Ecological study and preliminary culture of the sponge Candidaspongia a source of anticancer molecules

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1) Paper Accepted with Major Revision
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Letter from AACL journal

cristian coroian <cristian_coroian@yahoo.com> 22 Maret 2019 02.12
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Dear author,

On behalf of AACL Journal, we are glad to announce you that your article entitled: Preliminary Explant of Sponge Candidaspongia for Production of Potent Anticancer Molecules, Candidaspongiolides has fulfill the evaluation process and now has the rating of: ACCEPTANCE WITH MAJOR MODIFICATIONS.

Reviewer 1 wrote:

The introductive part is valuable, including many scientific references from the international literature.

Materials and methods are well presented, presenting all the intermediate work steps required in the experiment.

Statistical analysis (one-way analysis of variance ANOVA) and Tukey test is accepted for this type of research.

The Discussions and conclusion are well organized, clearly explained, valuable and predictable. The Discussions are very extensive and explicit.

The bibliography in the text is the same as the one at the end of the paper, except Mayer et al 2009 which appears only in the end of article not in text.

Reviewer 2 comments are attached in a PDF file.
Our suggestion is to highly improve the English language of entire manuscript.

Please consider to fulfill ALL the reviewer's suggestions in shortest time. Any issues you might have please do not hesitate to address them to us. In attachment you have the files.

Best,

Cristian Ovidiu Coroian, *PhD*

AACL Journal Editor

[Kutipan teks disembunyikan]

M31_FT_reviewer 2.pdf
289K

2) Attachment from the Reviewer 22 Maret 2019

AACL BIOFLUX
Aquaculture, Aquarium, Conservation & Legislation
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Preliminary Explant of Sponge Candidaspongia for Production of Potent Anticancer Molecules, Candidaspongiolides

Abstract: Sponge *Candidaspongia* sp. is a source compound class that active against various cell line at nanogram level called cadidaspongiolide. However, the scarcity of the sponge in nature and complex structure become the major obstacles to further development. Sponge culture is a proven method for bioactive compounds production. The study was conducted in Kupang, Nusa Tenggara Timur, Indonesia. The sponge abundance was observed using modified Line intercept transect method at 12 m and 20 m. Some sponge colonies were cut for culture and chemical analyses. Then, the recovery rate of the sponge was observed after 60 days. Sponge culture was carried out at 6 m, 12 m and 25 m depth for 60 days. Inventory of the *Candidaspongia* sp. showed that the sponge density at around 12 m depth is lower than those in 20 m depth. All of the sponges were survive after the cut, and fully recovered in 60 days. The length and width increments of the basal part were 0.25-2.1 cm/month and 0.5-1.75 cm/month, respectively. The sponges cultured at 12 m and 25 m depth have higher survival and growth rates than those at 6 m depth. Descriptively, the sponge cultured in deeper water have higher ethyl acetate extracts content than those of cultured at the shallower water. Sponge culture is a possible method for supply the candidaspongiolide for further studies.

Key Words: sponge, candidaspongia, anticancer, mariculture, natural stock

Introduction. Sponges have been proven as productive sources of various compounds classes with pharmacological potency as antibacterial, antifungal, anthelmintic, antimalarial, antiviral, anti-inflammatory, anticoagulant, antioxidant and antitumor (Monroe, 2010, Trianto et al., 2014, Balansa et al., 2017).

Candidaspongia is considerably a rare marine sponge producing a potent bioactive compound called candidaspongiolide, a unique 18-membered macrolide. Candidaspongiolide, have also been reported exhibited remarkable cytotoxicity in NCI (National Cancer Institute) 60-cells-panel with GI_{50} of 14 ng/mL. Originally, the compound was isolated from the sponge collected from Australian and Papua New Guinean water (Meragelman et al., 2007). The candidaspongiolide and its derivatives also exhibited activity against melanoma (UACC-257, LOXIMVI, and M14), breast (MCF7) and lung cancer (NCI-H460) cell lines (Whitson et al., 2011).

In 2011, we published two new derivatives of candidaspongiolide along with the known one isolated from the sponge collected from Indonesia. The compounds exhibited potent cytotoxicity with IC_{50} 37.0, 4.7 and 19.0 ng/mL, against NBT-T2 cells (Trianto et al., 2011).

However, the scarcity of the sponge in nature and their complex structures would be an obstacle for further bioassay and development of

the compounds as an anticancer drug. Meyer and co-workers noted that until 2010, among thousands of compounds isolated from marine organisms, those were only a few compounds entered a clinical trial. Material supply is the main problem besides the bioactivity and uniquely pharmacological profile.

Several methods commonly used to overcome the material supply problem including synthesis (total and semi-synthesis), mariculture, ex-situ culture, and fermentation (Mendola, 2003). Synthesis is the most preferred method for Pharmaceutical companies to produce a drug due its efficiency, economically, and robustly.

However, considering the candidaspongiolide has several stereocenters, total synthesis would be inefficient due to the longer pathway including protection and de-protection of some functional groups. Stereochemistry is a key for biological activity (Butler, 2004). The best synthesis method of the related compound, Tedanolide, has been achieved in 31 steps and gave only in 0.31 % overall yield from the starting material (Smith and Lee, 2007). Therefore, big-scale production of candidaspongiolide or its analogs via chemical synthesis may not be economical due to some starting materials are also expensive. Tedanolide, isolated from the Caribbean marine sponge *Tedania ignis,* has been reported to exhibit strong cytotoxicity at pico to the nanomolar range (Schmitz et al., 1984).

Sponges have been cultured for mass production for bath sponge and providing bioactive substances (Milanese et al., 2003). Sipkema and co-worker showed that cultured of sponge *Lissodendoryx* sp. and *Dysidea avara* to produce anticancer compounds halichondrin B and avarol, respectively (Sipkema et al., 2005). Muller and co-workers (2000) successfully produced avarol from *Dysidea avara* via cell culture that is known as primmorphs. To the best our knowledge, this study is the first effort to culture the sponge *Candidaspongia sp.*

Material and Methods.

Stock assessment. The survey was conducted by Line Intercept Transect (LIT) method with a slight modification (Cleary et al., 2005). The sponges were observed along transects (6 x 100 m) placed in about 20 and 12 m depth with observation area around 3m on both sides.

Sponge collection. The sponges for explant were collected by hand during the survey. The sponges were cut the upper part of the colony to let the basal part re-growth for future stock (Mendola, 2003).

Sponge culture. The sponge was cultured *in situ* in Kupang Bay, East Nusa. Before the culture, the sponge colonies were tied at 12 m depth in a net for acclimation. After four days, the sponges were cut into ± 4 cm x 5 cm. Totally 15 fragments were explanted in three different nets placed at 25, 12 and 6m depth (five fragments per net). The sponges growth rate were measured in the end culture periods. All the procedures applied base

on the methods proposed by de Caralt and co-workers (2003), Mendola (2003) and Osinga and co-workers (2003).

Monitoring recovery and growth rates sponges. The basal parts of the sponges were observed by SCUBA diving method to evaluate the recovery and the growth rates after 60 days incision. The height, width, and the number of new branches were recorded. The ruler was used for measuring the height and width increment.

Extraction of the sponges

The harvested sponges were cut into small pieces and extracted with methanol for 24 H with triplicates. The extract was filtered with filter paper and concentrated with a rotary evaporator under vacuum. Then, the extract was subjected to the separatory funnel using ethyl acetate and water to provide the organic and water fractions (Trianto et al., 2011)

Data analysis. The growth rate and chemical contents data were analysed with one-way analysis of variance (ANOVA) and Tukey test to indicate whether the treatments have significant effect or not.

Results

Candidaspongia inventory and collection. The sponge inventory was conducted using modified *Line Intercept Transect Method* at 20 and 12 m depths. The average sponge densities were 1.7 and 0.3 colonies transect at 20 m and 12 m, respectively as shown in Table 1 and 2.

Table 1. The sponge *Candidaspongia* sp. colonies observed at 20 m depth.

Station	Colony(s) number	Average height (cm)	Average width (cm)
I	2	18.75	10.70
II	2	15.20	9.00
III	2	14.20	7.30
IV	0	-	-
V	1	19.00	13.20
VI	1	10.00	5.10
VII	2	10.20	7.00
Total colonies	12	13.55	8.39
Average	1.7		

However, four small colonies of the sponges were observed at 10 m depth under the port out of the line transect. Light intensity probably plays an important role in the larval settlement. However, the hypothesis still needs to be proven with further study.

Table 2. The sponge *Candidaspongia* sp. colonies observed at 12 m depth.

Station	Colony (s) number	Average height (cm)	Average width (cm)
I	1	12.10	8.30
II	0		
III	0		
IV	0		
V	0		
VI	0		
VII	1	8.20	10.40
Total colonies	2	10.15	9.35
Average	0.3		

Monitoring of sponges survival and growth after cutting. The sponge *Candidaspongia* sp. was observed by SCUBA diving method to evaluate the recovery rate and the growth after cutting. The basal part of the sponges was 100 % survive and could grow well (see Table 3). The average height and width increments were 1.83 cm and 2.85 cm, respectively.

Table 3. The sponge *Candidaspongia* sp. growth after cutting in 60 days incision.

Colony no.	Growth		Number of new lobes
	ΔHeight (cm)	ΔWidth (cm)	
1	0.5	1	5
2	3.5	3.6	0
3	0.5	4.2	4
4	3.5	2.9	0
5	3.0	1	2
6	3.0	2.5	2
Average	1.83	2.85	

Sponge culture. The sponge explanted at 25 m, 12 m and 6 m depth were observed *in situ* by SCUBA diving. The sponge cultured at 6 m has lower survival rate and negative growth rate, while the sponge cultured at 12 m and 25 m depths have higher survival and growth rates as shown in Table 4.

ANOVA test indicated that there is a significant effect of the treatments. Tukey HSD test showed that there is a significantly different growth rate among sponges cultured at 6 m and both 12 m and 25 m, but there are no differences between 12 m and 25 m (see Table 5 and 6).

Table 4. The average growth and survival cultured sponges after 60 days.

Depth	Growth	Survival

	ΔHeight (cm)	ΔWidth (cm)	cm^2	rate (%)
25 m	1.50	2.25	3.375	80
12 m	1.40	1.50	2.100	80
6 m	-0.75	-1.50	- 1.125	40

Table 5. ANOVA test of the sponges growth rates.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	19.350	2	9.675	6.300	.027
Within Groups	10.750	7	1.536		
Total	30.100	9			

Table 6. Tukey HSD test showed the differences growth rate among the sponge cultured at 6 m to the ones at 12 m and 20 m depth.

