Marine Sponge, *Xestospongia* sp., under Natural Conditions to Produce Anticancer Compound, Renieramycin M

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บทคัดย่อ

ฟองน้ำทะเลสีน้ำเงินของไทยชนิดหนึ่งชื่อ *Xestospongia* sp. (c.f. *Neopetrosia* sp.) สามารถผลิตสารต้านมะเร็งที่มีประสิทธิภาพสูง คือ Renieramcin M (RM) ปัจจุบันสารนี้กำลังอยู่ในขั้นตอนการศึกษาในระดับคลินิกก่อนจะพัฒนาเป็นสารต้านมะเร็งชนิดใหม่ เพื่อเตรียมสาร RM ให้เพียงพอต่อการศึกษาระดับคลินิกและความต้องการของตลาดยาในอนาคต ฟองน้ำชนิดนี้จึงถูกเลี้ยงในสภาพธรรมชาติเพื่อประเมินการผลิตสาร RM และเก็บข้อมูลการเจริญเติบโต โดยเลี้ยงบริเวณแนวชายฝั่งบริเวณจังหวัดตรังด้วยวิธีการแขวนในแนวดิ่งเป็นเวลา 10 เดือน ใช้วัสดุยึดเกาะ 3 ชนิด ประกอบด้วย เชือกพลาสติก ชิ้นซีเมนต์ และท่อโพลีไวนิลคลอไรด์ (PVC) ตามลำดับ ผลการทดลองพบว่าฟองน้ำสร้างสารและสะสมสาร RM สูงสุดพบในเดือนกันยายน (0.42 ไมโครกรัมต่อมิลลิกรัมเนื้อเยื่อ) แต่ไม่พบว่ามีการผลิตสารในช่วงฤดูร้อน (กุมภาพันธ์-เมษายน) และช่วงเริ่มต้นฤดูมรสุม (พฤษภาคม-กรกฎาคม) การเจริญเติบโตของฟองน้ำทะเลสีน้ำเงินไม่มีความแตกต่างกันทางสถิติที่ระดับความเชื่อมั่นร้อยละ 95 การเจริญเติบโตสูงสุดพบในเดือนมิถุนายน จากผลการทดลองสรุปได้ว่าอุณหภูมิและความเค็มที่เปลี่ยนไปในฤดูมรสุมทำให้ฟองน้ำใช้พลังงานในการสังเคราะห์และสะสมสาร RM ในเนื้อเยื่อมากกว่าเพื่อการเจริญเติบโต

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Received: 30 April 2020, Revised: 2 July 2020, Accepted: 3 July 2020

คำสำคัญ: *Xestospongia* sp. การเลี้ยงสภาพธรรมชาติ Renieramycin M การสะสม การเจริญเติบโต

Abstract

A Thai blue marine sponge, *Xestospongia* sp. (c.f. *Neopetrosia* sp.), can produce highly effective anticancer compounds; namely, Renieramycin M (RM). Currently, this compound is in clinical trials processes before developing it as a new anticancer agent. To provide sufficient RM for clinical trials and a prospective pharmaceutical market, a blue *Xestospongia* sponge was cultured under natural conditions to assess the RM production and record the growth. The sponge was cultured near the coastline of the Trang Province Sea area by the long line method for 10 months. Three substrates, i.e., a plastic rope, cement pieces, and a polyvinylchloride (PVC) pipe, respectively, were used. Results revealed that the sponge produced maximum and accumulated RM in September (0.42 µg/mg tissue), but it was not observed during summer (February-April) and the early monsoon season (May-July). The growth of a blue sponge did not exhibit any statistical difference at the 95% confidence interval. The maximum growth was observed in June. Based on the result, it can be concluded that changes in the temperature and salinity in the monsoon season cause the sponges to utilize its energy for synthesizing and accumulating RM in their tissues rather than utilizing for its growth.

