

TOXICITY TEST OF *Gracilaria* sp. AGAINST *Aspergillus niger* van Tieghem AND THE PHYTOCHEMICAL ANALYSIS

Nevy Widya Pangestika, I Gede Putu Wirawan*, and I Ketut Suada

Faculty of Agriculture, Udayana University
Jl. PB. Sudirman, Denpasar, Bali 80232, Indonesia

*Corresponding author: igpwirawan@unud.ac.id

ABSTRACT

The purpose of this study was to know the effectiveness of *Gracilaria* sp. ethanol extract to inhibit the growth of *Aspergillus niger*. The results showed that the ethanol extract of *Gracilaria* sp. was not effective to inhibit the growth of *A. niger*. The minimum inhibitory concentration test (MIC) was carried out using extracts with 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7 %, 0.8 %, 0.9%, 1%, 2% and 0% percentage. The MIC results showed that the minimum extract inhibiting *A. niger* was 0.8%. The results of the antifungal activity test showed that the ethanol extract of *Gracilaria* sp. was fungistatic against *A. niger*. On the third day incubation, the 4% extract inhibited the growth of fungi with an average diameter of 5 mm. The fungal colony test was carried out using extract with 0.5%, 1%, 2%, 4% and 0% concentration, and the results showed that extract with 4% concentration can inhibit fungi colony growth by 69%. Phytochemical analysis was conducted using the UV-Vis Spectrophotometry, and the results showed that the ethanol extract of *Gracilaria* sp. contained 366.33 mg/100g/GAE phenol, 2041.47 mg/100g flavonoids, and 3041.60 mg/100g/TAE tannins. Tannins are suspected to be the most dominant fungistatic compound with the largest amount.

Keywords: antifungal, seaweed., *Aspergillus*, UV-Vis.

INTRODUCTION

Aspergillus niger is a fungi that is often used in the food industry as it has hydrolytic and oxidative enzymes that are useful for breaking down plant lignocellulose. *A. niger* is one of the fungi that has been labeled with GRAS (generally recognized as safe) by the USDA (United States Department of Agriculture). But *A. niger* has been found to be an opportunistic reason for infection if inhaled by humans and cause

aspergillosis, and this fungi is also associated with several important plant diseases. This fungi causes spoilage in some fruits, vegetables and other food products, resulting in huge economic loss. Plant diseases caused by *A. niger* include black rot of onions, crown rot of peanuts, tuber rot of yam, stem rot of dracaena, black mold rot of cherry, kernel rot of maize, fruit rot of grapes, fruit rot of banana, rot of tomatoes, and mango rotting. *A. Niger* can be found in soil,

water, air, and decaying plant material (Gautam *et al.*, 2011).

A. niger is generally known as a post-harvest fungi that grows well in the tropical climate. This fungi causes mycotoxin contamination which causes discoloration, loss of quality, and decreased commercial value of agricultural products. *A. niger* is also reported to contain ochratoxin A, fumonisin B₂, and aflatoxin (Al-Abdallah, 2009).

Fungi growth can be inhibited by using a fungicide. In general, the fungicide used by farmers is a chemical fungicide. The use of chemicals continuously has a negative impact on the environment so there is a need for substitution into more natural materials.

Nowadays, marine organisms such as macroalgae are one of the natural resources that can be explored as they contain broad-spectrum antifungal activity (Harman *et al.*, 2004). Besides, macroalgae are also known to stimulate the growth of vegetables, fruits, and other plant parts with their metabolite content. The compound was extracted from different macroalgae families, such as green, brown, and red algae which are estimated to have 40,000 compounds (Raven *et al.*, 1992).

Gracilaria sp. is one of seaweeds or macroalgae widespread in Bali which

has been consumed for generations as vegetables. Based on previous research by Suada (2012), *Gracilaria acutata* has the ability to inhibit the growth of *Fusarium oxysporum* f.sp *vanillae*.

As an effort to explore its benefits as an antifungal agent, it is important to know the content of secondary metabolites of *Gracilaria* sp. The method used to determine the secondary metabolite content of plants is phytochemical analysis. The phytochemical analysis is a branch of pharmacognition that studies plant chemical analysis methods. Plant chemistry has now developed into a branch of science itself. This branch of science lies between the organic chemistry of natural materials and plant biochemistry which are interrelated between the two that studies the diversity of organic compounds contained in plants, chemical structures, biosynthesis, changes in metabolism, scientific distribution, and biological functions (Harborne, 1984).