		Subset for alpha = 0.05	
Culture Depth	N	1	2
6	2	-1.5000	
12	4		1.5000
25	4		2.2500
Sig.		1.000	.748

Means for groups in homogeneous subsets are displayed.

(a) **(b)** **(c)**

Figure 1.Sponge *Candidaspongia* sp (a), explanted at 6 m depth showed loss of biomass, on the other hand, the explant at 12 m (b), and 25 m (c) growth well.

Chemical analysis. The sponges were extracted with methanol PA in Laboratory of Biotechnology, Department of Marine Sciences, Diponegoro University. Then, the crude extracts were separated into ethyl acetate and water fraction using a test tube. The result is shown in Table 7.

Table 7. Ethyl acetate and water extracts from cultured and natural sponge *Candidaspongia* sp.

No.	Sponge	Sponge	Ethyl acetate	Water extract

	Code	wet weight (g)	extract			
			Weight (mg)	Concent. %	Weight (mg)	Concent. %
1	C 25	16.56	0.20	1.24	0.30	1.82
2	C 10	14.97	0.14	0.93	0.26	1.63
3	N20	20.48	0.09	0.46	0.20	0.96
4	N12	26.70	0.05	0.17	0.61	2.30

Note: C-25: Cultured sponge at 25 m, C-10: Cultured sponge at 10 m, N-20: Natural sponge at 20 m, C-10: Natural sponge at 12 m.

Discussion. Sponge *Candidaspongia* sp. is known as a source of bioactive compounds with unique structure and high potent as anticancer drug candidate called candidaspongiolide (Meragelman et al., 2007, Trianto et al., 2011, Whitson et al., 2011). However, the scarcity of the sponges in nature and structural complexity of the candidaspongiolide have been hampering the development of the compounds into a commercial drug. Synthesis, the most preferred method by Pharmaceutical companies, is an unsuitable method for mass production of the candidaspongiolides due to compounds containing many stereo-centers.

To develop a mass production method, we conduct initial research in Kupang water including the sponge inventory, recovery rate after cutting, and mariculture. Based on our observation, the sponge density in nature is quite low where at around 20 m depth the average sponge density is 1.7 colonies per 100 m line transect length or 17 colonies per km transect length (see Table 1). Even, the sponge density at around 15 m depth is as low as 0.3 colonies per 100 m transect length or 3 colonies per km length (see Table 2). However, a group of colonies could be found at 10 m below the port, a protected area either from strong current or light intensity. The situation leads to the assumption that strong current and light intensity are limiting factors for the sponge growth. The sponge grows on hard substratum such as dead coral. Current is an important factor for the sponge growth because current brings the nutrient and oxygen, and at same time flush the CO2 and metabolisms product away. However, the strong current may damage the sponge colony. The sponge has photo sensory that sensitive to the certain light wave (Muller et al., 2006).

All of the sponges were survive after cutting, and they have fully recovered after 60 days. However, the growth rates of the basal parts were varied among the colonies. The highest length and wide increments were 4.2 cm and 3.5 cm in 60 days, respectively. The fastest grew on the basal part with uncut lamellae, for example, the growing direction on sponge no. 2 was down the side where the hanging lamella was uncut. And, the lowest length and wide increments is 0.5 cm and 1.0 cm in 60 days, respectively (see Table 3). However, the sponges that fully cut developed growing strategy by increasing the number of lamellae instead of higher the colony.

Even though *Candidaspogia* sp. maintains its colony basic shape, lamella, but it may become a complex branching lamellar colony with various thickness. The complexity of growth rate measurement is increase with natural habitat the sponge in deep water that causes restraining working period. This situation leads to obstruction for accurate growth rate measurements.

Mariculture is a possible method for mass production the sponge; even though, the further study still needed to standardize and to improve the method. The sponges explanted at 12 m and 25 m depth have 80 % survival rate. However, the sponges explanted at 5 m depth has only 40 % survival rate (see Table 4). The explant with four side wound could not survive due to loss of the mass and died or untied and driven away by the current. The explants grown in 5 m depth indicated the loss of mass cause the sponge dead or lost (see Figure 1).

Descriptively, the sponge explanted in deeper water have higher average growth rate than those explanted at the shallower water. The average growth rate of sponges explanted at 25 m, 12 m, and 6 m depth were 3.375 cm^2, 2.1 cm^2, and -1.125 cm^2 respectively. Size of the sponge explanted at 6 m decreased due to loss of biomass. However, further analyses with ANOVA did not show any significance between the treatments. Presumably, the number of samples were too small for ANOVA analyses, and some data were lost due to the sponge dead that increased the bias of the data. Increasing the number of samples is not easy regarding the natural stock of the Candidaspongia.

There are many factors related to a depth that may affect *Candidaspongia* sp. live and growth such as pressure, light intensity, nutrient, current, and sedimentation rate. We observed four small colonies of the sponge Candidaspongia that grow at 11 m depth below the harbor, a small and protected area either from current or direct sunlight. Based on our observation, there are only few *Candidaspongia* sp. that grow in open area, and among them usually grow under the hard coral or crevices. The further environmental study is needed to reveal the key factor of the sponge growth rate.

Water depth affects not only the growth rate and survival rate but also the chemical content. The ethyl acetate extracts of the natural and explanted sponges were affected by the depth. The extracts content were higher in the deeper water. Our previous research showed that the anticancer compounds, candidaspongiolide, and its analogs, were obtained from organic fraction (Trianto et al., 2011). It is presumably that biosynthesis of the compounds was highly affected by pressure. However, the ethyl acetate concentration on explanted sponges was lower than this natural one. It can be explained that the explant is still using higher energy for recovery and growth. The development of mass production of candidaspongiolide via sponge culture is still in a preliminary study that needs many strategies to overcome the problem regarding the environmental factors and the number of explants. However, all of the difficulties are in proportion to the potency of the sponge as a source of the anticancer drug candidate.

Conclusion. The sponge *Candidaspongia* sp density in deeper water is higher than in the lower water. All of the sponges were survive after the cut, and they have fully recovered in 60 days. The highest length and wide increments were 4.2 cm and 3.5 cm for 60 days, respectively. The lowest length and wide increments are 0.5 cm and 1.0 cm for 60 days, respectively.

The sponges explanted at 25 m and 12 m depth have higher survival rate than the sponges explanted at 6 m depth. Descriptively, the sponge explanted in deeper water have higher average growth rate and ethyl acetate extract than those explanted at the shallower water. However, further analyses with ANOVA did not show any significance between the treatments.

Acknowledgments

We thank Yusup, S.Kel of The Nature Conservation Kupang for assisting in field work. We also indebted BKKPN-Kupang for providing Diving Equipment. This research was partially funded by PNBP-DIPONEGORO UNIVERSITY with contract number: 261.14/UN7.5/ ST/ 2012.

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3) Mail from the author about the Review 22 Maret 2019

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Letter from AACL journal

Agus Trianto <agustrianto.undip@gmail.com> 22 Maret 2019 03.51
Kepada: cristian coroian <cristian_coroian@yahoo.com>

Dear Dr. Cristian,

Many thanks for your fast response. I will revise the manuscript as soon as possible, hopefully, It can be finished next tuesday.

Bsst regards,

Dr. Agus Trianto

Department of Marine Sciences

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50275

[Kutipan teks disembunyikan]

4) Revision Sent
25 Maret 2019

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Letter from AACL journal

Agus Trianto <agustrianto.undip@gmail.com> 25 Maret 2019 10.46
Kepada: cristian coroian <cristian_coroian@yahoo.com>

Dear Dr. Cristioan Coroian,

I have revised the manuscript as suggested by the reviewers as shown in the attachment. The manuscript title is Preliminary Explant of Sponge Candidaspongia for Production of Potent Anticancer Molecules, Candidaspongiolides.

Hopefully, the paper can be published soon.

Best regards,

Dr. Agus Trianto

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[Kutipan teks disembunyikan]

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5) Second Review
30 Maret 2019

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Letter from AACL journal

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Balas Ke: cristian coroian <cristian_coroian@yahoo.com>
Kepada: Agus Trianto <agustrianto.undip@gmail.com>

Dear Author,

There are still some minor changes to be done for finishing the manuscript.
In atachment you will find a PDF file. Please WORK ON THAT FORMAT!
Very important to complete those requirements!

Best regards,

Cristian-Ovidiu Coroian, *PhD*

[Kutipan teks disembunyikan]

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6) Comments Second Review
30 Maret 2019

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Ecological study and preliminary culture of the sponge Candidaspongia a source of anticancer molecules

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There is missing the corresponding author!

Abstract: Sponge *Candidaspongia* sp. is a source of candidaspongiolide, a very potent anticancer macrolide that active against various cell lines at nanogram level. However, low abundance of the sponge in nature and structurally complex of candidaspongiolide have become the major obstacles for the drug development. This study aims to assess the feasibility of the production of the anticancer compounds, the candidaspongiolides, using sponge culture. The study was conducted in Kupang, Nusa Tenggara Timur, Indonesia. The sponge abundance was observed using modified Line Intercept Transect method at 12 m and 20 m. Some sponge colonies were cut for culture and chemical analyses. Then, the recovery rate of the sponge was observed after 60 days. Sponge culture was carried out at 6 m, 12 m and 25 m depth for 60 days. Inventory of the *Candidaspongia* sp. showed that the sponge density at around 12 m depth is lower than those in 25 m depth. All of the sponges were survive after the cut and fully recovered in 60 days. The length and width increments of the basal part were 0.25-2.1 cm/month and 0.5-1.75 cm/month, respectively. The sponges cultured at 12 m and 25 m depth have higher survival and growth rates than those at 6 m depth. Descriptively, the sponge cultured in deeper water have higher ethyl acetate extracts content than the sponge cultured at the shallower water. Sponge mariculture is a possible method to supply candidaspongiolide for further studies.

Key Words: Ethyl acetate, candidaspongia, anticancer, mariculture**,** natural stock

Introduction. Sponges have been proven as productive sources of various compounds classes with pharmacological potency as antibacterial, antifungal, anthelmintic, antimalarial, antiviral, anti-inflammatory, anticoagulant, antioxidant and antitumor (Monroe 2010; Trianto et al 2014; Balansa et al 2017). Candidaspongia is a rare marine sponge that produces a potent anticancer compound called candidaspongiolide, a unique 18-membered macrolide. Candidaspongiolides have also been reported as exhibited remarkable cytotoxicity in NCI (National Cancer Institute) 60-cells-panel with GI_{50} of 14 ng/mL. Originally, the compound was isolated from the sponge collected from Australian and Papua New Guinean waters (Meragelman et al 2007). Candidaspongiolide and its derivatives also exhibited activity against melanoma (UACC-257, LOXIMVI, and M14), breast (MCF7) and lung cancer (NCI-H460) cell lines (Whitson et al 2011).