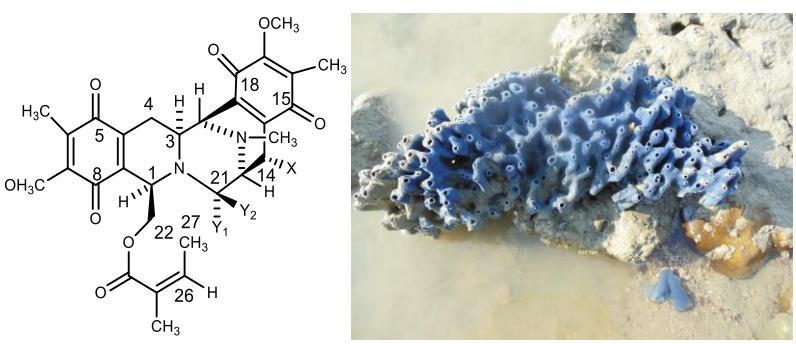
Keywords: *Xestospongia* sp., Culture, Natural condition, Renieramycin M, Accumulation, Growth

Introduction

Several anticancer agents are derived from marine sponges. A number of these molecules are being evaluated in clinical trials, but some of these molecules are present in limited quantities, e.g., cytarabine, vidarabine and discodermolide (Singh and Thakur, 2016). These drugs can produce by industrial synthesis, which is only possible after the completion of clinical trials. Moreover, the chemical synthesis strategy also must be confirmed; this process is lengthy and incurs high costs. Therefore, aquaculture is an alternative way for lead molecules production due to it is not just only facile, but also low invested method. Several experiments conducted worldwide verified that the culture of sponges by the sea-based method can be a prototype for the development of marine farming in the near future, including the culture of a sponge *Discodermia dissolute* in the Caribbean sea to produce an antitumor metabolite, discodermolide (Ruiz

et al., 2013); an anti-inflammatory compound, manoalide, from *Luffeariella veridabilis* (Epstein *et al*., 2007); *Dysidea avara* and *Chondrosia reniformis* in the Mediterranean and Indian Sea, respectively; and the mass production of *Stylissa massa* in the Indo-Pacific sea region (Osinga *et al*., 2010; Sankar *et al*., 2016).

In the Andaman Sea, a blue marine sponge, *Xestospongia* sp. *(*c.f. *Neopetrosia* sp.), (Figure 1) was found to be distributed in the west coast part of Thailand (Kieattisak *et al*., 2017). This sponge produces a group of anticancer drugs, namely renieramycins, with chemical structures and biological activities similar to the current anticancer drugs such saframycin, naphthyridomycins, quinocarcins, and ecteinascidin 743 (Daikuhara *et al*., 2009). Renieramycin M (RM) is a member of this class of compounds, which exhibits highly potent antitumor activity against several cell lines, including human colon (HCT166, DLD_1), breast cancer cells (MDA-MB-$_{436}$ and $T_{47}D$), and human lung cancer 460 at nanomolar concentrations (Halim *et al*., 2011; Pinkhien *et al*., 2016; Sirimangkalakitti *et al*., 2017; Tun *et al*., 2019). Currently, the amount of RM is insufficient for using in clinical trials and market demand.



A (1a) : X=OH, Y_1, Y_2=H_2 E (1e) : X=Y_2=H,Y_1=OH K(1k) : X=Y1=CH_2COCH_3,Y_2=H

B (1b) : X=OC_2H_5,Y_1,Y_2=H_2 F (1f) : X =OCH_3,Y_1=OH,Y_2=H M : X=Y_2=H,Y_1=CN

C (1c) : X=OH,Y_1,Y_2=O G (1g) : X=H,Y_1,Y_2=O O (1o) : X=OH,Y_1=CN, Y_2=H

D (1d) : X=OC_2H_5,Y_1,Y_2=0 J (1a) : X=Y_2=H,Y_1=CH_2COOCH_3

Figure 1 A blue marine sponge and chemical structure of Renieramycins.
Source: Suwanborirux *et al*. (2003)

Materials and Methods

1. Animal material

1.1 Identification of a sponge was done by Asst. Dr. Darumas Udomsak in following to the method described in "Systema Porifera: A Guide to the Classification of

Sponges" (Hooper and van Soest, 2002). Voucher specimen (SA200560) was preserved in 75% MeOH and deposited at the Department of Marine Science, Faculty of Sciences and Fishery Technology, Rajamangala University of Technology, Trang campus. A blue marine sponge, *Xestospongia* sp., in this study is only oxea (Figure 2D and Figure 2B). In the ectosomal layers, there are spicules covering membrane, comprising oxea bundle. Ectosomal layers are sticky when touched (Figure 2C) (Desqueyroux-Faundez and Valentine, 2002). Although, this blue marine sponge is very similar with *Neopetrosid* sponges. It contains of oxea as megascleres that are longer than 200 µm, while those of *Neopetrosid* sponges are shorter than 200 µm. This *Xestospongia* sponge differs from other *Xestospongia* species such as *X. exigua* in the Indo-Pacific, which has been recognized as *Xestospongia exigua* (Van Soest *et al*., 2005). *X. testudinaria*; *X. bergquistia* and sponges in the Caribbean, such as *X. muta*. Both *X. testudinaria* and *X. bergquistia* are volcano-shaped while the Caribbean *X. muta* is barrel shaped (Fromont, 1991). Previous specimens have not been same with the referred *Xestospongia* spp. This blue marine sponge found inhibiting on the rocks and/or dead coral substrata. Colony is massive or sub-massive with finger-liked extension of the surface lobes. The coloration is light blue, some colonies are navy blue. The oscules are numerous and mostly found on the apices of the surface lobes (Figure 2A).

