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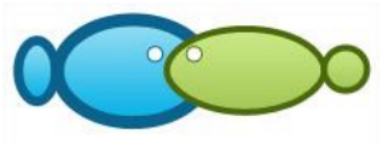

Bioactivity of β-sitosterol isolated from hydroid *Aglaophenia cupressina* Lamoureoux as antifungal in *Fusarium oxysporum*

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Abstract. *Fusarium oxysporum* is a fungus that often infects foodstuffs such as wilted and rotten vegetables. This causes losses for farmers in marketing their crops. This research is a follow-up study that aims to study the bioactivity and mechanism of action of β-sitosterol isolate from the hydroid *Aglaophenia cupressina* Lamoureoux in inhibiting or killing the fungus *F. oxysporum*. The method used for antifungal testing uses the agar diffusion method to determine the damage to fungal morphology using a Scan Electron Microscop (SEM). Pure culture of *F. oxysporum* mushroom with isolate code InaCC F641 was obtained from Indonesia Culture Collection (InaCC) LIPI, positive control using ketoconazole, and negative control using distilled water. The concentrations of β-sitosterol used were 20 ppm, 40 ppm and 60 ppm. The results obtained that β-sitosterol compound has antifungal properties at a concentration of 40 ppm and 60 ppm which is bactericidal against the fungus *F. oxysporum* with an inhibitory diameter of 18.30 mm which was formed at 48 hours of observation at a concentration of 40 ppm. Observation 72 hours formed inhibition diameter of 19.15 mm. While at a concentration of 60 ppm with 48 hours of observation, it formed an inhibition zone of 20.43 mm and at 72 hours it became 21.56 mm. The results of SSEM showed that concentrations of 40 ppm and 60 ppm of β-sitostrol damaged the cell walls of the fungus *F. oxysporum*.

Key Words: *β-sitosterol, hydroid Aglaophenia cupressina Lamoureoux, antifungal, Fusarium oxysporum*

Introduction. *Fusarium oxysporum* wilt is a disease of many types of plants, especially vegetables such as mustard greens, lettuce, tomatoes and chilies and other types. This disease is a problem and gets a lot of attention for vegetable commodities because it is consumed by the wider community and has high economic value. The distribution and dominance of *F. oxysporum* in the soil is quite high, making it quite difficult to control the cause of this disease (Margarida Ana Sampaio et al., 2020). Fungi are able to survive in the soil in the form of mycelium, microconidium, macroconidium or chlamydospores (Srinivas C et al., 2019). Aref Hanan Hassa (2020) and Made I Sudantha (2021) stated that the fungus *Fusarium* sp. able to survive in the soil for long periods of time even in the absence of a host plant.

Affected plants show symptoms of leaf veins that tend to be yellow in mature leaves then gradually the leaves become wilted (G. Kenneth Pegg et al., 2019). The fungus *F. oxysporum* resides in woody vessels and at the base of the stem is covered in a white thread-like braid and rotting bark (J. Francisco de Lamo and Frank L. W. Takken, 2020). Wilt disease caused by this fungus develops rapidly in moist soil conditions, especially in vegetables grown in the rainy season, and is transmitted through spores, mainly through the flow of water (Nath N. et al., 2017).

Many control efforts have been carried out by farmers by watering using synthetic pesticides but have not given satisfactory results. In addition, it also uses chemical compounds such as fertilizers, pesticides and other chemical compounds continuously at high doses which has been proven to cause increasingly complex problems, especially in

food safety (Temitope Elizabeth Alori and Olubukola Oluranti Babalola., 2018 ; M. Geraldin W. Lengai et al. al., 2019).

Schira Mario et al (2011) and Ons Lena et al., (2020) stated that the use of fungicides on fruits and vegetables after harvest has decreased due to increased pathogen resistance in several types of fungicides. In addition, the community's positive view of the dangers of using fungicides in post-harvest treatment of vegetables tends to increase. Biological control of postharvest disease is an alternative to effective control of several pathogens. Postharvest pathogen control is aimed at reducing pathogen infection, destroying inoculum or eradicating infection to maintain or extend the life of harvested vegetables and fruits (Kassim Alaika et al., 2020).

