EFFICACY OF ONCHIDIID SLUG (ONCHIDIIUM TYPHAE) ETHANOLIC EXTRACT AGAINST BACTERIAL AND FUNGAL GROWN IN BIOFILM CULTURES

Bambang Wijianto[a]*, Hasyrul Hamzah[b]

Article History: **Received:** 27.09.2022 **Revised:** 27.10.2022 **Accepted:** 06.11.2022

Abstract: *Staphylococcus aureus, Escherichia coli*, and Candida *albicans* are opportunistic species responsible for clinical infection by producing biofilms. In the form of biofilm, they are more resistant to various antimicrobial agents. This study at to determine the biofilm activity of onchidiid slug extract *against S.aureus, E.coli*, and *C.albicans*. The microtiter broth method was used to measure the inhibitory and eradicating ability of *S.aureus, E.coli,* and *C.albicans* biofilms. In samples of onchidiid slug ethanol extract at a level of 1%, the *S. aureus* biofilm was inhibited by 63.89%. The MBIC value is at a level of 0.5%. The inhibitory activity on *E.coli* biofilm was 85% at 1%, with the MBIC value at 0.5%. Onchidiid slug ethanol extract samples also showed inhibitory activity on the *C.albicans* biofilm of 67.95% at a level of 1% with an MBIC value of 0.25%. The test results showed that extract could be developed as a new antibiofilm candidate for treating *S.aureus, E.coli*, and *C.albicans* biofilm infections.

Keywords: *Onchidium typhae*, antibiofilm, Onchidiid slug extract ethanolic

[a]. Department of Pharmaceutical Chemistry, Faculty of Medicine, Universitas Tanjungpura, Pontianak, 78124, Indonesia.
[b]. Faculty of Pharmacy, Universitas Muhammadiyah Kalimantan Timur, Samarinda, Kalimantan Timur, 75124, Indonesia.

***Corresponding Author**

E-mail:bam.wijianto@gmail.com

DOI: 10.31838/ecb/2022.11.10.015

INTRODUCTION

Wound healing is a natural physiological reaction to tissue injury—wound healing results from the collaboration of many cell strains and their products [1–4]. Efforts to restore the lesion begin at the inflammatory stage. Ultimately, they result in a repair consisting of the substitution of specialized structures caused by collagen deposition and regeneration, which corresponds to the processes of cell proliferation and posterior differentiation through pre-existing cells in the tissue and stem cells [5,6]. Most chronic wound infections are treated with antibiotics. *S.aureus* and *E.coli* are aerobic pathogens, including *C.albicans* [7,8]. They are pathogenic microorganism found predominantly in chronic wound cases. They are found in the skin tissue of diabetic wound patients. In addition, it is also known that *S.aures, E.coli,* and *C.albicans* are the cause of infection in postoperative wounds [9,10]. They have a higher potential for producing biofilms [11].

A biofilm is a unit of microbial cell surface covered by a matrix of extracellular polymeric substances. Biofilms are constantly evolving and are influenced by internal and external processes. Biofilms consist of microbial cells and extracellular polymeric substances (EPS) [12–14]. EPS can cover 50% to 90% of the total organic carbon of the biofilm and can be considered the primary matrix material of the biofilm. EPS can differ in chemical and physical properties but is mainly composed of polysaccharides. Some polysaccharides are neutral or polyanionic, such as the EPS of Gram-negative bacteria. In the biofilm formation process, uronic acids, such as D-glucuronic, D-galacturonic, and mannuronic or pyruvic acids, react with polymeric yarns and provide greater binding strength. In some Gram-positive bacteria, such as Staphylococci, the chemical composition of EPS may be very different and is primarily cationic [15]. Coagulase-negative bacterial mucus consists of teichoic acid mixed with small amounts of protein. EPS is also highly hydrated because it can incorporate large amounts of water into the structure by hydrogen bonding. EPS can be hydrophobic, although most are hydrophilic and hydrophobic and vary in solubility. Biofilm is a significant virulence factor contributing to chronic wound infection [16]. Bacteria in the biofilm can withstand antibiotics because the antibiotics fail to penetrate the biofilm. Biofilm causes the eradication of bacteria to be hampered, making the infected skin tissue hard to heal. In addition, the process of re-epithelialization of skin tissue is hampered due to biofilms developing in these tissues.