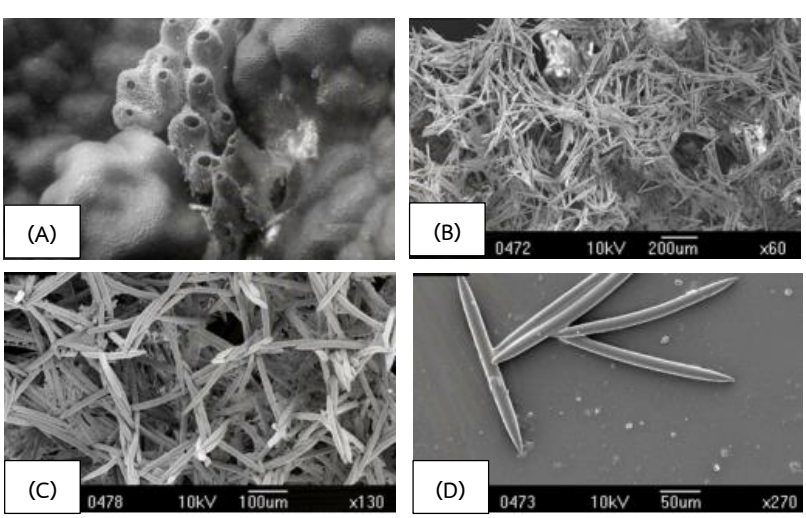


Figure 2 Morphology of *Xestospongia* sp.

Remark: (A) A Thai blue marine sponge, *Xestospongia* sp. coexisting with the hard coral, *Porites lutea*. (B) Choanosomal skeleton, showing a highly dense network of multispicular tracts. (C) Longitudinal section through the surface, showing ectosomal tangential disordered network of spicule brushes. (D) Oxeas.

1.2 Mother plant preparation

Mother plants of the *Xestospongia* sp. were collected from the Andaman Sea coastline area of Trang Province, Thailand. The specimens were preserved in a cage at a depth of 3 m for 1 month. Before use, the RM exist in mother plant was measured by high performance liquid chromatography (HPLC) (Belarbi *et al*., 2003).

1.3 Explant plant preparation

Sponge specimens with a size of 2 × 2 cm. were cut from the mother plant and then attached on substrates using a nylon rope (Duckworth and Peterson, 2013). There were 4 rows for one type of substrate, in which one row contained three attached positions; at the end of row was connected to a small weighing box as illustrated in Figure 3. The weighing box is a supporting of a rope balancing in case of strong currents and big wave attacking.

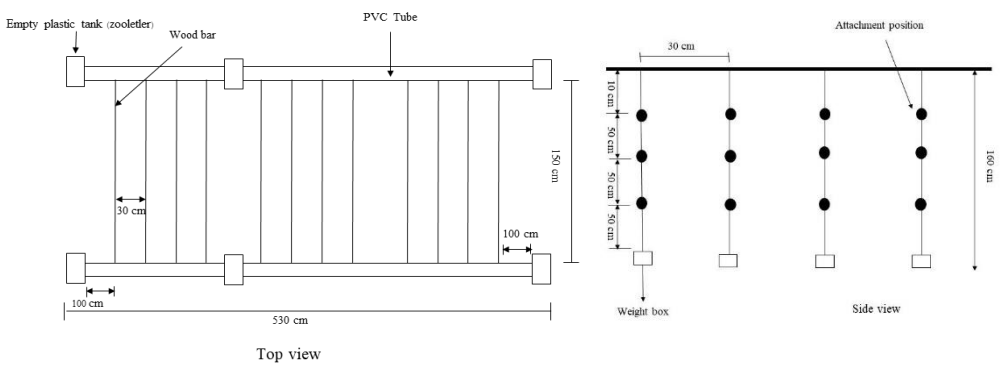


Figure 3 Scheme of the culture system.