The results of Johannes (2008) research by isolating bioactive compounds from Hydroid *A. cupressina* L. a marine invertebrate animal found β-sitosterol compounds with potential as antifungals. Olusola Clement Ogidi et al (2021), steroid compounds used as medicinal and antifungal substances are β-sitosterol. β-sitosterol in the form of white crystals (clear), melting point 138°C-139°C, with the chemical formula $C_{29}H_{50}O$. In this study, the bioactivity and mechanism of action of β-sitosterol isolate from the hydroid *A. cupressina* Lamoureoux will be studied in inhibiting or killing the fungus *Fusarium oxysporum*.

Material and Method. The method used is an experimental method using the hydroid *A. cupressina* Lamoureoux and the solvent n-hexane for maceration (NN Azwanida, 2015) then partitioned using chloroform and ethyl acetate as eluents, respectively. Each solvent will selectively separate the chemical content group. Fractionation and isolation using KKG, KKV and TLC with various combinations of eluents, then purification of the isolates was carried out. Structure elucidation by UV, IR spectroscopy method. The bioactivity test used pure culture of the fungus *F. oxysporum* with isolate code InaCC F641 obtained from the Indonesia Culture Collection (InaCC) LIPI, positive control using ketoconazole, and negative control using aquades. β-sitosterol concentrations used were 20 ppm, 40 ppm and 60 ppm. The method used was agar diffusion using NaCl, Potato Dextrose Agar (PDA) medium (Merck), ketoconazole (PT. Alpharma), 70% alcohol, distilled water, lactophenol and aluminum foil.

Preparation of test solution and control solution. The β-sitosterol compound was weighed as much as 0.60 mg and dissolved in 10 ml of distilled water to obtain a solution with a concentration of 60 ppm. Furthermore, the test solution was made with a concentration of 40 and 20 ppm. The positive control used ketoconazole with a concentration of 60 ppm, and the negative control used distilled water.

Preparation of test mushroom suspension. Test mushrooms that have been rejuvenated, suspended and diluted using a sterile 0.9% NaCl solution and then homogenized. The transmittance of the suspension was measured at 75% by using a spectrophotometer as a blank using 0.9% NaCl at a wavelength of 580 nm.

Inhibitory test of β-sitosterol compounds. The test was carried out using the agar diffusion method using a blank disc with an inner diameter of 6 mm, an area diameter of 8 mm, and a height of 10 mm. Sterile sabouraud dextrose agar (SDA) medium was cooled to 40ºC-45ºC. Then it was poured aseptically into a 10 ml petri dish and allowed to solidify as a base layer. After solidifying, 1 ml of each test mushroom suspension was added into 5 ml of sabouraud dextrose agar (SDA) medium, then homogenized and poured over the base layer and left half solid as a seed layer. After that, the blank disc was placed aseptically with sterile tweezers on the surface of the medium with a distance of 2-3 cm from the edge of the petri dish, and left at room temperature.

Each blank disc was filled with 0.25 ml of β-sitosterol compound isolated from the hydroid *A. cupressina* Lamoureoux with concentrations of 20, 40, and 60 ppm, respectively. Similarly, the ketoconazole solution as a positive control and aquadest as a negative control was poured 0.25 ml each using a micropipette. Then it was incubated at 37ºC for 48 hours and 72 hours.

Diameter measurement of inhibition zone. Observations were made by measuring the diameter of the fungal growth inhibition around the blank disc using a caliper. Measurements were made at incubation for 48 hours and 72 hours, respectively to determine the ability of these hydroid bioactive compounds to inhibit the growth of the test fungi.

Effect on cell morphology by scanning electron microscopy (sem) (yang-shi tang et al, 2014). The analysis of cell morphology damage was intended to study the changes in morphology and cell structure of the fungus *F. oxysporum* due to the metabolic effect of hydroid. The changes observed were changes in cell appearance and cell wall thickness observed through a Scanning Electron Microscope (SEM). The SEM method was carried out with a suspension of test bacteria cells that had been treated with β-sitosterol which was incubated for 24 hours in a shaking incubator with a speed of 150 rpm at 37ºC. After being centrifuged at 3500 rpm for 15 minutes, the supernatant was discarded and pellets were taken and then fixed with 2.5% glutaraldehyde in (0.1 M sodium cacodilate buffer pH 7.2) left for 1.5 hours, then washed with cacodilate buffer 0.05 M pH 7.2 for 20 minutes for each treatment.