In 2011, we identified two new derivatives of the candidaspongiolide along with the known one isolated from the sponge collected in Indonesia. The compounds exhibited potent cytotoxicity with IC_{50} 37.0, 4.7 and 19.0 ng/mL, against NBT-T2 cells (Trianto et al 2011). However, low abundance of the sponge in nature and structurally complex of candidaspogiolide have become the major obstacles for the drug development. This study aims to assess the feasibility of the production of the anticancer compounds, the

candidaspongiolides, using sponge culture. Mayer et al (2010) noted that among thousands of bioactive compounds isolated from marine organisms, those were only a few compounds entered a clinical trial. Material supply is the main problem besides the bioactivity and the pharmacological profile.

There are several methods that are commonly used to supply the bioactive compounds including chemical synthesis, mariculture, closed system culture, and fermentation (Mendola 2003). The most preferred method to produce a drug is chemical synthesis because of its efficiency, economically, and robustly. However, considering the candidaspongiolide has several stereocenters, total synthesis would be impractical due to the longer pathway. Stereochemistry is a key to activity in biological systems (Butler 2004). The best synthesis method of one of the related compounds, tedanolide, has been achieved in 31 steps and gave only 0.31% of overall yield from the starting material (Smith & Lee 2007). Therefore, big-scale production of candidaspongiolide or its analogs via chemical synthesis may not be an economical method due to the high price of some starting materials. Tedanolide, isolated from the Caribbean marine sponge *Tedania ignis,* has been reported to exhibit strong cytotoxicity at pico to the nanomolar range (Schmitz et al 1984).

Sponges have been cultured for mass production of bath sponge and providing bioactive substances (Milanese et al 2003). Sipkema et al (2005) showed that culture of sponges *Lissodendoryx* sp. and *Dysidea avara* was able to produce anticancer compounds as halichondrin B and avarol, respectively. Muller et al (2000) successfully produced avarol from *Dysidea avara* via a cell culture that is known as primmorphs. To the best of our knowledge, this study is the first effort to culture the sponge *Candidaspongia* sp.

Material and Methods.

Candidaspongia inventory. The survey was conducted by Line Intercept Transect (LIT) method with a slight modification (Cleary et al 2005). The sponges were observed along transects (6 x 100 m) placed in 12 and 20 m depth with observation area around 3 m on both sides.

Sponge collection. The sponge colonies for explant and extract were collected by hand during the survey. The upper part of the sponge colonies was cut to let the basal part (about 5 cm height) re-growth for future stock (Mendola 2003).

Sponge culture. The sponge was cultured *in situ* in Kupang Bay, East Nusa Tenggara, Indonesia. Before the culture, the sponge colonies were tied at 12 m depth in a net for acclimation. After four days, the sponge colonies were cut into ±3 cm x 5 cm. 15 fragments were explanted in three different nets placed at 6, 12, and 25 m depths (five fragments per net). The sponge's growth rate were measured at the end of culture period. All the procedures applied were based on the methods proposed by de Caralt et al (2003), Mendola (2003), and Osinga et al (2003).

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Monitoring of the recovery and growth rates of the sponges. The basal parts of the sponge colonies were observed by SCUBA diving method to evaluate the recovery and the growth rates after 60 days from cutting. The height, width, and the number of new branches were recorded. The ruler was used for measuring the height and width increment.

Extraction of the sponges. The harvested sponges were cut into small pieces and extracted with methanol for 24 hours with triplicates. The extract was filtered with filter paper and concentrated with a rotary evaporator under vacuum. Then, the extract was subjected to the separatory funnel using ethyl acetate and water to provide the organic and water fractions (Trianto et al 2011).

Data analysis. The growth rate and the ethyl acetate (EA) extract contents data were analyzed with the Mann-Whitney test, to indicate whether the treatments have a

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significant effect or not. Mann-Whitney test is a non-parametric test that was chosen due to the data are not distributed normally and homogenously.

Results

Candidaspongia inventory and collection. The sponge inventory was conducted using a modified Line Intercept Transect Method at 12 and 20 m depths. The average sponge densities were 0.3 and 1.7 colonies transect at 12 m and 20 m respectively, as shown in Tables 1 and 2.

Table 1

The sponges of *Candidaspongia* sp. colonies observed at 12 m depth

Station	*Colony(s) number*	*Average height (cm)*	*Average width (cm)*
I	1	12.10	8.30
II	0		
III	0		
IV	0		
V	0		
VI	0		
VII	1	8.20	10.40
Total colonies	2	10.15	9.35
Average	0.3		

Table 2

The sponges of *Candidaspongia* sp. colonies observed at 20 m depth

Station	*Colony(s) number*	*Average height (cm)*	*Average width (cm)*
I	2	18.75	10.70
II	2	15.20	9.00
III	2	14.20	7.30
IV	0	-	-
V	1	19.00	13.20
VI	1	10.00	5.10
VII	2	10.20	7.00
Total colonies	12	13.55	8.39
Average	1.7		

However, four small colonies of the sponges were observed at 10 m depth under the port out of the line transects. Light intensity probably plays an important role in the larval settlement. However, the hypothesis still needs to be proven with further study.

Monitoring of sponge colonies survival and growth rates after the cut. The sponges of *Candidaspongia* sp. colonies were observed by SCUBA diving method to evaluate the recovery rate and the growth after the cut. The basal parts of the sponge were survive 100% and grown well (see Table 3). The average height and width increments were 1.83 cm and 2.85 cm, respectively.

Table 3

The basal parts of sponge *Candidaspongia* sp. grown after 60 days from cut at around 20 m depth

Colony no.	*Growth*		*Number of new lobes*
	Δ Height (cm)	*Δ Width (cm)*	
1	0.5	1	5
2	3.5	3.6	0
3	0.5	4.2	4
4	3.5	2.9	0
5	3.0	1	2
6	3.0	2.5	2
Average	1.83	2.85	2.17

Sponge culture. The survival and growth rates of the sponge explanted at 6, 12 and 25 m depths were observed *in situ* by SCUBA diving. The sponge cultured at 6 m has lower survival and growth rates, while the sponge cultured at 12 and 25 m depths have higher survival and growth rates as shown in Table 4.

Table 4

The average growth and survival cultured sponges after 60 days

Depth	*Growth*			*Survival rate (%)*
	ΔHeight (cm)	*ΔWidth (cm)*	*cm^2*	
6 m	-0.75	-1.50	- 1.125	40
12 m	1.40	1.50	2.100	80
25 m	1.50	2.25	3.375	80

ANOVA test indicated that depth has a significant effect on the sponge colonies growth. The further test, Tukey HSD test, showed that the sponge growth rate at 6 m was significantly different with a sponge growth rate at 12 and 25 m, but there are no differences of the growth rates between 12 and 25 m (see Table 5 and 6).

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Comment [A5]: You also have to mention and describe this test into Statistical Data Analysis section (material and methods).

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Depth	*ΔL (cm)*	*ΔW (cm)*	*ΔA (%)*	*SR(%)*
6 m	-1,0	-2,0	-73,3	40
	-0,5	-1,0	-40,0	
Average	-0,8	-1,5	-56,7	
12 m	0,5	0,0	10,0	80
	2,0	1,0	113,3	
	2,0	3,0	260,0	
	1,0	2,0	100,0	
Average	1,4	1,5	120,8	
25 m	1,5	1,0	73,3	80
	1,0	1,5	80,0	
	2,0	2,5	203,3	
	1,5	4,0	203,3	
Average	1,5	2,3	140,0	

Note: ΔL: Length increment, ΔW: Wide increment, ΔA: Area increment

(a) **(b)** **(c)**

Figure 1. The sponge *Candidaspongia* sp. explanted at 6 m depth showed loss of biomass (a), while the explants at 12 m (b), and 25 m (c) grew well.

Chemical analysis. The sponges were extracted with methanol PA in laboratory of Biotechnology, department of Marine Sciences, Diponegoro University. Then, the crude extracts were separated into ethyl acetate (EA) and water fraction using a test tube. The result is shown in Table 7.

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Table 7

Ethyl acetate and water extracts from cultured and natural sponges of *Candidaspongia* sp.

No.	*Sponge code*	*Sponge wet weight (g)*	*Ethyl acetate extract*		*Water extract*	
			Weight (mg)	*Content (%)*	*Weight (mg)*	*Content (%)*
1	C-12	14.97	0.14	0.93	0.26	1.63
2	C-25	16.56	0.20	1.24	0.30	1.82
3	N-12	26.70	0.05	0.17	0.61	2.30
4	N-20	20.48	0.09	0.46	0.20	0.96

Note: C-12: Cultured sponge at 12 m, C-25: Cultured sponge at 25 m, N-12: Natural sponge cultured at 12 m, N-20: Natural sponge cultured at 20 m.

Discussion. Sponge *Candidaspongia* sp. is known as a source of bioactive compounds with unique structure and high potent as anticancer drug candidate called candidaspongiolide (Meragelman et al 2007; Trianto et al 2011; Whitson et al 2011). However, the scarcity of the sponges in nature and structural complexity of the candidaspongiolide have been hampering the development of the compounds into a commercial drug. Synthesis, the most preferred method by pharmaceutical companies, is an unsuitable method for mass production of the candidaspongiolides due to compounds containing many stereo-centers. Tadpecth et al (2017) showed the synthesis of a macrolide greensporone C using 16 steps with overall yield 3%. Synthesis of the (-)-hortonone C has also provided a yield as low as 1 % with 11 steps (Niroula et al 2017). Light has a great effect on the metabolism rate for the sponge-associated microorganisms since spicule can be used as light transduction for the microorganisms live inside the colony (Brümmer et al 2016). In turn, the metabolites will give an impact to the host.

To develop a mass production method, we conduct initial research for the sponge culture in Kupang water including the sponge inventory, recovery rate after cutting, and mariculture. Based on our observation, the sponge density in nature is quite low. The average sponge density is 1.7 colonies per 100 m line transect length or 17 colonies per km tt around 20 m depth. Even, the sponge density at around 15 m depth is as lower as 0.3 colonies per 100 m transect length or 3 colonies per km length. However, a group of small sponge colonies could be found at 10 m below the port, a protected area either from strong current or light intensity. The situation leads to the assumption that strong current and light intensity may be the limiting factors for sponge growth. Larval swimming behavior is highly affected by light and temperature. The stronger light intensity reduces the swimming periods of the larval of sponge *Hymeniacidon perlevis* (Xue et al 2009).