2. Cultivation method

The long line method was employed in this experiment. Three substrates, such as polyvinyl chloride (PVC) pipe, cement pieces, and plastic ropes, respectively, were used. The cultivation cage was constructed using PVC pipes with a diameter of 15 cm, and 200-L empty plastic tanks were used as buoyancy support (Figure 3).

3. Environmental factor determination

Environmental factors such as salinity, temperature, dissolved oxygen (DO), pH, transparency, and dissolved silicate were determined each month. Salinity, DO, pH, and temperature were measured using a multi-probe instrument (YSI 556MPS, California, USA); transparency was determined by Secchi disc; and dissolved silicate was analyzed by the method described in the APHA 23rd edition.

4. Growth determination

Growth was assessed each month by direct dimensional measurements, which all of those values were calculated as the average size (Duckworth and Peterson, 2013).

5. RM analysis

RM was extracted according to a method reported by Suwanborirux *et al*. (2003). Briefly, a specimen was cut into fine pieces and accurately weighed to the nearest 150 mg. A sample was macerated with 10 mM potassium cyanide in a phosphate buffer solution (6.00 ml, pH 7.0) for 5 h before extraction using 24 ml of methanol. After centrifugation, the liquid methanol was partitioned using ethyl acetate, followed by drying under vacuum to obtain a solid extract. A milligram of extract was dissolved in 1 ml of methanol for subjecting to HPLC (Dionex Ultimate 3000, Munich, Germany). HPLC was performed under the following conditions: a C18 reverse-phase column at 25°C using a mixture of methanol and water (7:3, v/v) as the mobile phase at a flow rate of 1 ml/min with a photodiode array detector. The RM concentration was calculated by the Chromeleon software (version 6.8 SR_7) compared to a standard.

6. Cytotoxic assay

Cytotoxicity against African green monkey kidney (Vero cell, ATCCCCL-81) of sponge extracts was determined by the green fluorescent protein (GFP) method as described in our previous study (Kieatisak *et al*., 2017).

7. Statistical analysis

The completely randomized design (CRD) was employed to perform experiments comprising three treatments (type of substrate), and each treatment was conducted in triplicate. Mean values were compared by the Mann-Whitney U test. All statistical values were calculated by SPSS software (version 6).

Results

The growth of a blue sponge varies according to the type of substrate and season (Figure 4), furthermore the observation of growth of sponges on cement pieces is interesting. However, statistical analysis revealed that the growth do not differ in all substrates at 95% confident interval (Table 1). The blue sponge attached on cement and a PVC pipe exhibited growth throughout the experiment period (Figure 3), but the sponge attached on plastic ropes was pulled by the water current. The plastic ropes were greasy when tied together with the sponge; therefore, it slips away by the strong water current. The growth of the blue sponge varied according to the season: The blue

sponge grew well in summer (February-April), but its growth decreased in the late monsoon season (May-October). Environmental parameters such as water quality (Table 2) measured during the experimental period revealed that three key parameters widely fluctuate, including salinity (29.00-31.00 ppt), water temperature (29.50-31.50°C), and pH (7.54-9.37), respectively.

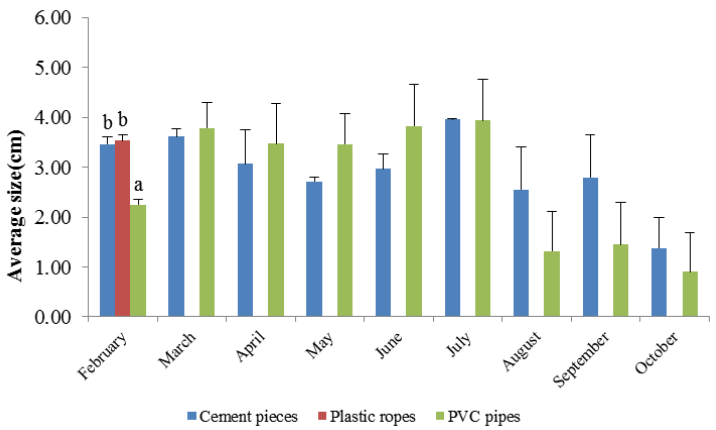


Figure 4 Growth of a blue sponges on three different subtrates.
Remark: Specimens attached on plastic ropes were lost.