Fixation with 1% osmium tetraoxide in cacodilate buffer 0.05%, pH 7.2 for 1-2 minutes then washed with distilled water (DDH_2O) for 2 minutes each, hydrated with ethanol at various concentrations of 25, 50, 75 and 100 %, each for 10 minutes. Specimens were taken and passed through a 0.2 m membrane to be glued to aluminum stubs and coated with gold through a vacuum process (6-7 Pa) for 20 minutes. The sample was observed under a SEM.

Data analysis. The data obtained from the inhibition test were analyzed descriptively which were presented in the form of tables and figures 3.

Results. β-sitosterol in the form of white crystals (clear), melting point 138°C-139°C. The IR spectrum (KBr) showed absorption at a wave number of 3433 cm-1, an indication of the presence of a hydroxyl group supported by the presence of an absorption peak at 1050 cm^{-1} indicating the presence of C-O. The absorption at 2956, 2938 and 2869 cm^{-1} was from methyl and methylene, the absorption at 1634 cm^{-1} was from the C=C stretching indicating the presence of an olefin group, and the C-H bending at 1465 cm^{-1}.

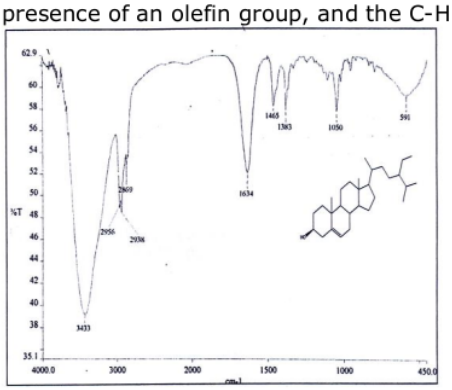

Figure 1. IR spectrum of β-sitosterol compound (Johannes, 2008)

Table 1

IR Spectrum of β-sitosterol compound

Wave number (cm^{-1})	*Band Shape*	*Intensity*	*Suspected Functional Group*
3433	Wide	Strong	Strain of the OH (Hydroxyl) group stretching
2956	Wide	Medium	Strain of C-H group stretch
2938	Sharp	Medium	Strain of aliphatic C-H Group Stretch From CH2
2969	Sharp	Medium	Strain of the aliphatic C-H group stretching of CH3
1634	Wide	Medium	Strain of Stretch C=C
1465	Wide	Medium	The bending of the C-H group
1050	Sharp	Medium	Strain of C-O stretch

From the table above, it shows that the compound is β-sitosterol ($C_{29}H_{50}O$)

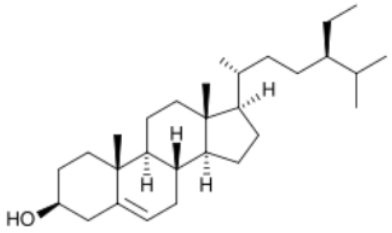

Figure 2. Structure of β-sitosterol compound (Johannes, 2008)

Bioactivity test of β -sitosterol compounds. The antifungal test results on *F. oxysporum* are shown in table 2.

Table 2

Results of bioactivity test of β -sitosterol compound Hydroid *A.cupressina* L. isolate against the fungus *F. oxysporum*

No.	*Concentration*	*Average of Inhibition Zone (mm) F. oxysporum*	
		48 hours	*72 hours*
1.	β-sitosterol 20 ppm	11.20	10.50
2.	β-sitosterol 40 ppm	18.30	19.15
3.	β-sitosterol 60 ppm	20.43	21.56
4.	Ketoconazole (positive control)	20.30	21.50
5.	Aquadest (negative control)	00.00	00.00