Natural products derived from marine organisms have become a source of increasingly important biologically active compounds. One of these marine organisms is the sea slug of the genus *Onchidium* (family *Onchidiidae*). Onchidiid slugs (*Onchidium thyphae*) are widely distributed in the waters of West Kalimantan, inhabiting shallow coastal waters and mangrove forests. In West Kalimantan, onchidiid slugs are considered economically important because of their high economic value as an export commodity. Its main ingredients, such as polyketides, terpenoids, steroids, alkaloids, and amino acids, have activity as antibacterial [17–19]. Onchidiid slug meat has a high protein content of 67.88% with a low-fat content of 3.17% [17,20]. Studies on onchidiid slug for antibiofilm against *S.aureus, E.coli*, and *C.albicans* have not yet been reported. Evaluation activity of biofilm from onchidiid

slugs extract is important to explore the potential of these marine biotas as antibiofilm compounds for topical treatment of chronic wound infections.

MATERIAL AND METHODS

Instrument: LAF, incubator (Memmert IN55, Schwabach-Germany), multichannel SOCOREX Acura micropipette (Switzerland), microtiter plate reader (Optical device Ivymen 2100-C, Spain), spectrophotometry (UV Genesys 10 experiment, 335903) (Thermo scientific Spectronic, United States of America), autoclave (Sakura, Japan), Buchi 23022A010 Rotary Evaporator (Poland), KERN-Moisture Analyser-DLB 160-3A (UK), IWAKI Microplate Multi Well Plate 96 wells Flat Bottom.

Materials: Onchidiid Slug collect from Sambas waters, West Kalimantan. *aureus* (ATCC 25923) and *E. coli* (ATCC 25922), for the antifungal assay was performed using *Candida albicans* (ATCC 102310). Other materials were 1% DMSO, NaCl, 0.5 McFarland standard, sterile distilled water, Brain Heart Infusion (BHI) media, phosphate buffer saline (PBS) solution, and 1% crystals violet.

Preparation of extract ethanolic onchidiid slug: The onchidiid slug came from West Kalimantan waters with a dimension length of 5-7 cm. The fresh onchidiid slug was washed thoroughly to remove the adhering mud and dirt. The following process is cleaning the mucus. The mucus is removed by boiling for 30 minutes while still stirring. Onchidiid slugs also are clean of innards and dirt. Onchidiid slug meat was dried in the oven for 1x24 hours at 60ºC. Pollination of the sample is done by grinding the dried sample. Onchidiid slug powder is macerated with an ethanol solvent to obtain a thick extract ready to be tested.

Chromatography profile: Silica gel G60 F254 plate was used as stationary phase. Chamber is filled with 20 mL of hexane, ethyl acetate, and methanol mixture (1:2:2) as mobile phase. The eluted plate was observed under 254 nm and 366 nm UV lights. Stain reagents used were Dragendorff for alkaloid, AlCl3 5% for flavonoid, FeCl3 5% for tannin, and Liebermann-Bouchard for steroid.

Preparation of antibacterial and antifungal assay: *S.aureus* and *E.coli* ware cultured in Mueller Hinton agar (MHA) medium, then incubated for 72 hours at 37ºC. Sabouraud Dextrose Broth (SDB) was used as a medium for culturing *C.albicans*. It was incubated for 72 hours at 37ºC. Cell density was adjusted equivalent to the McFarland standard 0.5 ~ 1.5 x 108 CFU/ml) the optical density of the culture suspension to 0.1 at 600 nm [17,21].

Antibacterial and antifungal assay: The microdilution method measured the inhibitory activity of *S.aureus* and *E.coli*. The ethanolic extract of onchidiid slug was 1.0% w/v, 0.5% w/v, 0.25% w/v, and 0.125% w/v on a 96-well microplate added microbial cultures. Chloramphenicol 1% w/v and fluconazole 1% w/v were used as control. The percent inhibition was determined by observing the clarity of the solution—sample absorbance measured at a wavelength of 595 nm [17,22,23].

Antifungal Assay: Clinical and Laboratory Standard Institute (CLSI) guidelines (2007) were used as a reference in measuring the inhibitory ability of onchidiid slug extract (slug i.e 1% w/v, 0.5% w/v, 0.25% w/v, and 0.125% w/v) against *C.albicans*. Fluconazole 1% w/v was used as control. The samples were incubated at 37ºC for 72 hours. Sample was measured in triplicate at 595nm [17,22].