Table 1 Statistical analysis of growth of a blue sponge

Growth	n	Mean Rank	Mann-Whitney U test	*p*-value
Cement pieces	9	9.72	38.5	0.86
PVC pipes	9	9.28		
Total	18			

Remark: Specimens attached on plastic ropes were lost.

Twenty milligrams of blue sponge specimens was collected each month, and after extraction, the crude extract was subjected to HPLC analysis for analyzing the RM consistency. HPLC chromatograms (Figure 5) revealed that RM peaks are observed at a retention time (Rt) of 22.5 min; the same value also was observed for the crude extract of the sponge collected in August–November. RM accumulated in the tissue of the blue sponge determined by HPLC revealed that the sponge does not synthesize RM during summer (February-April) and the start of the monsoon season (May-July), but it was synthesized and accumulated in the sponge's tissue in the late monsoon season, as shown by the values in Table 3. Cytotoxicity analysis against Vero cells of the total extract from a blue sponge revealed a moderately value IC_{50} at 10 µg/ml.

Discussion

Based on the experiment, the blue sponge exhibited high biomass production in March and July, while it decreased during August-October; however, a certain pattern was not observed (Figure 4). The structure of sponge contains the pores which are connected all over the body to form water circulating cannels; the outside suspended food and other materials are carried through the body by water current. Therefore, the growth of the sponge is generally related to the surrounding water quality (Schiefenhövel and Kunzman, 2012). Water quality pattern of this study accorded to season variation as illustrate in figure. Dissolved oxygen in seawater reduces the toxicity of some chemical components that are deposited in an aquiferous system and controls the energy budget (Thomassen and Riisgard, 1995). Oxygen consumption of marine sponges varies according to their species; nonetheless, it is in the general range of 0.2-25 $\mu molO_2h^{-1}/cm^3$; below this range, the growth rate decreases (Belarbi *et al*., 2003). However, previous data describing the oxygen consumption in a blue *Xestospongia* sponge are not available. In general, the average DO value of seawater is ~5mg/ml at 30 °C (Duckworth and Peterson, 2013). This value revealed that there is no critical value. According to this experiment, oxygen concentration values for the culture area were in the range of 6.58-8.78 at a temperature range of 29.4-31.52°C, which was greater than the saturation point of standard sea water (Figure 6 and Table 2). Temperature is a key parameter for sponge growth, because it has important functions in the metabolic process in marine sponges by controlling the production of free amino acid and ions. Changes in the water temperature (Figure 6) in the culture area were caused by two factors: seasonal variation and water currents; however, it fluctuated in a narrow range (29.4-31.50°C), which corresponded to the tolerance values for a *Xestospongia* sponge. The highest growth (average size of 3.96 cm) was recorded in July at 29.40°C because a sponge grows well at temperatures slightly less than the ambient temperature (29-34°C), while it grows to a lesser extent when the temperature increases; this result is also according to that found in a sponge, *Mycale hentscheli* (Carballo *et al*., 2010). However, effect of temperature to growth of sponges is still rear report found, furthermore it vary in species. Generally, growth of sponge involve regeneration rate, wound healing and pumping rate of ostia (Osinga *et al*., 1999). Silica is used to synthesize spicules in marine sponges, particularly *Xestospongia* sponge (order Haplosclerida), which contains 62.3% siliceous spicules (Osinga *et al*., 1999).

Table 2 Environmental parameters in the sponge culture area.

Parameters	February	March	April	May	June	July	August	September
pH	8.24±0.27^{ab}	9.03±0.56^{b}	7.65±0.01^{a}	7.38±0.25^{a}	7.71±0.10^{a}	7.50±0.30^{a}	7.49±0.10^{a}	8.59±0.39^{b}
Transparency (m)	2.60±0.10^{b}	1.20±0.10^{a}	1.54±0.04^{c}	0.84±0.04^{c}	0.90±0.00^{d}	0.95±0.05^{de}	1.34±0.04^{de}	1.45±0.05^{cd}
Dissolved oxygen (mg/l)	7.54±0.29^{b}	6.76±0.18^{a}	8.29±0.15^{c}	8.48±0.06^{cd}	8.98±0.03^{e}	8.87±0.11^{de}	8.79±0.03^{de}	7.22±0.08^{b}
Silicate (mg/l)	0.64±0.08^{abc}	0.58±0.02^{abc}	0.41±0.12^{ab}	0.36±0.02^{a}	0.47±0.04^{ab}	0.76±0.11^{c}	0.65±0.22^{bc}	0.67±0.02^{bc}
Salinity (ppt)	31.11±0.84^{ab}	32.00±1.00^{b}	31.50±0.50^{ab}	30.50±0.50^{ab}	31.70±0.70^{b}	29.50±0.50^{a}	31.50±0.50^{ab}	31.00±1.00^{ab}
Temperature (°C)	31.50±0.26^{bc}	30.60±0.10^{ab}	31.00±0.50^{bc}	31.30±0.10^{bc}	31.80±0.50^{c}	29.60±0.20^{a}	31.60±0.30^{bc}	30.70±0.70^{b}