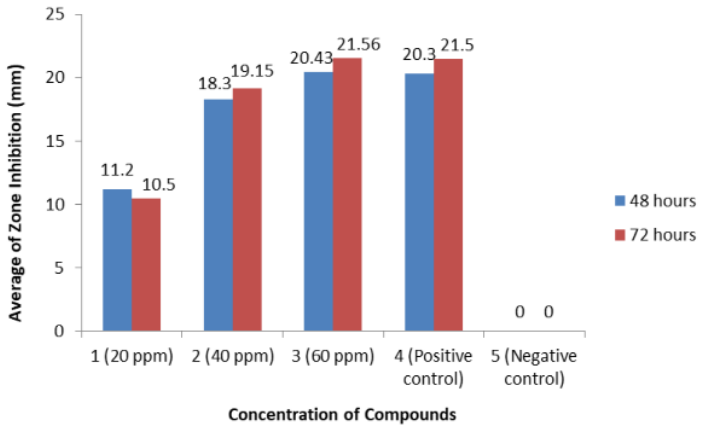

Figure 3. Histogram of inhibition diameter of β-sitosterol compound against the fungus *F.oxysporum* .

The results of the inhibition zone measurements in table 2 and figure 3 show the antifungal bioactivity of β-sitosterol against the fungus *F. oxysporum* with an average diameter of the inhibition zone at 48 hours of incubation the largest at a concentration of 60 ppm of 20.43 mm, and 72 hours of incubation the inhibition zone being 21.56 mm. .

For a concentration of 20 ppm, the inhibition zone formed at 48 hours was 18.30 mm and at 72 hours incubation was 19.15 mm. Concentrations of 60 ppm and 40 ppm showed fungicidal properties against *F. oxysporum* , while concentrations of 20 ppm at 48 hours incubation showed a much smaller diameter of the inhibition zone of 11.20 mm and 72 hours of incubation reduced to 10.50 mm.

Ketoconazole as a positive control with a concentration of 60 ppm at 48 hours incubation showed an inhibition zone of 20.30 mm and at 72 hours incubation the inhibition zone became 21.50 mm, while the negative control used aquadest as a solvent which did not form an inhibitory zone at 48 hours and 72 hours incubation.

The difference in the area of inhibition was influenced by the variation in the concentration given to the fungal isolate. According to D. Faraja. Gonelimali et al., (2018), the difference in the size of the inhibition zone for each concentration can be caused by differences in the content of the active substance, the reaction between the active ingredient and the medium and the incubation temperature.

Likewise, according to Huan Yuchen et al., (2020) suggested that what causes antimicrobial inhibition is due to interference with fungal cell membranes, inhibiting enzyme work, interfering with protein and nucleic acid synthesis, or inhibiting cell wall synthesis.

The mechanism of inhibition that occurs by the activity of β-sitosterol compounds at a concentration of 20 ppm against the fungus *F. oxysporum* is fungistatic characterized by a reduction in the diameter of the inhibition zone at 72 hours incubation. Fungal cell walls are composed of chitin compounds although this reaction does not damage the main structure of chitin and only reacts with structures that are outside the ring (CH_2-OH). This condition causes the reaction between chitin and the active group to not affect the integrity of the fungal cell wall because it does not damage the backbone structure of chitin, so this compound cannot kill the growth of fungal cells and is only an inhibitor.

Weiss Katharina et al., (2014) said that if the inhibition area is no longer clear after the next day or in other words the clear zone overgrown with fungus again, it means that the compound is fungistatic because it is only able to inhibit the growth of the fungus and does not kill the fungus. If the antifungal concentration is increased to a higher concentration then the fungistatic properties will change to a fungicide according to the statement of F.B Maria Morais-Braga et al., (2017).

It can be seen that β-sitosterol with a concentration of 40 ppm and 60 ppm is fungicidal, because the inhibition zone formed at 48 hours of incubation increased at 72 hours of incubation. The positive control used was ketoconazole which has proven its effectiveness in inhibiting the growth of fungal cells. Positive control was used to see whether the response to death of the test microbes was really caused by efficacious chemicals (Köhl Jürgen et al., 2019). The negative control was used to see whether the response to death really came from the sample and was not caused by technical factors of treatment (A. Cossarizza et al., 2017). In this study, the negative control used was distilled water which is a polar solvent (Silaban Hertina., 2021).

Effect on cell morphology by scanning electron microscopy (sem). *Based on the* results of the inhibition zone measurements, a concentration of 60 ppm β-sitosterol was used to see the effect of these compounds on the *F. oxysporum* fungus through Scanning Electron Microscopy (SEM).