Antibiofilm assay of extract ethanolic onchidiid slug

5 µL of microbial suspension (107 CFU/mL) and 75 µL of media containing the test extract with concentration series (0.125%, 0.25%, 0.5%, and 1% w/v) were added to each well. In addition, 20 µL of aquadest, 75 µL of media control, ethanol control, and drug control in other wells were given as a comparison of results. Once ready, the plates were incubated for 24 and 48 hours.

After completion of incubation, the plate was washed using sterile distilled water three times to remove nonadherent cells. Then, 100 µL of 1% crystal violet was added to each well filled with the test sample and allowed to stand for 15 minutes. After that, the crystal violet solution was then discarded on the plate and washed again using sterile distilled water three times. After washing, 100 µL of 96% ethanol was added to each well filled with the test sample and then measured with a microreader.

A medium without microbial growth was used as a control medium, and a microbial suspension was used as growth control. A microbial suspension was used as a controlled drug, which was given chloramphenicol 1% and fluconazole 1% w/v. The plates were incubated at 37°C for 24 and 48 hours. Then the plate was washed with distilled water three times and dried at room temperature.

125 µL of 1% crystal violet solution was added to each well to color the biofilm that had been formed, dead and live cells, which are also components of the biofilm. The plate was then incubated at room temperature for 15 minutes. After incubation, the plate was washed with running water three times to remove the remaining crystal violet, and 200 µL of 96% ethanol was added to each well to dissolve the formed biofilm. Optical Density (OD) readings on a microplate reader at 620 nm wavelength. The test was carried out with three replications. Data obtained from the analysis of biofilm inhibition in the form of OD values of each concentration of the test compound and control without the test compound (growth-control) obtained from reading with a microplate reader [22].

To determine the percent inhibition of test, the calculation of the OD value from the results of research analysis using the following equation:

$$\frac{(OD_{negative\ control\ mean} - OD_{test\ sample\ mean})}{OD_{negative\ control\ mean}} X\ 100$$

The sample level inhibiting at least 50% of biofilm formation is MBIC50 (Minimal Biofilm Inhibition Concentration) [14].

The effect of extract ethanolic onchidiid slug was also examined against the preformed biofilm of the *aureus (ATCC 25923, E. coli (ATCC 25922),* and *Candida albicans (ATCC 102310)* using the previously described method [15]. Biofilms were inoculated on 96 well-plates. After incubation at 37ºC for 48h, each of the plate wells were washed three times with 150 µL of sterile distilled water to get rid of nonadherent cells. 100 µL of media containing various concetrations of extract

ethanolic onchidiid slug (1% w/v, 0.5% w/v, 0.25% w/v, 0.125 % w/v) and incubated at 37ºC for 48 hours. Following the incubation, the plates were washed three times with 200 mL sterile PBS to remove adhering cells. The staining strategies are described above. The percentage of *minimum of biofilm degradation concentration* (MBEC) was calculated, as described before [16].

RESULTS AND DISCUSSION

The onchidiid slug tested in this study was collected from the waters of Sambas, West Kalimantan, with the appropriate length. The sample is to choose the right size (5-7 cm) and fresh to get the maximum active composition. The moisture content of onchidiid slug powder was measured through a moisture balance. The result of measuring the water content was 5.37%. This result meets the requirements where the good water content is ≤10%. The result allows the onchidiid slug powder to be resistant during storage to fungi and other microorganisms.

The content of the compounds in the extract can be separated based on the nature of the polarity. Knowing the chromatogram profile makes the active compound content identification process more accessible. The mobile phase used is hexane-ethyl acetate-methanol with a 1:2:2 ratio. This combination can separate the spots on the KLT plate. The results are shown in Figure 1. The results of the staining reagent showed orange spots with Dragendorff and purple spots with Liebermann-Bourchard after heating. In line with the previous study, secondary metabolites in the ethanolic extract of onchidiid slug are alkaloids and steroids.

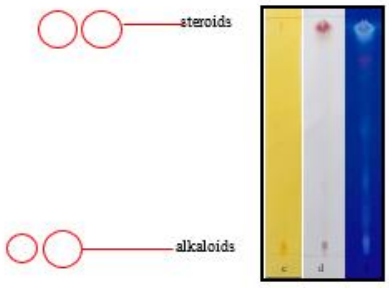


Figure 1. Profile chromatography of onchidiid slug methanol extract; c) sprayed with Dragendorff; d) sprayed with Liebermann Buchard; e) after sprayed with LB

Antibacterial and antifungal effect of Onchidiid slug against S. aureus, E. coli dan C. albicans

The antibacterial and antifungal assay was carried out using the microdilution method. The assay results on 1% methanol extracts showed inhibitory activity against *S. aureus*, *E. coli*, and *C. albicans*. The inhibition value against *S. aureus*, *E. coli*, and *C. albicans* was 82.0±0.01%; 85.8±0.01%; 84.9±0.01%, respectively (Figure 2).