This study is conducted using Spectrophotometry UV-Vis method. The advantage of using this spectrophotometric method is very simple to determine the number of substances that are very small. Spectrophotometry implies the measurement of the distance of light

energy by a chemical system as a function of the wavelength of radiation, as well as the measurement of solitude absorption at a certain wavelength (Hasibuan, 2015).

MATERIALS AND METHODS

Macroalgae material and preparation of the extract

Fresh *Gracilaria* sp. was collected from Serangan Beach, Denpasar. Then wash the sample to clean the impurities with running water, then chopped to reduce the size. Then, dry the cleaned sample in the oven for 3-7 days with a temperature of 40°C until obtaining the constant weight. Extraction of the algae is carried out using maceration as follows: the finely powdered algae material (500 g) is macerated using 96% ethanol (2 L) at room temperature for three days with regular shaking. After the filtration, the organic solvent was evaporated under vacuum with the temperature of 50°C to obtain dry ethanol extract. The crude extract then stored at the temperature of -20°C.

Media Preparation

Media preparation is conducted by dissolving 3.9 g of PDA powder and 100 mg of *chloramphenicol* in 100 ml distilled water. Homogenize the solution

using a magnetic stirrer, then autoclave at 121°C for 15 minutes. Pour 20 ml PDA media into a sterile petri dish.

Minimum Inhibitory Concentration Test

A total of 500 µl of fungi suspension was mixed with 20 ml of a thin PDA (medium temperature around 42-45°C) and shake horizontally until evenly mixed in a petri dish. After the PDA is freezing, make three diffusion wells using cork borer, then fill each diffusion well with extract of the concentration: 0.0; 0.1; 0.2; 0.3; 0.4; 0.5; 0.6; 0.7; 0.8; 0.9 and 1% as much as 20 µl using a micropipette (Dewi *et al.*, 2019).

Inhibition Zone Test

A total of 0.5 ml of fungi spore suspension of the conidia with the concentration of 2×10^5 conidia/ml was put into a petri dish, then poured with 20 ml PDA which was still liquid. The culture is shaken until the conidia are evenly mixed into all media. After the media is freezing, make the diffusion well with cork borer Ø 5 mm, and fill the diffusion well with 20 µl extract, the concentrations are as follows: 0; 0.5; 1.0; 2.0; 4.0%. The inhibition categories are determined according to the Davis Stout category (1971).

Table 1. Determination of extract inhibitory power according to Davis Stout category:

Inhibition zone	Inhibition power
≤5 mm	Weak
6-10 mm	Moderate
11-20 mm	Strong
>20 mm	Very Strong

Fungi's Colonies Inhibition Test

Colony inhibition test refers to Hutasoit *et al.*, 2013. 20 ml PDA media which is still liquid is poured into a petri dish that contains an extract with a concentration of 0; 0.5; 1.0; 2.0; 4.0%. The petri dish is shaken until the media and extract are evenly mixed and freezing. After the media is frozen, make a diffusion well Ø 5 mm and then the well is filled with *A. niger* colonies that have been bred before. Measurement of the diameter of the colony is conducted when the fungi on the control have grown evenly on the petri dish.

Determination of the Total Phenolic Contents

The total phenolics of the extract were determined using Folin-Ciocalteu reagent. Sample and standards readings were made using a UV-Vis spectrophotometry at 743 nm. The test sample (10 mg) was mixed with ethanol 96% (10 ml). 1 ml of the sample was mixed with Folin-Ciocalteu's phenol

reagent. Shake the solution and leave it for 4-8 minutes. Then the solution was added with 4 ml 7% Na₂CO₃ then added with sterile aquadest to the limit marker. Then it was incubated for 2 hours and the absorbance of the solution was measured at a wavelength of 743 nm with a blank solution of distilled water and a Folin-Ciocalteu reagent. The obtained results were measured as an equivalent mg phenol gallic acid/100 mg fresh sample (Herlinda, 2016).

Determinations of Total Flavonoids Contents

Dissolve 15 mg extract in 10 ml ethanol to obtain a 1500 ppm solution. Then pipette 1 ml solution, add with 1 ml of 2% AlCl₃ solution, and 1 ml of 120 mM potassium acetate solution. Then the sample was incubated for one hour at room temperature, absorbance was determined by the UV-Vis Spectrophotometry method at a maximum wavelength of 450 nm (Stankovic, 2011).

Determination of Total Tannins Contents

Dissolve 0.5 g sample with 10 ml aquabidest. Then pipette 1 ml solution and put in a 10 ml container that has been filled with 7.5 ml of aquabidest. Then add 0.5 ml Folin Denis's reagent and incubate

for 3 minutes. Then, add 1 ml of saturated Na₂CO₃ solution. After incubating for 15 minutes, read the absorption at the maximum wavelength (Mukhriani *et al.*, 2014).