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Current is an important factor for the sponge growth because current brings the nutrient and oxygen, and at the same time flush the CO_2 and metabolisms products away. However, the strong current may damage the sponge colony. The sponge has photo sensory that are sensitive to the certain light wave (Muller et al 2006). The sponge grows on hard substratum such as dead coral.

All of the sponge colonies were survive after the cut, and they have fully recovered after 60 days. However, the growth rates of the basal parts were varied among the colonies. The highest length and wide increments were 4.2 cm and 3.5 cm in 60 days, respectively. The fastest grew on the basal part with uncut lamellae, for example, the growing direction on sponge no. 2 was down the side where the hanging lamella was uncut. And, the lowest length and wide increments is 0.5 cm and 1.0 cm in 60 days, respectively (see Table 3). However, the sponges that were fully cut developed a growing strategy by increasing the number of lamellae instead of increase the colony size.

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Even though the *Candidaspogia* sp. maintains its colony basic shape as lamella, but it may become a complex branching lamellar colony with various thickness. The complexity of growth form has correlated with water depth and the protected level of the natural habitat the sponge.

Mariculture is a feasible method for mass production of the sponge; even though, the further study still needed to standardize and to improve the method. The sponges explanted at 12 m and 25 m depth have an 80% survival rate. However, the sponges explanted at 6 m depth had only a 40% survival rate). The explant with four side incision could not survive due to loss of the mass and died or untied and driven away by the current. The explants grown in 6 m depth indicated the loss of mass because the sponge dead or lost (see Figure 1). Louden et al (2007) reported the *in situ* culture of sponges *Rhopaloeides odorabile,* and *Coscinoderma* sp. have survival rates of 65 % and 90% for 78 days. The sponge has also dead up to 3% naturally, and up to 7% caused by accidentally fishing (Butler et al 2017). The total growth of *R. odorabile* (146.0±40.3%) and *Coscinoderma* sp. (195.9±39.8%) was not significantly different over the 21 month experimental period but was highly variable between explants from the same individual.

Descriptively, the sponge explanted in deeper water has a higher average growth rate than those explanted at the shallower water. The average growth rate of sponges explanted at 25 m, 12 m, and 6 m depth were 3.375 cm^2, 2.1 cm^2, and -1.125 cm^2, respectively. Size of the sponge explanted at 6 m decreased due to loss of biomass. However, further analyses with ANOVA showed a significant effect between the culture depth. There are many factors related to a depth that may affect *Candidaspongia* sp. live and growth such as pressure, light intensity, nutrient, current, and sedimentation rate. We observed four small colonies of the sponge Candidaspongia that grow at 10 m depth below the harbor, a small and protected area either from current or direct sunlight. Based on our observation, there are only few *Candidaspongia* sp. that grow in open area, and among them usually, grow under the hard coral or crevices. Further environmental study is needed to reveal the key factor of the sponge growth rate.

Our previous research showed that the anticancer compounds the candidaspongiolide and its analogs were obtained from organic fraction (Trianto et al 2011). So, in this study, we pay more attention to the ethyl acetate content in the sponge. The EA extract content in the transplanted and the natural sponges were not significantly different (Mann-Whitney test, U=6.00). However, water depth affects not only the growth rate and survival rate but also the chemical content. The EA extracts of the natural and explanted sponges were affected by the depth. The extracts content in transplanted sponges were higher in the deeper water (Mann-Whitney test, U=0.00), on the other hand, the extract contents in natural sponges were higher in the lower water (Mann-Whitney test, U=0.00). Naturally, sponges produce bioactive compounds that support their survival, including from bacterial infection. The marine sponge reported produces the antibacterial compounds to protect the colony (Yu et al 2017).

The development of large-scale production of the candidaspongiolide via sponge culture is still in a preliminary study that needs many strategies to overcome the problem regarding the environmental factors and the number of explants. However, the difficulties are in proportion to the potency of the sponge as a source of the anticancer drug

candidate. Sponge culture is a promising method to overcome the bottleneck in drug development and to avoid the over-exploitation of wild population (Pérez-López et al 2014).

Conclusion. The sponge *Candidaspongia* sp. density in deeper water is higher than in the lower water. All of the sponges were survive after the cut, and they have fully recovered in 60 days. The growth rate of the explants were 10%-60% and 73%-203% at 12 m and 25 m respectively. The explants in 6 m have a negative growth rate.

The sponges explanted at 12 and 25 m depth have higher survival and growth rates than the sponge explanted at 6 m depth. The sponge explanted in deeper water has higher EA extract than those explanted at the shallower water.

Acknowledgments

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7) Second Review Revised 10 Mei 2019

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Dear Dr. Cristian Coroian,

Herein, I am sending the revised manuscript entitled: "Ecological Study and Preliminary Culture of The Sponge Candidaspongia, A Source of Anticancer Molecules" in the attachment. I do hope, the manuscript is already fit the journal standard, and can be published soon.

Thanks for your patience and assistance.

Best regards,

Dr. Agus Trianto

Department of Marine Sciences

Fact. of Fisheries and Marine Sciences, Diponegoro University

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8) Third Review
11 Juni 2019

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9) Comments on Third Review
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Ecological study and preliminary culture of the sponge Candidaspongia a source of anticancer molecules

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Abstract: Sponge *Candidaspongia* sp. is a source of candidaspongiolide, a very potent anticancer macrolide that active against various cell lines at nanogram level. However, low abundance of the sponge in nature and structurally complex of candidaspongiolide have become the major obstacles for the drug development. This study aims to assess the feasibility of the production of the anticancer compounds, the candidaspongiolides, using sponge culture. The study was conducted in Kupang, Nusa Tenggara Timur, Indonesia. The sponge abundance was observed using modified Line Intercept Transect method at 12 m and 20 m. Some sponge colonies were cut for culture and chemical analyses. Then, the recovery rate of the sponge was observed after 60 days. Sponge culture was carried out at 6 m, 12 m and 25 m depth for 60 days. Inventory of the *Candidaspongia* sp. showed that the sponge density at around 12 m depth is lower than those in 25 m depth. All of the sponges were survive after the cut and fully recovered in 60 days. The length and width increments of the basal part were 0.25-2.1 cm/month and 0.5-1.75 cm/month, respectively. The sponges cultured at 12 m and 25 m depth have higher survival and growth rates than those at 6 m depth. Descriptively, the sponge cultured in deeper water have higher ethyl acetate extracts content than the sponge cultured at the shallower water. Sponge mariculture is a possible method to supply candidaspongiolide for further studies.

Key Words: Ethyl acetate, candidaspongia, anticancer, mariculture**,** natural stock

Introduction. Sponges have been proven as productive sources of various compounds classes with pharmacological potency as antibacterial, antifungal, anthelmintic, antimalarial, antiviral, anti-inflammatory, anticoagulant, antioxidant and antitumor (Monroe 2010; Trianto et al 2014; Balansa et al 2017). Candidaspongia is a rare marine sponge that produces a potent anticancer compound called candidaspongiolide, a unique 18-membered macrolide. Candidaspongiolides have also been reported as exhibited remarkable cytotoxicity in NCI (National Cancer Institute) 60-cells-panel with GI_{50} of 14 ng/mL. Originally, the compound was isolated from the sponge collected from Australian and Papua New Guinean waters (Meragelman et al 2007). Candidaspongiolide and its derivatives also exhibited activity against melanoma (UACC-257, LOXIMVI, and M14), breast (MCF7) and lung cancer (NCI-H460) cell lines (Whitson et al 2011).

In 2011, we identified two new derivatives of the candidaspongiolide along with the known one isolated from the sponge collected in Indonesia. The compounds exhibited potent cytotoxicity with IC_{50} 37.0, 4.7 and 19.0 ng/mL, against NBT-T2 cells (Trianto et al 2011). However, low abundance of the sponge in nature and structurally complex of candidaspogiolide have become the major obstacles for the drug development. This study aims to assess the feasibility of the production of the anticancer compounds, the

candidaspongiolides, using sponge culture. Mayer et al (2010) noted that among thousands of bioactive compounds isolated from marine organisms, those were only a few compounds entered a clinical trial. Material supply is the main problem besides the bioactivity and the pharmacological profile.

There are several methods that are commonly used to supply the bioactive compounds including chemical synthesis, mariculture, closed system culture, and fermentation (Mendola 2003). The most preferred method to produce a drug is chemical synthesis because of its efficiency, economically, and robustly. However, considering the candidaspongiolide has several stereocenters, total synthesis would be impractical due to the longer pathway. Stereochemistry is a key to activity in biological systems (Butler 2004). The best synthesis method of one of the related compounds, tedanolide, has been achieved in 31 steps and gave only 0.31% of overall yield from the starting material (Smith & Lee 2007). Therefore, big-scale production of candidaspongiolide or its analogs via chemical synthesis may not be an economical method due to the high price of some starting materials. Tedanolide, isolated from the Caribbean marine sponge *Tedania ignis,* has been reported to exhibit strong cytotoxicity at pico to the nanomolar range (Schmitz et al 1984).

Sponges have been cultured for mass production of bath sponge and providing bioactive substances (Milanese et al 2003). Sipkema et al (2005) showed that culture of sponges *Lissodendoryx* sp. and *Dysidea avara* was able to produce anticancer compounds as halichondrin B and avarol, respectively. Muller et al (2000) successfully produced avarol from *Dysidea avara* via a cell culture that is known as primmorphs. To the best of our knowledge, this study is the first effort to culture the sponge *Candidaspongia* sp.

Material and Methods.

Candidaspongia inventory. The survey was conducted by Line Intercept Transect (LIT) method with a slight modification (Cleary et al 2005). The sponges were observed along transects (6 x 100 m) placed in 12 and 20 m depth with observation area around 3 m on both sides.

Sponge collection. The sponge colonies for explant and extract were collected by hand during the survey. The upper part of the sponge colonies was cut to let the basal part (about 5 cm height) re-growth for future stock (Mendola 2003).