In the Onchidiidae family, terpenoids and their derivatives such as Onchidal, Aspermeroterpene A-C, Furanasperterpene A and B, and 11-acetoxy-terretonin E are responsible for the antibacterial activity (Figure 3). Many studies have found that terpenoid compounds in onchidiid slugs provide inhibition of bacteria and fungi [17,18]. The KLT results also showed that the ethanolic extract of the onchidiid slug contained terpenoid compounds.

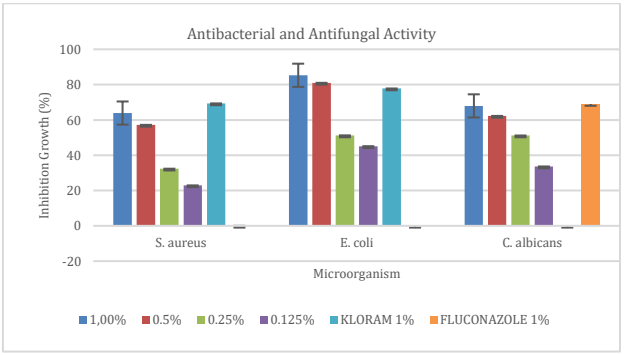
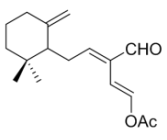
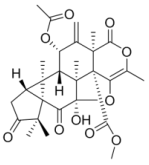


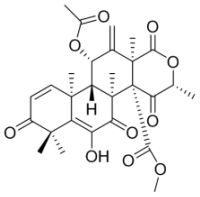
Figure 2. The antibacterial and antifungal activity of the ethanol extract of Onchidiid Slug



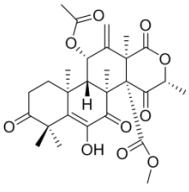
Onchidal



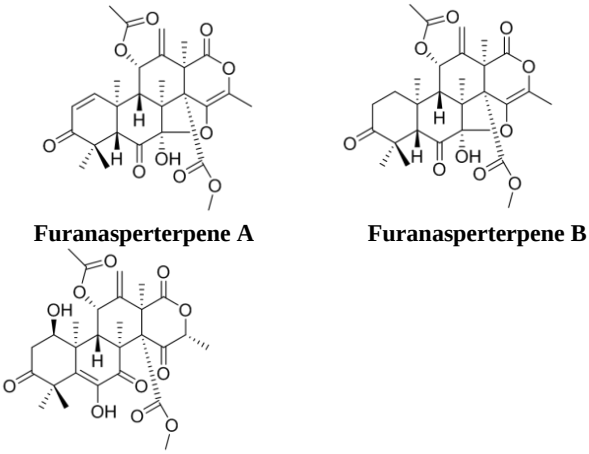
Aspermeroterpene A



Aspermeroterpene B



Aspermeroterpene C



Furanasperterpene A **Furanasperterpene B**

11-acetoxy-terretonin E

Figure 3. Structures of terpenoids and their derivates from Onchidiidae family

The results obtained on antifungal testing using microdilution showed that at 1% w/v concentration of ethanol extract Bajakah Tampala was able to provide inhibitory activity against *C. albicans* as much as 82.31% ± 0.01, whereas Fluconazole as positive control gave 88.10% ± 0.01 growth inhibition, respectively. This result provide evidence that the ethanol extract of Bajakah Tampala has an antifungal activity against *C. albicans*, and the activity was dose dependent.

Antibiofilm result of extract ethanolic onchidiid slug

This study measured the antibiofilm ability of the ethanolic extract of the onchidiid slug on the S. Aureus, E. Coli, and C. albicans biofilms formation. The results showed that the ethanolic extract of the onchidiid slug could inhibit the formation of S. Aureus, E. Coli, and C. albicans biofilms by 50%. The results of the biofilm assay of the onchidiid slug ethanolic extract are shown in Figure 4.