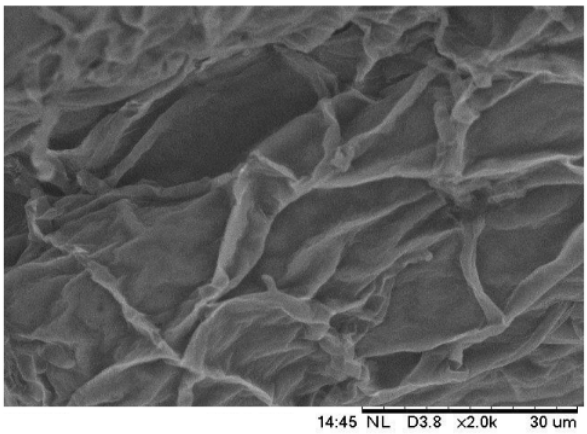

Figure 4. SEM results of β-sitosterol compound isolate from hydroid *A. cupressina* Lamoureoux against the fungus *F. oxysporum* (magnification 3000x).

The SEM results also showed that the cell wall of the fungus *F. oxysporum* had an irregular shape. Antifungal compounds work in addition to inhibiting the work of ergosterol, antifungals also work in inhibiting the synthesis of fungal cell walls, interfering with the function of cell membranes, inhibiting the synthesis of nucleic acids, inhibiting protein synthesis, inhibiting cell nuclear division, and as an inhibitor of the metabolic system in fungal cells (K. Tryphon Mazu et al., 2016 and Saleh Mohammed Al Aboody and Suresh Mickymaray., 2020).

Conclusions. The β-sitosterol compound isolated from the hydroid *A. cupressina* Lamoureoux was fungicidal against the fungus *F. oxysporum* at a concentration of 40 ppm and 60 ppm damaging the fungal cell wall.

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References

A . Cossarizza., Chang, H.D., Radbruch, A., Andrä, Annunziato F., Bacher ., .Barnaba V., 2017 Guidelines for the use of flow cytometry and cell sorting in immunological studies. Europian Journal Of Immunology.

Aref Hanan Hassan. 2020. Biology and Integrated Control of Tomato Wilt Caused by Fusarium oxysporum lycopersici: A Comprehensive Review under the Light of Recent Advancements. Journal of Botany Research. Vol.3., Pages 84-99.

C.Srinivasa C , D.Nirmala Devi, K.Narasimha Murthy, Chakrabhavi Dhananjaya Mohan, T.R.Lakshmeesha, Bhim Pratap Singhe Naveen Kumar Kalagatur, S.R.Niranjana, AbeerHashem, Abdulaziz A.Alqarawi, BabyTabassum, Elsayed FathiAbd_Allah, S.Chandra Nayaka, Rakesh K.Srivastava. 2019. Fusarium oxysporum f. sp. lycopersici causal agent of vascular wilt disease of tomato: Biology to diversity– A review. Saudi Journal of Biological Sciences. Volume 26, Issue 7, Pages 1315-1324 .

D. Faraja. Gonelimali, Jiheng Lin, Wenhua Miao, Jinghu Xuan, Fedrick Charles, Meiling Chen, Shaimaa R. Hatab. 2018. Action of Some Plant Extracts Against Food Pathogens and Spoilage Microorganisms. J. Front. Microbiol., | https://doi.org/10.3389/fmicb.2018.01639.

G. Kenneth Pegg , Lindel M. Coates, Wayne T. O'Neill, David W. Turner. 2019. The Epidemiology of Fusarium Wilt of Banana. J. Front. Plant Sci., | https://doi.org/10.3389/fpls.2019.01395.

Huan Yuchen , Qing Kong, Haijin Mou , Huaxi Yi., 2020. Antimicrobial Peptides: Classification, Design, Application and Research Progress in Multiple Fields . J. Front. Microbiol., |https://doi.org/10.3389/fmicb.2020.582779. Köhl Jürgen.

J. Francisco de Lamo and Frank L. W. Takken. 2020. Biocontrol by Fusarium oxysporum Using Endophyte-Mediated Resistance. J. Front. Plant Sci., | https://doi.org/10.3389/fpls.2020.00037.