Experimental Design

This study uses a completely randomized design (CRD) with three repetitions in each treatment.

RESULTS AND DISCUSSION

The MIC results showed that the minimum extract inhibiting *A. niger* was 0.8%. The lowest inhibition zone diameter is 1.3 mm in 0.8% extract and the highest inhibition zone diameter is 2.8 mm in 2%

Statistical Analyses

The obtained data were analyzed quantitatively using analysis of variance (ANOVA) at the 5% level and continued with the Duncans Multiple Range Test (DMRT) at the 5% level if there were significant differences.

extract observed on the 3rd day after incubation. Ethanol extract of *Gracilaria* sp. below 0.8% concentration does not have inhibition against *A. niger*, but for the 0.8% concentrations and above have inhibition against *A. niger* growth.

Table 2. Inhibition zone of *Gracilaria* sp. extract against *A. Niger*

No.	Extract concentration (%)	Inhibition zone diameter (mm)			Average (mm)
		I	II	III	
1.	0.0	0.00	0.00	0.00	0.00 ^a
2.	0.1	0.00	0.00	0.00	0.00 ^a
3.	0.2	0.00	0.00	0.00	0.00 ^a
4.	0.3	0.00	0.00	0.00	0.00 ^a
5.	0.4	0.00	0.00	0.00	0.00 ^a
6.	0.5	0.00	0.00	0.00	0.00 ^a
7.	0.6	0.00	0.00	0.00	0.00 ^a
8.	0.7	0.00	0.00	0.00	0.00 ^a
9.	0.8*	2.00	1.50	0.50	1.33 ^b
10.	0.9	0.50	3.00	1.50	1.66 ^c
11.	1.0	2.50	2.00	1.00	1.83 ^c
12.	2.0	4.50	1.50	2.50	2.83 ^d

*MIC value of *Gracilaria* sp. against *A. niger*

Note: Numbers followed by the same letters in the same column are not significantly different based on the DMRT test at the 5% level.

The results of the antifungal activity test using the diffusion well method showed that the crude extract of *Gracilaria* sp. able to inhibit the growth of *A. niger* fungi on PDA insignificantly and its inhibition is

weak. On the first, second, and third days of crude extracts of *Gracilaria* sp. can still inhibit the growth of *A. niger*, but on the fourth day, *Gracilaria* sp. has no toxic properties against *A. niger* at all.

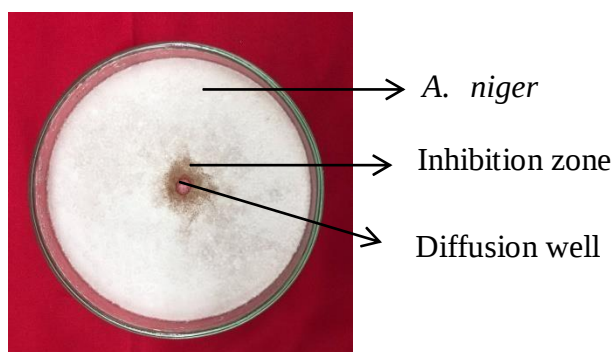


Fig. 1. Inhibition zone of *Gracilaria* sp. against *A. niger* at the concentration of 4%

The study showed that the crude extract of *Gracilaria* sp. except for control treatment and treatment with concentration 0.5% have weak inhibitory zones (<5mm) based on Davis and Stout category with the inhibition power of 0.4%. The results of this study indicate that the crude extract of *Gracilaria* sp. is not yet fungicidal because it still provides the opportunity for the fungi cells to germinate.

Fungi's colony inhibition test of *Gracilaria* sp. crude extract against *A. niger* colonies showed that the higher the concentration of the extract given, the smaller the growth of fungal colonies or the greater inhibition of fungal colony growth. The most active extract inhibited was the extract with a concentration of 4% with inhibition of fungi colonies reaching 69%.

Then the inhibition decreases along with the decreasing concentration of the extract, this shows that the extract which diffuses into the fungi cells also increases and causes the growth of the fungi to be disrupted.