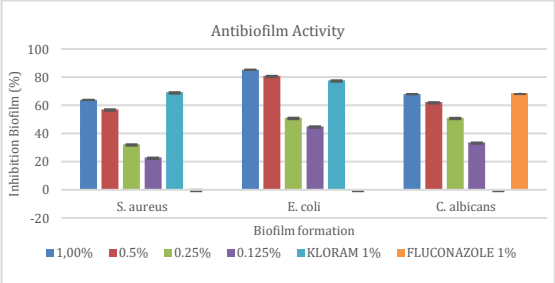


Figure 4. Antibiofilm Effect of Onchidiid slug ethanolic extract against S. aureus, E. coli, and C. albicans

The ethanolic extract samples of onchidiid slug showed inhibitory activity on the biofilms of S. aureus, E. coli, and C. albicans by 63.89%, 85.3%, and 67.95%, respectively, at 1% levels. This antibiofilm activity occurred because the extract could penetrate the EPS biofilm matrix. The EPS matrix was damaged and caused the matrix's S. aureus, E. coli, and C. albicans biofilms to break down to lysis. In line with studies results, the ethanolic extract of onchidiid slug can be developed towards a dosage formulation as a wound medicine which is mainly caused by bacterial infection[24].

CONCLUSION

The onchidiid slug ethanolic extract has an antibacterial, antifungal, and antibiofilm activity against S. aureus, E. coli, and C. albicans. The ethanolic extract of onchidiid slug can be developed as a wound medicine and, at a time, as an anti-biofilm.

Acknowledgment: We would like to thank all parties who support this study.

Ethical Approval: This research has passed the ethical clearance with No.700/UN22.9/PG/2022.

Funding Details: DIPA BLU UNTAN No. 023.17.2.677517/2022, 17 November 2022.

Conflict Of Interest: The author declares that there is no conflict of interest and all have an equal share in the content of this article.

Informed Consent: The research focused on the antibiofilm base on local wisdom Borneo Island, Onchidiid Slug (*Onchidiium typhae*) against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans.*

Authorship

B. Wijianto : main idea of study, writing manuscript, extraction sample, final approval, data analysis

H. Hamzah : main idea of research, biofilm testing, data analysis

REFERENCES

i. Raziyeva, K.; Kim, Y.; Zharkinbekov, Z.; Kassymbek, K.; Jimi, S.; Saparov, A. 2021. Immunology of Acute and Chronic Wound Healing. *Biomolecules*. *11*, 700. https://doi.org/ 10.3390/biom11050700.

ii. Thiruvoth, F.M.; Mohapatra, D.P.; Kumar, D.; Chittoria, S.R.K.; Nandhagopal, V. 2015. Current Concepts in the Physiology of Adult Wound Healing. *Plastic and Aesthetic Research*. *2*, 250–256. https://doi.org/ 10.4103/2347-9264.158851.

iii. Cañedo-Dorantes, L.; Cañedo-Ayala, M. Skin Acute Wound Healing: A Comprehensive Review. 2019. *International Journal of Inflammation*. *2019*, 3706315. https://doi.org/10.1155/2019/3706315.

iv. Tottoli, E.M.; Dorati, R.; Genta, I.; Chiesa, E.; Pisani, S.; Conti, B. 2022. Skin Wound Healing Process and New Emerging Technologies for Skin Wound Care and Regeneration. *Pharmaceutics*. *12*, 735. https://doi.org/10.3390/pharmaceutics12080735.

v. Dexheimer, V.; Frank, S.; Richter, W. 2012. Proliferation as a Requirement for In Vitro Chondrogenesis of Human Mesenchymal Stem Cells. *Stem Cells and Development*. *21*, 2160–2169. https://doi.org/10.1089/scd.2011.0670.

vi. Zhang, B.; Luo, Q.; Deng, B.; Morita, Y.; Ju, Y.; Song, G. 2018. Construction of Tendon Replacement Tissue Based on Collagen Sponge and Mesenchymal Stem Cells by Coupled Mechano-Chemical Induction and Evaluation of Its Tendon Repair Abilities. *Acta Biomaterialia*. *74*, 247–259. https://doi.org/10.1016/j.actbio.2018.04.047.

vii. Negut, I.; Grumezescu, V.; Grumezescu, A. 2018. Treatment Strategies for Infected Wounds. *Molecules*. *23*, 2392. https://doi.org/10.3390/molecules23092392.

viii. Puca, V.; Marulli, R.Z.; Grande, R.; Vitale, I.; Niro, A.; Molinaro, G.; Prezioso, S.; Muraro, R.; Di Giovanni, P. 2021. Microbial Species Isolated from Infected Wounds and Antimicrobial Resistance Analysis: Data Emerging from a Three-Years Retrospective Study. *Antibiotics*. *10*, 1162. https://doi.org/10.3390/antibiotics10101162.