Johannes E., 2008. Isolasi, Karakterisasi dan Uji Bioaktivitas Metabolit Sekunder dari Hydroid Aglaophenia cupressina Lamoureoux Sebagai Bahan Dasar Antimikroba.Thesis. Program PascaSarjana Universitas Hasanuddin. Makassar.

K. Tryphon Mazu, Barbara A. Bricker, Hernan Flores-Rozas, Seth Y. Ablordeppey. 2016. The Mechanistic Targets of Antifungal Agents: An Overview. Mini Rev Med Chem. ; 16(7): 555–578.

Kassim Alaika , Tilahun S. Workneh , Mark D. Laing., 2020. A review of the postharvest characteristics and pre-packaging treatments of citrus fruit J. AIMS Agriculture and Food. Volume 5, Issue 3: 337-364.

M. Geraldin W.Lengai, James W.Muthomi, Ernest R.Mbega. 2019. Phytochemical activity and role of botanical pesticides in pest management for sustainable agricultural crop production. J. Scientific African. Vol. 7.

Made I Sudantha . 2021. Characterization and virulence of Fusarium oxysporum f. sp. cubense cause wilt disease in banana plants and its biological control using endophytic fungi Trichoderma spp. at West Nusa Tenggara, Indonesia. IOP Conf. Ser.: Earth Environ. Sci. 886 012016.

Margarida Ana Sampaio, Susana de Sousa Araújo, Diego Rubiales, Maria Carlota Vaz Patto. 2020. Fusarium Wilt Management in Legume Crops. J. Agronomy, 10(8), 1073; https://doi.org/10.3390/agronomy10081073.

Nath N. , A. U. Ahmed, F. M. Aminuzzaman. 2017. Morphological and physiological variation of Fusarium oxysporum f. sp. ciceri isolates causing wilt disease in chickpea. International Journal of Environment, Agriculture and Biotechnology (IJEAB) Vol-2, Issue-1, http://dx.doi.org/10.22161/ijeab/2.1.25.

Olusola Clement Ogidi, Ayokunbi Elizabeth Ojo, Oluwatayo Benjamin Ajayi-Moses, Oluwatoyin Modupe Aladejana, Oluwakemi Abike Thonda, Bamidele Juliet Akinyele. Synergistic antifungal evaluation of over-the-counter antifungal creams with turmeric essential oil or Aloe vera gel against pathogenic fungi. J. BMC Complementary Medicine and Therapies volume 21, Article number: 47.

Ons Lena, Dany Bylemans, Karin Thevissen, Bruno P. A. Cammue ., 2020. Review Combining Biocontrol Agents with Chemical Fungicides for Integrated Plant Fungal Disease Control.

Rogier Kolnaar, Willem J. Ravensberg. 2019. Mode of Action of Microbial Biological Control Agents Against Plant Diseases: Relevance Beyond Efficacy. J. Front. Plant Sci., https://doi.org/10.3389/fpls.2019.00845.

Saleh Mohammed Al Aboody and Suresh Mickymaray., 2020. Settings Open Access Review Anti-Fungal Efficacy and Mechanisms of Flavonoids. J.Antibiotics , 9(2), 45; https://doi.org/10.3390/antibiotics9020045.

Schira Mario, Salvatore d'aquino, Paolo Cabra., 2011. Control of Postharvest Diseases of Fruit by Heat and Fungicides: Efficacy, Residue Levels, and Residue Persistence. A Review. Journal of Agricultural and Food Chemistry 59(16):8531-42.

Silaban Hertina., 2021. The Effect of Various Concentrations of Ethanol Extract of the Leaves of Paederia foetida L. on the Growth of Escherichia Coli Bacteria. Journal of Drug Delivery and Therapeutics.

Temitope Elizabeth Alori and Olubukola Oluranti Babalola., 2018. Microbial Inoculants for Improving Crop Quality and Human Health in Africa. Front. Microbiol., | https://doi.org/10.3389/fmicb.2018.02213.

Yang-Shi Tang ,Wei Zhang, Rebecca Soffe, Sofia Nahavandi, Ravi Shukla, Khashayar Khoshmanesh., 2014. High Resolution Scanning Electron Microscopy of Cells Using Dielectrophoresis. J. Plos one. https://doi.org/10.1371/journal.pone.0104109.

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