Sponge culture. The sponge was cultured *in situ* in Kupang Bay, East Nusa Tenggara, Indonesia. Before the culture, the sponge colonies were tied at 12 m depth in a net for acclimation. After four days, the sponge colonies were cut into 3 cm x 5 cm (width x length). 15 fragments were explanted in three different nets placed at 6, 12, and 25 m depths (five fragments per net). The sponge's growth rate were measured at the end of culture period. All the procedures applied were based on the methods proposed by de Caralt et al (2003), Mendola (2003), and Osinga et al (2003).

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Monitoring of the recovery and growth rates of the sponges. The basal parts of the sponge colonies were observed by SCUBA diving method to evaluate the recovery and the growth rates after 60 days from cutting. The height, width, and the number of new branches were recorded. The ruler was used for measuring the height and width increment.

Extraction of the sponges. The harvested sponges were cut into small pieces and extracted with methanol for 24 hours with triplicates. The extract was filtered with filter paper and concentrated with a rotary evaporator under vacuum. Then, the extract was subjected to the separatory funnel using ethyl acetate and water to provide the organic and water fractions (Trianto et al 2011).

Data analysis. The growth rate and the ethyl acetate (EA) extract contents data were analyzed with the Mann-Whitney test, to indicate whether the treatments have a

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significant effect or not. Mann-Whitney test is a non-parametric test that was chosen due to the data are not distributed normally and homogenously. Statistical analyses were performed using SPSS ver.16 software.

Results

Candidaspongia inventory and collection. The sponge inventory was conducted using a modified Line Intercept Transect Method at 12 and 20 m depths. The average sponge densities were 0.3 and 1.7 colonies transect at 12 m and 20 m respectively, as shown in Tables 1 and 2.

Table 1

The sponges of *Candidaspongia* sp. colonies observed at 12 m depth

Station	*Colony(s) number*	*Average height (cm)*	*Average width (cm)*
I	1	12.10	8.30
II	0		
III	0		
IV	0		
V	0		
VI	0		
VII	1	8.20	10.40
Total colonies	2	10.15	9.35
Average	0.3		

Table 2

The sponges of *Candidaspongia* sp. colonies observed at 20 m depth

Station	*Colony(s) number*	*Average height (cm)*	*Average width (cm)*
I	2	18.75	10.70
II	2	15.20	9.00
III	2	14.20	7.30
IV	0	-	-
V	1	19.00	13.20
VI	1	10.00	5.10
VII	2	10.20	7.00
Total colonies	12	13.55	8.39
Average	1.7		

However, four small colonies of the sponges were observed at 10 m depth under the port out of the line transects. Light intensity probably plays an important role in the larval settlement. However, the hypothesis still needs to be proven with further study.

Monitoring of sponge colonies survival and growth rates after the cut. The sponges of *Candidaspongia* sp. colonies were observed by SCUBA diving method to evaluate the recovery rate and the growth after the cut. The basal parts of the sponge were survive 100% and grown well (see Table 3). The average height and width increments were 1.83 cm and 2.85 cm, respectively.

Table 3

The basal parts of sponge *Candidaspongia* sp. grown after 60 days from cut at around 20 m depth

Colony no.	*Growth*		*Number of new lobes*
	Δ Height (cm)	*Δ Width (cm)*	
1	0.5	1	5
2	3.5	3.6	0
3	0.5	4.2	4
4	3.5	2.9	0
5	3.0	1	2
6	3.0	2.5	2
Average	1.83	2.85	2.17

Sponge culture. The survival and growth rates of the sponge explanted at 6, 12 and 25 m depths were observed *in situ* by SCUBA diving. The sponge cultured at 6 m has lower survival and growth rates, while the sponge cultured at 12 and 25 m depths have higher survival and growth rates as shown in Table 4.

Table 4

The average growth and survival cultured sponges after 60 days

Depth	*Growth*			*Survival rate (%)*
	ΔHeight (cm)	*ΔWidth (cm)*	*cm^2*	
6 m	-0.75	-1.50	- 1.125	40
12 m	1.40	1.50	2.100	80
25 m	1.50	2.25	3.375	80

Table 4. The average growth and survival cultured sponges after 60 days.

Depth	**ΔL (cm)**	**ΔL (%)**	**ΔW (cm)**	**ΔW (%)**	**ΔA (%)**	**SR(%)**
6 m	-1,0	-20,0	-2,0	-66,7	-73,3	40
	-0,5	-10,0	-1,0	-33,3	-40,0	
Average	-0,8	-16,0	-1,5	-50,0	-56,7	
12 m	0,5	10,0	0,0	0,0	10,0	80
	2,0	40,0	1,0	33,3	113,3	
	2,0	40,0	3,0	100,0	260,0	
	1,0	20,0	2,0	66,7	100,0	
Average	1,4	28,0	1,5	50,0	120,8	
25 m	1,5	30,0	1,0	33,3	73,3	80
	1,0	20,0	1,5	50,0	80,0	
	2,0	40,0	2,5	83,3	203,3	
	1,5	30,0	4,0	133,3	203,3	
Average	1,5	30,0	2,3	76,7	140,0	

Note: ΔL: Length increment, ΔW: Width increment, ΔA: Area increment

ANOVA test indicated that depth has a significant effect on the sponge colonies growth. The further test, Tukey HSD test, showed that the sponge growth rate at 60 days for 6 m was significantly different with a sponge growth rate at 12 and 25 m, but there are no differences of the growth rates between 12 and 25 m (see Table 5).

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Table 5

The Tukey test of the growth cultured sponges expressed by the area increment for 60 days

Area Increment

	Depth	N	Subset	
			1	2
Tukey HSD[a,b]	6	4	-78.325	
	12	4		120.825
	25	4		139.975
	Significance		1.000	0.931

Means for groups in homogeneous subsets are displayed based on observed means.

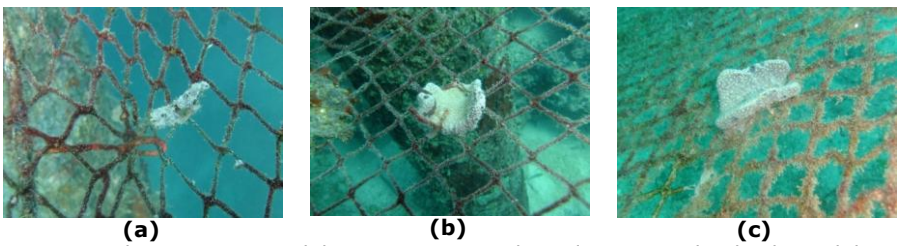
The error term is Mean Square (Error) = 5626.500.

a. Uses Harmonic Mean Sample Size = 4.000.

b. Alpha =0.05.

Depth	*ΔL (cm)*	*ΔW (cm)*	*ΔA (%)*	*SR(%)*
6 m	-1,0	-2,0	-73,3	40
	-0,5	-1,0	-40,0	
Average	-0,8	-1,5	-56,7	
12 m	0,5	0,0	10,0	80
	2,0	1,0	113,3	
	2,0	3,0	260,0	
	1,0	2,0	100,0	
Average	1,4	1,5	120,8	
25 m	1,5	1,0	73,3	80
	1,0	1,5	80,0	
	2,0	2,5	203,3	
	1,5	4,0	203,3	
Average	1,5	2,3	140,0	

Note: ΔL: Length increment, ΔW: Wide increment, ΔA: Area increment



(a) **(b)** **(c)**

Figure 1. The sponge *Candidaspongia* sp. explanted at 6 m depth showed loss of biomass (a), while the explants at 12 m (b), and 25 m (c) grew well.

Chemical analysis. The sponges were extracted with methanol PA in laboratory of Biotechnology, department of Marine Sciences, Diponegoro University. Then, the crude

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extracts were separated into ethyl acetate (EA) and water fraction using a test tube. The result is shown in Table 6.

Table 6

Ethyl acetate and water extracts from cultured and natural sponges of *Candidaspongia* sp.

No.	*Sponge code*	*Sponge wet weight (g)*	*Ethyl acetate extract*		*Water extract*	
			Weight (mg)	*Content (%)*	*Weight (mg)*	*Content (%)*
1	C-12	14.97	0.14	0.93	0.26	1.63
2	C-25	16.56	0.20	1.24	0.30	1.82
3	N-12	26.70	0.05	0.17	0.61	2.30
4	N-20	20.48	0.09	0.46	0.20	0.96

Note: C-12: Cultured sponge at 12 m, C-25: Cultured sponge at 25 m, N-12: Natural sponge cultured at 12 m, N-20: Natural sponge cultured at 20 m.

The Mann-Whitney U test indicated that the EA extract concentration in natural and cultured is no significantly difference (see Table 7a). However, water depth gives significant effect to the EA extract concentration both in natural and cultured sponges (see Table 7b and c).

Table 7

The Mann-Whitney U test of ethyl acetate extracts of the *Candidaspongia* sp.
a. cultured vs natural sponge.
b. The Shallow water vs deep water natural sponge extracts. c. The Shallow water vs deep water culture sponge extracts

a	EA extract
Mann-Whitney U	*5,000*
Wilcoxon W	*15,000*
Z	*-,866*
Asymp. Sig. (2-tailed)	*,386*
Exact Sig. [2(1-tailed Sig.)]*	*,486*[b]

a. Grouping variable: Depth
b. Not corrected for ties.

b	EA extract
Mann-Whitney U	*.000*
Wilcoxon W	*3.000*
Z	*-1.549*
Asymp. Sig. (2-tailed)	*.121*
Exact Sig. [2(1-tailed Sig.)]*	*.333*[a]

a. Not corrected for ties.
b. Grouping Variable: Depth

c	EA extract
Mann-Whitney U	*.000*
Wilcoxon W	*3.000*
Z	*-1.549*
Asymp. Sig. (2-tailed)	*.121*
Exact Sig. [2(1-tailed Sig.)]*	*.333*[a]

a. Not corrected for ties.
b. Grouping Variable: Depth

Comment [A9]: Please do not use ABBREVIATIONS into table! Also wsa is 2*? What is 1-tailed sig.?

Discussion. Sponge *Candidaspongia* sp. is known as a source of bioactive compounds with unique structure and high potent as anticancer drug candidate called candidaspongiolide (Meragelman et al 2007; Trianto et al 2011; Whitson et al 2011). However, the scarcity of the sponges in nature and structural complexity of the candidaspongiolide have been hampering the development of the compounds into a commercial drug. Synthesis, the most preferred method by pharmaceutical companies, is an unsuitable method for mass production of the candidaspongiolides due to compounds containing many stereo-centers. Tadpecth et al (2017) showed the synthesis of a macrolide greensporone C using 16 steps with overall yield 3%. Synthesis of the (-)-hortonone C has also provided a yield as low as 1 % with 11 steps (Niroula et al 2017). Light has a great effect on the metabolism rate for the sponge-associated microorganisms since spicule can be used as light transduction for the microorganisms

live inside the colony (Brümmer et al 2016). In turn, the metabolites will give an impact to the host.