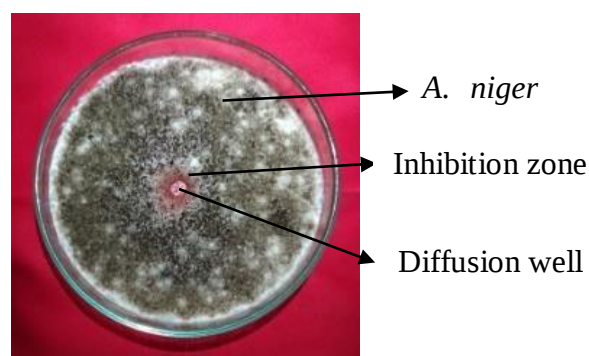


Fig. 2. Inhibition zone of *Gracilaria* sp. against *A. niger* at the concentration of 4% after three days

Table 3. Inhibition zone of *Gracilaria* sp. against *A. niger* third day after inhibition

No.	Extract concentration (%)	Inhibition zone (mm)			Avr (mm)	Inhibition power (%)
		I	II	III		
1.	0	0	0	0	0	0
2.	0.5	0	0	0	0	0
3.	1	0	0	0	0	0
4.	2	0	0	0	0	0
5.	4	0	15	0	5	0.4

Table 4. Inhibition zone of *Gracilaria* sp. against *A. niger* Colonies

No.	Extract concentration (%)	Colonies diameter (mm)			Avr (mm)	Inhibition power (%)
		I	II	III		
1.	0	82.50	67.00	63.00	70.83 ^e	0
2.	0.5	50.00	52.00	38.00	46.66 ^{cd}	34
3.	1	44.00	46.00	45.50	45.16 ^{bc}	36
4.	2	34.00	35.50	35.50	35.00 ^{ab}	50
5.	4	20.00	23.00	21.16	21.38 ^a	69

Note: Numbers followed by the same letters in the same column are not significantly different based on the DMRT test at the 5% level.

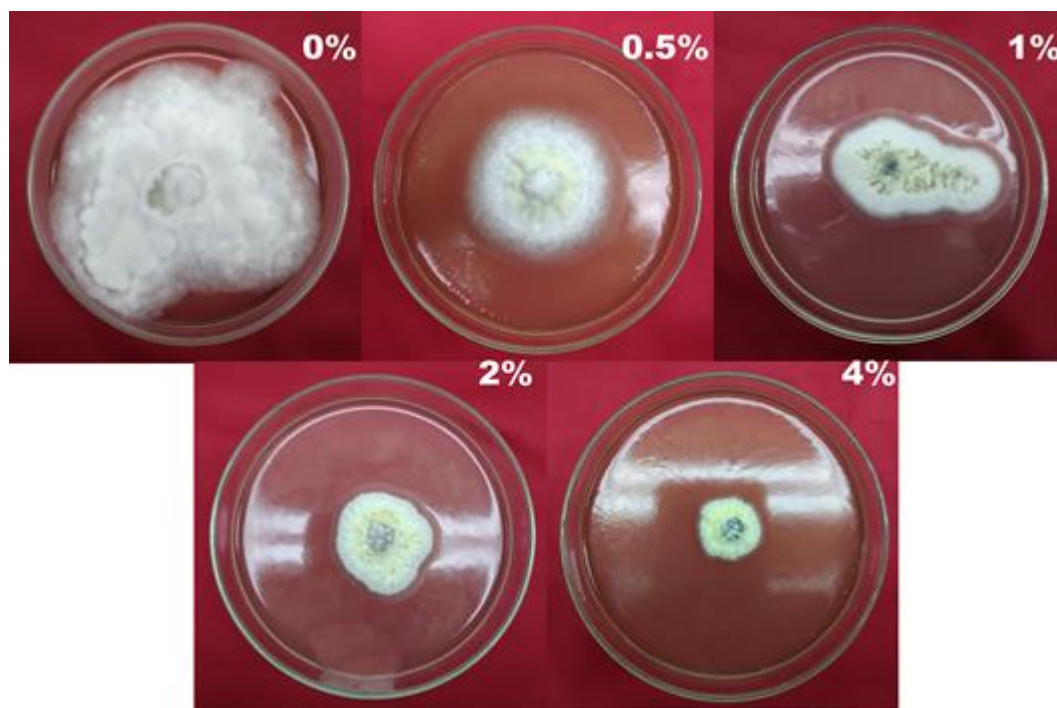


Fig. 3. Inhibition power of *Gracilaria* sp. against *A. niger* colonies

The phenol content obtained was expressed as an equivalent mg of gallic acid/100 mg of a fresh sample. The value of total phenolic content is expressed in Gallic

Equivalents (GAE) per 100 g dry weight of *Gracilaria* sp. ethanol extract. The results showed the total phenol ethanol extract of *Gracilaria* sp. was 366.33 mg/100g/GAE.

Table 5. Phytochemical analysis of *Gracilaria* sp.