Remark: Mean values with the same superscripts are not significantly different ($p < 0.05$).

Different superscripts like ^{a,b,c,d,e} are depicted for significant different at $p < 0.05$ based on turkey test.

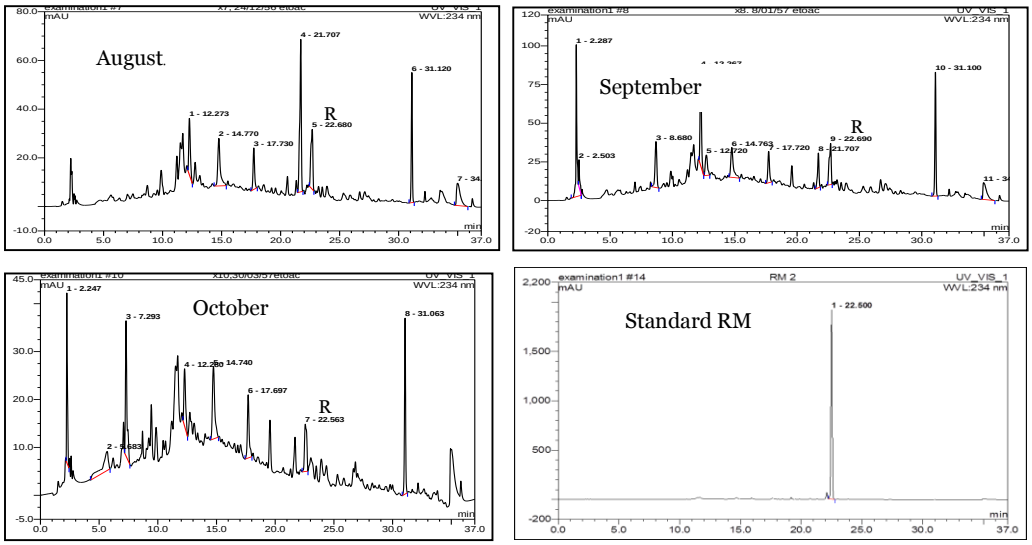


Figure 5 HPLC chromatogram showing RM consistency in sponge's specimens.

Table 3 RM accumulation in a blue sponge's tissue during cultivation peroid.

Months	RM concentration (µg/g tissue)
February	0.00±0.00[a]
March	0.00±0.00[a]
April	0.00±0.00[a]
May	0.00±0.00[a]
June	0.00±0.00[a]
July	0.00±0.00[a]
August	0.39±0.06[cd]
September	0.42±0.18[d]
October	0.22±0.11[bc]
November	0.15±0.05[ab]

Remark: Mean values with the same superscripts are not significantly different ($p < 0.05$). Different superscripts like [a,b,c,d] are depicted for significant different at $p < 0.05$ based on turkey test.

Spicule generation occurs under high concentrations of dissolved silica, leading to a high growth rate; on the other hand, the growth of sponge was interrupted under low concentrations of dissolved silica as reported for *Halichondria panacea* (Frøhlich and Barthel, 1997).