To develop a mass production method, we conduct initial research for the sponge culture in Kupang water including the sponge inventory, recovery rate after cutting, and mariculture. Based on our observation, the sponge density in nature is quite low. The average sponge density is 1.7 colonies per 100 m line transect length or 17 colonies per km at around 20 m depth. Even, the sponge density at around 15 m depth is as lower as 0.3 colonies per 100 m transect length or 3 colonies per km length. However, a group of small sponge colonies could be found at 10 m below the port, a protected area either from strong current or light intensity. The situation leads to the assumption that strong current and light intensity may be the limiting factors for sponge growth. Larval swimming behavior is highly affected by light and temperature. The stronger light intensity reduces the swimming periods of the larval of sponge *Hymeniacidon perlevis* (Xue et al 2009).

Current is an important factor for the sponge growth because current brings the nutrient and oxygen, and at the same time flush the CO_2 and metabolisms products away. However, the strong current may damage the sponge colony. The sponge has photo sensory that are sensitive to the certain light wave (Muller et al 2006). The sponge grows on hard substratum such as dead coral.

All of the sponge colonies survived after the cut, and they have fully recovered after 60 days. However, the growth rates of the basal parts were varied among the colonies. The highest length and wide increments were 4.2 cm and 3.5 cm in 60 days, respectively. The fastest grew on the basal part with uncut lamellae, for example, the growing direction on sponge no. 2 was down the side where the hanging lamella was uncut. And, the lowest length and wide increments is 0.5 cm and 1.0 cm in 60 days, respectively (see Table 3). However, the sponges that were fully cut developed a growing strategy by increasing the number of lamellae instead of increase the colony size.

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Even though the *Candidaspogia* sp. maintains its colony basic shape as lamella, but it may become a complex branching lamellar colony with various thickness. The complexity of growth form has correlated with water depth and the protected level of the natural habitat the sponge.

The sponges were able to survive after cut, however, the smaller size will reduce the survival rate (Duckworth & Wolff 2007). Naturally, sponge mortality was mostly caused by sedimentation and diseases, even though, sponges have predators but they do not cause mortality on the sponge (Bell et al 2017; Wulff 2006a). Wulff (2006) reported that erect sponge has 70% survival rate post damage by hurricane. Sponge has also change the morphology for adaptation to the ecological condition (Wulff 2006b).

Mariculture is a feasible method for mass production of the sponge; even though, the further study still needed to standardize and to improve the method. The sponges explanted at 12 m and 25 m depth have an 80% survival rate. However, the sponges explanted at 6 m depth had only a 40% survival rate). The explant with four side incision could not survive due to loss of the mass and died or untied and driven away by the current. The explants grown in 6 m depth indicated the loss of mass because the sponge dead or lost (see Figure 1). Louden et al (2007) reported the *in situ* culture of sponges *Rhopaloeides odorabile,* and *Coscinoderma* sp. have survival rates of 65 % and 90% for 78 days. The sponge has also dead up to 3% naturally, and up to 7% caused by accidentally fishing (Butler et al 2017). The total growth of *R. odorabile* (146.0±40.3%) and *Coscinoderma* sp. (195.9±39.8%) was not significantly different over the 21 month experimental period but was highly variable between explants from the same individual.

Descriptively, the sponge explanted in deeper water has a higher average growth rate than those explanted at the shallower water. The average growth rate of sponges explanted at 25 m, 12 m, and 6 m depth were 3.375 cm^2, 2.1 cm^2, and -1.125 cm^2, respectively. Size of the sponge explanted at 6 m decreased due to loss of biomass. However, further analyses with ANOVA showed a significant effect between the culture depth. There are many factors related to a depth that may affect *Candidaspongia* sp. live and growth such as pressure, light intensity, nutrient, current, and sedimentation rate. We observed four small colonies of the sponge Candidaspongia that grow at 10 m depth

below the harbor, a small and protected area either from current or direct sunlight. Based on our observation, there are only few *Candidaspongia* sp. that grow in open area, and among them usually, grow under the hard coral or crevices. Further environmental study is needed to reveal the key factor of the sponge growth rate.

Our previous research showed that the anticancer compounds the candidaspongiolide and its analogs were obtained from organic fraction (Trianto et al 2011). So, in this study, we pay more attention to the ethyl acetate content in the sponge. The EA extract content in the transplanted and the natural sponges were not significantly different (Mann-Whitney test, U=6.00). However, water depth affects not only the growth rate and survival rate but also the chemical content. The EA extracts of the natural and explanted sponges were affected by the depth. The extracts content in transplanted sponges were higher in the deeper water (Mann-Whitney test, U=0.00), on the other hand, the extract contents in natural sponges were higher in the lower water (Mann-Whitney test, U=0.00). Naturally, sponges produce bioactive compounds that support their survival, including from bacterial infection. The marine sponge reported produces the antibacterial compounds to protect the colony (Yu et al 2017).

The development of large-scale production of the candidaspongiolide via sponge culture is still in a preliminary study that needs many strategies to overcome the problem regarding the environmental factors and the number of explants. However, the difficulties are in proportion to the potency of the sponge as a source of the anticancer drug candidate. Sponge culture is a promising method to overcome the bottleneck in drug development and to avoid the over-exploitation of wild population (Pérez-López et al 2014).

Conclusion. The sponge *Candidaspongia* sp. density in deeper water is higher than in the lower water. All of the sponges were survive after the cut, and they have fully recovered in 60 days. The growth rate of the explants were 10%-60% and 73%-203% at 12 m and 25 m respectively. The explants in 6 m have a negative growth rate.

The sponges explanted at 12 and 25 m depth have higher survival and growth rates than the sponge explanted at 6 m depth. The sponge explanted in deeper water has higher EA extract than those explanted at the shallower water.

Acknowledgments

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Agus Trianto <agustrianto.undip@gmail.com> 16 Juni 2019 17.04
Kepada: cristian coroian <cristian_coroian@yahoo.com>

Dear Dr. Cristian Coroian,

Thanks for the information. I believe the manuscript you returned to me was from the older version. Anyhow, I am sending the revised manuscript as shown in the attachment.
I have to explain that the symbol in the statistical tables was originally from the SPSS output, I just changed the table format. Hopefully, the manuscript has already fit with your journal standard.

Best regards,

Dr. Agus Trianto

Department of Marine Sciences

Fact. of Fisheries and Marine Sciences, Diponegoro University

Jl. Prof. Soedharto, SH Tembalang

Semarang- INDONESIA

50275

[Kutipan teks disembunyikan]

AACL-CANDIDASPONGIA-Agus Trianto-UNDIP-R4.doc
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11) Input from the Editor
20 Juni 2019

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Letter from AACL journal

cristian coroian <cristian_coroian@yahoo.com> 20 Juni 2019 17.11
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Cristian-Ovidiu Coroian, *PhD*

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12) Comments on the Input
20 Juni 2019

AACL BIOFLUX
Aquaculture, Aquarium, Conservation & Legislation
International Journal of the Bioflux Society

Ecological study and preliminary culture of the sponge Candidaspongia a source of anticancer molecules

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Abstract: Sponge *Candidaspongia* sp. is a source of candidaspongiolide, a very potent anticancer macrolide that active against various cell lines at nanogram level. However, low abundance of the sponge in nature and structurally complex of candidaspongiolide have become the major obstacles for the drug development. This study aims to assess the feasibility of the production of the anticancer compounds, the candidaspongiolides, using sponge culture. The study was conducted in Kupang, Nusa Tenggara Timur, Indonesia. The sponge abundance was observed using modified Line Intercept Transect method at 12 m and 20 m. Some sponge colonies were cut for culture and chemical analyses. Then, the recovery rate of the sponge was observed after 60 days. Sponge culture was carried out at 6 m, 12 m and 25 m depth for 60 days. Inventory of the *Candidaspongia* sp. showed that the sponge density at around 12 m depth is lower than those in 25 m depth. All of the sponges were survive after the cut and fully recovered in 60 days. The length and width increments of the basal part were 0.25-2.1 cm/month and 0.5-1.75 cm/month, respectively. The sponges cultured at 12 m and 25 m depth have higher survival and growth rates than those at 6 m depth. Descriptively, the sponge cultured in deeper water have higher ethyl acetate extracts content than the sponge cultured at the shallower water. Sponge mariculture is a possible method to supply candidaspongiolide for further studies.

Key Words: Ethyl acetate, candidaspongia, anticancer, mariculture**,** natural stock

Introduction. Sponges have been proven as productive sources of various compounds classes with pharmacological potency as antibacterial, antifungal, anthelmintic, antimalarial, antiviral, anti-inflammatory, anticoagulant, antioxidant and antitumor (Monroe 2010; Trianto et al 2014; Balansa et al 2017). Candidaspongia is a rare marine sponge that produces a potent anticancer compound called candidaspongiolide, a unique 18-membered macrolide. Candidaspongiolides have also been reported as exhibited remarkable cytotoxicity in NCI (National Cancer Institute) 60-cells-panel with GI_{50} of 14 ng/mL. Originally, the compound was isolated from the sponge collected from Australian and Papua New Guinean waters (Meragelman et al 2007). Candidaspongiolide and its derivatives also exhibited activity against melanoma (UACC-257, LOXIMVI, and M14), breast (MCF7) and lung cancer (NCI-H460) cell lines (Whitson et al 2011).

In 2011, we identified two new derivatives of the candidaspongiolide along with the known one isolated from the sponge collected in Indonesia. The compounds exhibited potent cytotoxicity with IC_{50} 37.0, 4.7 and 19.0 ng/mL, against NBT-T2 cells (Trianto et al 2011). However, low abundance of the sponge in nature and structurally complex of candidaspogiolide have become the major obstacles for the drug development. This study aims to assess the feasibility of the production of the anticancer compounds, the

candidaspongiolides, using sponge culture. Mayer et al (2010) noted that among thousands of bioactive compounds isolated from marine organisms, those were only a few compounds entered a clinical trial. Material supply is the main problem besides the bioactivity and the pharmacological profile.