No.	Sample	Phenolic total (mg/100g/GAE)	Flavonoid (mg/100mg)	Tannins (mg/100 g TAE)
1.	<i>Gracilaria</i> sp.	366.33	2041.47	3041.60

Quantitative analysis method of total flavonoid compounds using UV-Vis spectrophotometry as flavonoids have a conjugated aromatic system that shows a strong absorption band in the ultraviolet spectrum and visible light spectrum. Based on the results of this study, the levels of flavonoids obtained was 2041.47 mg/100 g. study, the levels of flavonoids obtained was 2041.47 mg/100 g.

The determination of tannin content is measured using a tannin standard curve. The tannin standard used was tannic acid. Tannic acid was chosen as it is a hydrolyzed tannins class so that it can be used as a comparison in measuring total tannin levels (Supriyanto, 2011). From this study, it was found that every 100 g of extract containing tannin was 3041.60 mg/100g TAE.

CONCLUSION

This study showed that the ethanol extract of *Gracilaria* sp. exhibited weak

antifungal activity against *A. niger* with the MIC value of 0.8 and inhibitory zones (<5mm), and the results showed that the ethanol extract of *Gracilaria* sp. contained 366.33 mg/100g/GAE phenol, 2041.47 mg flavonoids, and 3041.60 mg/100g/TAE tannins. Tannins were suspected to be the most dominant fungi static compound with the largest amount.

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REFERENCES

- Al-Abdallah, A.H.A. 2009. Production of aflatoxins by *Aspergillus flavus* and *Aspergillus niger* strains isolated from seeds of pulses. J. Food Agric. Environ. 7(1): 33-39.
- Davis, W. W. dan T. R. Stout. 1971. Disc Plate Method of Microbiological Antibiotic Assay. Applied Microbiology. 22(4): 20-25.
- Dewi, N. L. P. S. S., D. N. Suprpta., I. K. Suada. 2019. Uji Efektivitas Ekstrak

- Daun Cengkeh (*Syzygium aromaticum* L.) terhadap *Phytophthora palmivora* Penyebab Penyakit Busuk Buah Kakao (*Theobroma cacao* L.). *Jurnal Agroekoteknologi Tropika*. 8(4): 80-86.
- Gautam, A. K., S. Sharma., S. Avasthi., R. Bhaduria. 2011. Diversity, Pathogenicity and Toxicology of *A. Niger*: An Important Spoilage Fungi. *Research Journal of Microbiology*. 6(3): 270-280.
- Harborne, J. 1984. *Phytochemical Methods A Guide to Modern Techniques of Plant Analysis*, Second Edition. New York: Chapman and Hall.
- Harman, G.E., C.R. Howell, A. Viterbo, I. Chet., M. Lorito. 2004. *Trichoderma* species opportunist avirulent plants symbionts. *Nat. Rev. Microbiol.* 2(1): 43-56.
- Hasibuan, Elliwati. 2015. *Pengenalan Spektrofotometri pada Mahasiswa yang Melakukan Penelitian di Laboratorium Terpadu Fakultas Kedokteran USU. Karya Tulis Ilmiah. Pranata Laboratorium Perguruan Tinggi. Fakultas Kedokteran. Universitas Sumatera Utara.*
- Herlinda, A., A. Malik., A. Najib. 2016. Penetapan Kadar Fenolik Total dari Ekstrak Etanol Bunga Rosella (*Hibiscus sabdariffa* L.) Berwarna Ungu Menggunakan Spektrofotometri UV-Vis. *Jurnal Fitofarmaka Indonesia*, 3(1): 210-249.
- Lubis, S. S. 2015. *Penapisan Bakteri Laut Penghasil Antimikroba dari Pesisir Serdang Begadai Sumatera Utara. Elkawnie: Journal of Islamic Science and Technology.* 1(1): 60-78.
- Mukhrani. 2014. *Ekstraksi. Pemisahan Senyawa, dan Identifikasi Senyawa Aktif. Jurnal Kesehatan.* 7(2): 45-63.
- Raven, P. H., R. F. Evert, S. E. Eichorn. 1992. *Biology of Plants*, 5th ed. New York: Worth Publishers.
- Stankovic, M. S. 2011. Total phenolic content, flavonoid concentration and antioxidant activity of *Marrubium peregrinum* L. Extracts. *Kragujevac J Sci*, 33 (1): 63-72.
- Suada, I. K. 2012. *Keberagaman Aktivitas Antifungi Biota Laut Terhadap Fusarium oxysporum f.sp. vanillae Penyebab Busuk Batang Vanili. Jurnal Bumi Lestari*, 12(1): 66-70.