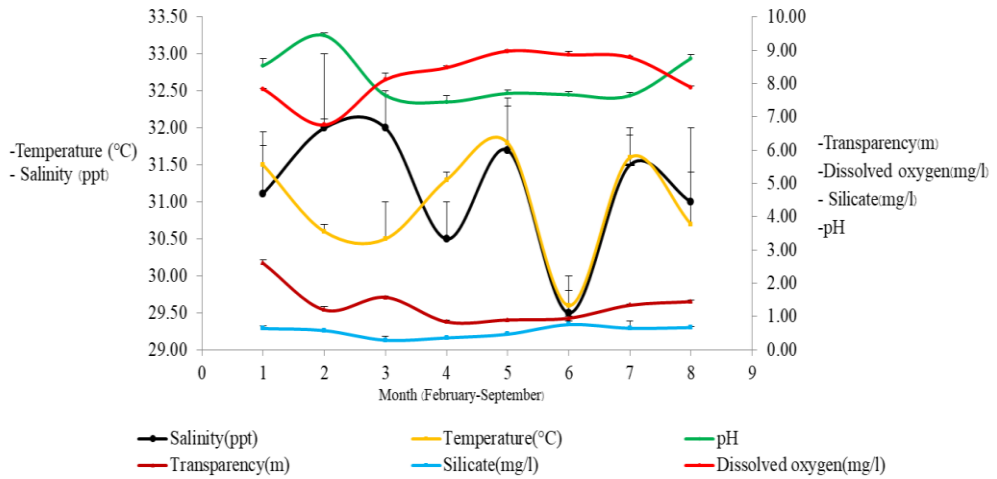


Figure 6 Water qualities pattern in the cultural area.

In this experiment, the concentration of silica in seawater was in the range of that in normal seawater; thus, it is not a limiting factor for sponge growth. Salinity relates to osmoregulation system, they can grow at salinity around 26-46 ppt. varying in species (Osinga *et al*., 2010). Transparency involve organic particle in sea water that deposit in water current cannel cause toxic and interrupt water flow in a sponge (Osinga *et al*., 2010).

A blue *Xestospongia* sponge produced RM and other derivatives of anticancer metabolites during the monsoon season (August-October) (Table 2); the maximum RM synthesized and accumulated in the sponge's tissue was observed in October (Figure 8). This phenomenon can be explained by environmental stress. During the monsoon season, the cultivation area was affected by the flow of the river from the mainland, leading to seawater dilution; in addition, physicochemical factors such as pH, salinity, and water temperature were changed. The fluctuation in temperature and salinity during the monsoon season (Figure 6) resulted in stressful conditions for the blue sponge; hence, it produces secondary metabolites that accumulate in its tissue, while its growth performance decreases due to the balanced energy budget. A stress-induced defensive system via growth metabolism incorporates the energy budget, and sponges demand extremely high amounts of energy (133%) for growth, as opposed to the increase in biomass reported for *H. panacea* (Thomassen and Riisgard, 1995).

The moderately IC_{50} value (10 µg/ml) implied the high potential of renieramycins to apply for a new anticancer agent. As the previously noted, RM inhibited several cancer cell lines in concentrations of nanomolar (nM) levels, for example HCT116, QG56, NCI-H460 and DLD cells at IC_{50} 7.9, 19.0, 5.9 and 9.6 nM, respectively (Suwanborirux *et al*., 2003). Those of IC_{50} values revealed that RM inhibit the broad cancer cells, but do not inhibit normal cell lines. In this work, we do not isolate a pure RM for examine anticancer. In total extract contained several minor derivatives and contaminated metabolites (Figure 5) that will be interfered the measurement. This phenomenon was the same case as found in halichondrin B, an anticancer metabolite, which was synthesized *by Lissodendoryx* sp. (Sipkema *et al*., 2005).

Conclusions

A blue Thai marine sponge, *Xestospongia* sp., can be cultivated under natural conditions to increase biomass and produce an anticancer metabolite, RM, as well as other derivatives, as an alternative method. The blue sponge was cultured in the Trang coastline by the vertical long line method using three substrates, including cement, a plastic rope, and PVC pipe, respectively. Results suggested that the growth of a blue sponge varies according to season, albeit with no certain pattern. The growth fluctuated in a short range in summer and the start of the monsoon season (February-July), and then it suddenly decreased in the middle of the monsoon season (August-November), indicating that the water quality in the culture area changes, particularly salinity, DO, and dissolved silicate. Based on sponge's plants observation, we suggest that cement piece should be a suitable substrate for this blue sponge. On the cement substrate, the form of growth of sponge showed plump shape and gave brilliant blue color indicated the healthy condition as observed in original habitat. The maximum amount of RM was synthesized in the monsoon season in October when the growth decreased. The potential of RM synthesis was affected by stress induction and energy budget processes.

Acknowledgements

This work supported by RMUTSV annual year project and Scholarship provided by Walailak University Fund (Contract no. 17/2557, 05/2560) to Yokseng, K. Authors would like to thank all of those research funds.

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