There are several methods that are commonly used to supply the bioactive compounds including chemical synthesis, mariculture, closed system culture, and fermentation (Mendola 2003). The most preferred method to produce a drug is chemical synthesis because of its efficiency, economically, and robustly. However, considering the candidaspongiolide has several stereocenters, total synthesis would be impractical due to the longer pathway. Stereochemistry is a key to activity in biological systems (Butler 2004). The best synthesis method of one of the related compounds, tedanolide, has been achieved in 31 steps and gave only 0.31% of overall yield from the starting material (Smith & Lee 2007). Therefore, big-scale production of candidaspongiolide or its analogs via chemical synthesis may not be an economical method due to the high price of some starting materials. Tedanolide, isolated from the Caribbean marine sponge *Tedania ignis,* has been reported to exhibit strong cytotoxicity at pico to the nanomolar range (Schmitz et al 1984).

Sponges have been cultured for mass production of bath sponge and providing bioactive substances (Milanese et al 2003). Sipkema et al (2005) showed that culture of sponges *Lissodendoryx* sp. and *Dysidea avara* was able to produce anticancer compounds as halichondrin B and avarol, respectively. Muller et al (2000) successfully produced avarol from *Dysidea avara* via a cell culture that is known as primmorphs. To the best of our knowledge, this study is the first effort to culture the sponge *Candidaspongia* sp.

Material and Methods.

Candidaspongia inventory. The survey was conducted by Line Intercept Transect (LIT) method with a slight modification (Cleary et al 2005). The sponges were observed along transects (6 x 100 m) placed in 12 and 20 m depth with observation area around 3 m on both sides.

Sponge collection. The sponge colonies for explant and extract were collected by hand during the survey. The upper part of the sponge colonies was cut to let the basal part (about 5 cm height) re-growth for future stock (Mendola 2003).

Sponge culture. The sponge was cultured *in situ* in Kupang Bay, East Nusa Tenggara, Indonesia. Before the culture, the sponge colonies were tied at 12 m depth in a net for acclimation. After four days, the sponge colonies were cut into 3 cm x 5 cm (width x length). 15 fragments were explanted in three different nets placed at 6, 12, and 25 m depths (five fragments per net). The sponge's growth rate were measured at the end of culture period. All the procedures applied were based on the methods proposed by de Caralt et al (2003), Mendola (2003), and Osinga et al (2003).

Comment [A1]: How long the cultura period is? You did not ANSWER!

Monitoring of the recovery and growth rates of the sponges. The basal parts of the sponge colonies were observed by SCUBA diving method to evaluate the recovery and the growth rates after 60 days from cutting. The height, width, and the number of new branches were recorded. The ruler was used for measuring the height and width increment.

Extraction of the sponges. The harvested sponges were cut into small pieces and extracted with methanol for 24 hours with triplicates. The extract was filtered with filter paper and concentrated with a rotary evaporator under vacuum. Then, the extract was subjected to the separatory funnel using ethyl acetate and water to provide the organic and water fractions (Trianto et al 2011).

Data analysis. The growth rate and the ethyl acetate (EA) extract contents data were analyzed with the Mann-Whitney test, to indicate whether the treatments have a

Comment [A2]: ANOVA and Tuckey test have to be also be described in here!

significant effect or not. Mann-Whitney test is a non-parametric test that was chosen due to the data are not distributed normally and homogenously. Statistical analyses were performed using SPSS ver.16 software.

Results

Candidaspongia inventory and collection. The sponge inventory was conducted using a modified Line Intercept Transect Method at 12 and 20 m depths. The average sponge densities were 0.3 and 1.7 colonies transect at 12 m and 20 m respectively, as shown in Tables 1 and 2.

Table 1

The sponges of *Candidaspongia* sp. colonies observed at 12 m depth

Station	*Colony(s) number*	*Average height (cm)*	*Average width (cm)*
I	1	12.10	8.30
II	0		
III	0		
IV	0		
V	0		
VI	0		
VII	1	8.20	10.40
Total colonies	2	10.15	9.35
Average	0.3		

Table 2

The sponges of *Candidaspongia* sp. colonies observed at 20 m depth

Station	*Colony(s) number*	*Average height (cm)*	*Average width (cm)*
I	2	18.75	10.70
II	2	15.20	9.00
III	2	14.20	7.30
IV	0	-	-
V	1	19.00	13.20
VI	1	10.00	5.10
VII	2	10.20	7.00
Total colonies	12	13.55	8.39
Average	1.7		

However, four small colonies of the sponges were observed at 10 m depth under the port out of the line transects. Light intensity probably plays an important role in the larval settlement. However, the hypothesis still needs to be proven with further study.

Monitoring of sponge colonies survival and growth rates after the cut. The sponges of *Candidaspongia* sp. colonies were observed by SCUBA diving method to evaluate the recovery rate and the growth after the cut. The basal parts of the sponge were survive 100% and grown well (see Table 3). The average height and width increments were 1.83 cm and 2.85 cm, respectively.

Table 3

The basal parts of sponge *Candidaspongia* sp. grown after 60 days from cut at around 20 m depth

Colony no.	*Growth*		*Number of new lobes*
	Δ Height (cm)	*Δ Width (cm)*	
1	0.5	1	5
2	3.5	3.6	0
3	0.5	4.2	4
4	3.5	2.9	0
5	3.0	1	2
6	3.0	2.5	2
Average	1.83	2.85	2.17

Sponge culture. The survival and growth rates of the sponge explanted at 6, 12 and 25 m depths were observed *in situ* by SCUBA diving. The sponge cultured at 6 m has lower survival and growth rates, while the sponge cultured at 12 and 25 m depths have higher survival and growth rates as shown in Table 4.

Table 4

The average growth and survival cultured sponges after 60 days

Depth	*Growth*			*Survival rate (%)*
	ΔHeight (cm)	*ΔWidth (cm)*	*cm^2*	
6 m	-0.75	-1.50	- 1.125	40
12 m	1.40	1.50	2.100	80
25 m	1.50	2.25	3.375	80

Table 4. The average growth and survival cultured sponges after 60 days.

Depth	**ΔL (cm)**	**ΔL (%)**	**ΔW (cm)**	**ΔW (%)**	**ΔA (%)**	**SR(%)**
6 m	-1,0	-20,0	-2,0	-66,7	-73,3	40
	-0,5	-10,0	-1,0	-33,3	-40,0	
Average	-0,8	-16,0	-1,5	-50,0	-56,7	
12 m	0,5	10,0	0,0	0,0	10,0	80
	2,0	40,0	1,0	33,3	113,3	
	2,0	40,0	3,0	100,0	260,0	
	1,0	20,0	2,0	66,7	100,0	
Average	1,4	28,0	1,5	50,0	120,8	
25 m	1,5	30,0	1,0	33,3	73,3	80
	1,0	20,0	1,5	50,0	80,0	
	2,0	40,0	2,5	83,3	203,3	
	1,5	30,0	4,0	133,3	203,3	
Average	1,5	30,0	2,3	76,7	140,0	

Note: ΔL: Length increment, ΔW: Width increment, ΔA: Area increment

ANOVA test indicated that depth has a significant effect on the sponge colonies growth. The further test, Tukey HSD test, showed that the sponge growth rate at 60 days for 6 m was significantly different with a sponge growth rate at 12 and 25 m, but there are no differences of the growth rates between 12 and 25 m (see Table 5).

Comment [A3]: Is this an extra table?

Comment [A4]: You added this table as table 4, but in this case what to do with the above table which was before table 4? What SR is? I suppose survival rate. Note that under the table.

Comment [A5]: Put in there a value for P!

Table 5

The Tukey test of the growth cultured sponges expressed by the area increment for 60 days

Area Increment

	Depth	N	Subset	
			1	2
Tukey HSD[a,b]	6	4	-78.325	
	12	4		120.825
	25	4		139.975
	Significance		1.000	0.931

Means for groups in homogeneous subsets are displayed based on observed means.

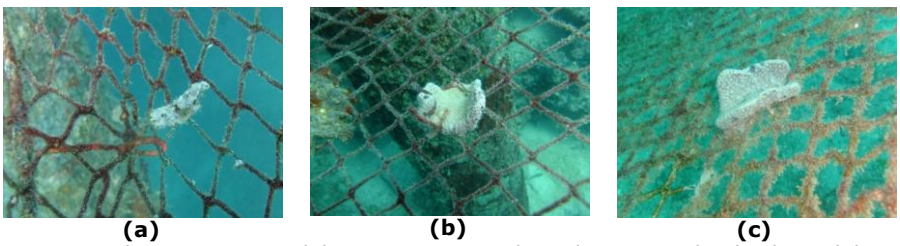
The error term is Mean Square (Error) = 5626.500.

a. Uses Harmonic Mean Sample Size = 4.000.

b. Alpha =0.05.

Depth	*ΔL (cm)*	*ΔW (cm)*	*ΔA (%)*	*SR(%)*
6 m	-1,0	-2,0	-73,3	40
	-0,5	-1,0	-40,0	
Average	-0,8	-1,5	-56,7	
12 m	0,5	0,0	10,0	80
	2,0	1,0	113,3	
	2,0	3,0	260,0	
	1,0	2,0	100,0	
Average	1,4	1,5	120,8	
25 m	1,5	1,0	73,3	80
	1,0	1,5	80,0	
	2,0	2,5	203,3	
	1,5	4,0	203,3	
Average	1,5	2,3	140,0	

Note: ΔL: Length increment, ΔW: Wide increment, ΔA: Area increment



(a) **(b)** **(c)**

Figure 1. The sponge *Candidaspongia* sp. explanted at 6 m depth showed loss of biomass (a), while the explants at 12 m (b), and 25 m (c) grew well.

Chemical analysis. The sponges were extracted with methanol PA in laboratory of Biotechnology, department of Marine Sciences, Diponegoro University. Then, the crude

Comment [A6]: From this table there is no evidence of a significant diference!

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extracts were separated into ethyl acetate (EA) and water fraction using a test tube. The result is shown in Table 6.

Table 6

Ethyl acetate and water extracts from cultured and natural sponges of *Candidaspongia* sp.

No.	*Sponge code*	*Sponge wet weight (g)*	*Ethyl acetate extract*		*Water extract*	
			Weight (mg)	*Content (%)*	*Weight (mg)*	*Content (%)*
1	C-12	14.97	0.14	0.93	0.26	1.63
2	C-25	16.56	0.20	1.24	0.30	1.82
3	N-12	26.70	0.05	0.17	0.61	2.30
4	N-20	20.48	0.09	0.46	0.20	0.96

Note: C-12: Cultured sponge at 12 m, C-25: Cultured sponge at 25 m, N-12: Natural sponge cultured at 12 m, N-20: Natural sponge cultured at 20 m.

The Mann-Whitney U test indicated that the EA extract concentration in natural and cultured is no significantly difference (see Table 7a). However, water depth gives significant effect to the EA extract concentration both in natural and cultured sponges (see Table 7b and c).

Table 7

The Mann-Whitney U test of ethyl acetate extracts of the *Candidaspongia* sp.
a. cultured vs natural sponge.
b. The Shallow water vs deep water natural sponge extracts. c. The Shallow water vs deep water culture sponge extracts

a	EA extract
Mann-Whitney U	*5,000*
Wilcoxon W	*15,000*
Z	*-,866*
Asymp. Sig. (2-tailed)	*,386*
Exact Sig. [2(1-tailed Sig.)]*	*,486*[b]

a. Grouping variable: Depth
b. Not corrected for ties.

b	EA extract
Mann-Whitney U	*.000*
Wilcoxon W	*3.000*
Z	*-1.549*
Asymp. Sig. (2-tailed)	*.121*
Exact Sig. [2(1-tailed Sig.)]*	*.333*[a]

a. Not corrected for ties.
b. Grouping Variable: Depth

c	EA extract
Mann-Whitney U	*.000*
Wilcoxon W	*3.000*
Z	*-1.549*
Asymp. Sig. (2-tailed)	*.121*
Exact Sig. [2(1-tailed Sig.)]*	*.333*[a]

a. Not corrected for ties.
b. Grouping Variable: Depth

Comment [A9]: Please do not use ABBREVIATIONS into table! Also wsa is 2*? What is 1-tailed sig.?

Discussion. Sponge *Candidaspongia* sp. is known as a source of bioactive compounds with unique structure and high potent as anticancer drug candidate called candidaspongiolide (Meragelman et al 2007; Trianto et al 2011; Whitson et al 2011). However, the scarcity of the sponges in nature and structural complexity of the candidaspongiolide have been hampering the development of the compounds into a commercial drug. Synthesis, the most preferred method by pharmaceutical companies, is an unsuitable method for mass production of the candidaspongiolides due to compounds containing many stereo-centers. Tadpecth et al (2017) showed the synthesis of a macrolide greensporone C using 16 steps with overall yield 3%. Synthesis of the (-)-hortonone C has also provided a yield as low as 1 % with 11 steps (Niroula et al 2017). Light has a great effect on the metabolism rate for the sponge-associated microorganisms since spicule can be used as light transduction for the microorganisms

live inside the colony (Brümmer et al 2016). In turn, the metabolites will give an impact to the host.

To develop a mass production method, we conduct initial research for the sponge culture in Kupang water including the sponge inventory, recovery rate after cutting, and mariculture. Based on our observation, the sponge density in nature is quite low. The average sponge density is 1.7 colonies per 100 m line transect length or 17 colonies per km at around 20 m depth. Even, the sponge density at around 15 m depth is as lower as 0.3 colonies per 100 m transect length or 3 colonies per km length. However, a group of small sponge colonies could be found at 10 m below the port, a protected area either from strong current or light intensity. The situation leads to the assumption that strong current and light intensity may be the limiting factors for sponge growth. Larval swimming behavior is highly affected by light and temperature. The stronger light intensity reduces the swimming periods of the larval of sponge *Hymeniacidon perlevis* (Xue et al 2009).

Current is an important factor for the sponge growth because current brings the nutrient and oxygen, and at the same time flush the CO_2 and metabolisms products away. However, the strong current may damage the sponge colony. The sponge has photo sensory that are sensitive to the certain light wave (Muller et al 2006). The sponge grows on hard substratum such as dead coral.

All of the sponge colonies survived after the cut, and they have fully recovered after 60 days. However, the growth rates of the basal parts were varied among the colonies. The highest length and wide increments were 4.2 cm and 3.5 cm in 60 days, respectively. The fastest grew on the basal part with uncut lamellae, for example, the growing direction on sponge no. 2 was down the side where the hanging lamella was uncut. And, the lowest length and wide increments is 0.5 cm and 1.0 cm in 60 days, respectively (see Table 3). However, the sponges that were fully cut developed a growing strategy by increasing the number of lamellae instead of increase the colony size.

Comment [A10]: Please re-arrange all this part, simply because is not a discussion, but is results! You did not considered what we asked for! Bad English still!

Even though the *Candidaspogia* sp. maintains its colony basic shape as lamella, but it may become a complex branching lamellar colony with various thickness. The complexity of growth form has correlated with water depth and the protected level of the natural habitat the sponge.

The sponges were able to survive after cut, however, the smaller size will reduce the survival rate (Duckworth & Wolff 2007). Naturally, sponge mortality was mostly caused by sedimentation and diseases, even though, sponges have predators but they do not cause mortality on the sponge (Bell et al 2017; Wulff 2006a). Wulff (2006) reported that erect sponge has 70% survival rate post damage by hurricane. Sponge has also change the morphology for adaptation to the ecological condition (Wulff 2006b).

Mariculture is a feasible method for mass production of the sponge; even though, the further study still needed to standardize and to improve the method. The sponges explanted at 12 m and 25 m depth have an 80% survival rate. However, the sponges explanted at 6 m depth had only a 40% survival rate). The explant with four side incision could not survive due to loss of the mass and died or untied and driven away by the current. The explants grown in 6 m depth indicated the loss of mass because the sponge dead or lost (see Figure 1). Louden et al (2007) reported the *in situ* culture of sponges *Rhopaloeides odorabile,* and *Coscinoderma* sp. have survival rates of 65 % and 90% for 78 days. The sponge has also dead up to 3% naturally, and up to 7% caused by accidentally fishing (Butler et al 2017). The total growth of *R. odorabile* (146.0±40.3%) and *Coscinoderma* sp. (195.9±39.8%) was not significantly different over the 21 month experimental period but was highly variable between explants from the same individual.

Descriptively, the sponge explanted in deeper water has a higher average growth rate than those explanted at the shallower water. The average growth rate of sponges explanted at 25 m, 12 m, and 6 m depth were 3.375 cm^2, 2.1 cm^2, and -1.125 cm^2, respectively. Size of the sponge explanted at 6 m decreased due to loss of biomass. However, further analyses with ANOVA showed a significant effect between the culture depth. There are many factors related to a depth that may affect *Candidaspongia* sp. live and growth such as pressure, light intensity, nutrient, current, and sedimentation rate. We observed four small colonies of the sponge Candidaspongia that grow at 10 m depth

below the harbor, a small and protected area either from current or direct sunlight. Based on our observation, there are only few *Candidaspongia* sp. that grow in open area, and among them usually, grow under the hard coral or crevices. Further environmental study is needed to reveal the key factor of the sponge growth rate.

Our previous research showed that the anticancer compounds the candidaspongiolide and its analogs were obtained from organic fraction (Trianto et al 2011). So, in this study, we pay more attention to the ethyl acetate content in the sponge. The EA extract content in the transplanted and the natural sponges were not significantly different (Mann-Whitney test, U=6.00). However, water depth affects not only the growth rate and survival rate but also the chemical content. The EA extracts of the natural and explanted sponges were affected by the depth. The extracts content in transplanted sponges were higher in the deeper water (Mann-Whitney test, U=0.00), on the other hand, the extract contents in natural sponges were higher in the lower water (Mann-Whitney test, U=0.00). Naturally, sponges produce bioactive compounds that support their survival, including from bacterial infection. The marine sponge reported produces the antibacterial compounds to protect the colony (Yu et al 2017).

The development of large-scale production of the candidaspongiolide via sponge culture is still in a preliminary study that needs many strategies to overcome the problem regarding the environmental factors and the number of explants. However, the difficulties are in proportion to the potency of the sponge as a source of the anticancer drug candidate. Sponge culture is a promising method to overcome the bottleneck in drug development and to avoid the over-exploitation of wild population (Pérez-López et al 2014).

Conclusion. The sponge *Candidaspongia* sp. density in deeper water is higher than in the lower water. All of the sponges were survive after the cut, and they have fully recovered in 60 days. The growth rate of the explants were 10%-60% and 73%-203% at 12 m and 25 m respectively. The explants in 6 m have a negative growth rate.

The sponges explanted at 12 and 25 m depth have higher survival and growth rates than the sponge explanted at 6 m depth. The sponge explanted in deeper water has higher EA extract than those explanted at the shallower water.

Acknowledgments

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Comment [A11]: Please arrange all the references as in the journal requirements! Follow the instructions!

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13) Author's Revision
28 Juni 2019

Gmail **Agus Trianto <agustrianto.undip@gmail.com>**

Letter from AACL journal

Agus Trianto <agustrianto.undip@gmail.com> 28 Juni 2019 14.35
Kepada: cristian coroian <cristian_coroian@yahoo.com>

Dear Dr. Cristian Coroian,

Sorry for the late response. I have been traveling abroad. I am sending the PDF with some comment for my draft revision. But, again I am very happy if you accept the last version of my manuscript in the word I sent because I revise a lot in the content. I apologize if my PDF is not satisfying you due to I am not so familiar with it.

Best regards,

Dr. Agus Trianto

Department of Marine Sciences

Fact. of Fisheries and Marine Sciences, Diponegoro University

Jl. Prof. Soedharto, SH Tembalang

Semarang- INDONESIA

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Dear Author,

We will send you back what modifications you have made, but there is still some changes you have not answered. The most important is that regarding the statistical part, as you mentioned in material and methods that you have used Mann/Withney test and other statistical tests. In this case, you have to use those for estabishing the statistical diffrences between your plots (6, 12 and 25 m). All these requests are noted on PDF file you have it attached. Also the references are not yet all properly completed.

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15) Author's Revision
23 Juli 2019

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Agus Trianto <agustrianto.undip@gmail.com> 23 Juli 2019 21.54
Kepada: cristian coroian <cristian_coroian@yahoo.com>

Dear Dr. Cristian,

Sorry for the inconvenience. I am attaching the manuscript both the PDF version for the answer to your questions and the version of the words due to it more convenient for editing.

Dr. Agus Trianto

Department of Marine Sciences

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Dear Author,

Within a few days you will receive the final form and payment conditions.
Best regards,

Cristian-Ovidiu Coroian, *PhD*

University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca
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[Kutipan teks disembunyikan]

17) Final Editor's Mail
4 Januari 2020

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Dear Author,

The manuscript has been arranged. One last request from you, because you have omitted: please put the contact addresses of all your co-authors.

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Best regards,

Cristian-Ovidiu Coroian, *PhD*