ix. Shaw, T.J.; Martin, P. 2009. Wound Repair at a Glance. *Journal of Cell Science*. *122*, 3209–3213. https://doi.org/10.1242/jcs.031187.

x. Ramirez-Acuña, J.M.; Cardenas-Cadena, S.A.; Marquez-Salas, P.A.; Garza-Veloz, I.; Perez-Favila, A.; Cid-Baez, M.A.; Flores-Morales, V.; Martinez-Fierro, M.L. 2019. Diabetic Foot Ulcers: Current Advances in Antimicrobial Therapies and Emerging Treatments. *Antibiotics*. *8*, 193. https://doi.org/10.3390/antibiotics8040193.

xi. Wolcott, R.D.; Rhoads, D.D.; Bennett, M.E.; Wolcott, B.M.; Gogokhia, L.; Costerton, J.W.; Dowd, S.E. 2010. Chronic Wounds and the Medical Biofilm Paradigm. *J Wound Care*. *19*, 45–53. https://doi.org/10.12968/jowc.2010.19.2.46966.

xii. Di Martino, P. 2018. Extracellular Polymeric Substances, a Key Element in Understanding Biofilm Phenotype. *AIMS Microbiology***.** *4*, 274–288. https://doi.org/10.3934/microbiol.2018.2.274.

xiii. Costa, O.Y.A.; Raaijmakers, J.M.; Kuramae, E.E. 2018. Microbial Extracellular Polymeric Substances: Ecological Function and Impact on Soil Aggregation. *Frontiers in Microbiology*. *9*. https://doi.org/10.3389/fmicb.2018.01636.

xiv. Karygianni, L.; Ren, Z.; Koo, H.; Thurnheer, T. 2020. Biofilm Matrixome: Extracellular Components in Structured Microbial Communities. *Trends in Microbiology*. *28*, 668–681. https://doi.org/10.1016/j.tim.2020.03.016.

xv. Donlan, R.M. 2002. Biofilms: Microbial Life on Surfaces. *Emerg. Infect. Dis.* *8*, 881–890. https://doi.org/10.3201/eid0809.020063.

xvi. Percival, S.L.; McCarty, S.M.; Lipsky, B. 2015. Biofilms and Wounds: An Overview of the Evidence. *Advances in Wound Care*. *4*, 373–381. https://doi.org/10.1089/wound.2014.0557.

xvii. Bambang Wijianto; Hasyrul Hamzah; Annisa Larasati Nurhidayah; Guci Intan Kemuning; Riyadh Aqilsya Amaryl Dyas. 2022. Characterization of Onchidiid Slug (Onchidium Typhae) West Kalimantan Waters as Antibacterials and Antifungal. *Borneo Journal of Pharmacy.* *5*, 35–41. https://doi.org/10.33084/bjop.v5i1.2936.

xviii. Wang, B.; Chen, D.; Yu, M.; Liu, Y.; Liu, P.; Zhang, X. 2021. A Review on Metabolites from Onchidium Genus: Chemistry and Bioactivity. *Chem. Biodiversity*. *18*. https://doi.org/10.1002/cbdv.202000580.

xix. Guan, J.; Shen, H.D.; Qian, J.; Zhang, K.X.; Zheng, P. 2013. Analysis and Evaluation of Nutritive Composition of Four Species of Onchidiidae. *Sci. Tech. Food Industry*. 349–354.

xx. Wijianto, B.; Nurhidayah, A.L.; Luliana, S. 2022. Standardization of secondary metabolites and heavy metal contamination assay on onchidiid slug (*Onchidium typhae*) West Kalimantan waters. *JFSP*. *8*, 199–206. https://doi.org/10.31603/pharmacy.v8i3.7296.

xxi. Wijianto, B.; Ritmaleni, R.; Purnomo, H.; Nurrochmad, A. 2019. In Silico and in Vitro Assay of HGV Analogue as Antibacterial. *Int J Pharm Pharm Sci*. *11*, 78–85. https://doi.org/10.22159/ijpps.2019v11i3.30581.

xxii. Hamzah, H.; Siregar, K.A.; Nurwijayanto, A.; Wahyuningrum, R.; Sari, S. 2021. Effectiveness of Oxalis Corniculata L. Ethanol Extract against MonoSpecies of Biofilm Staphylococcus Aureus. *Borneo Journal of Pharmacy*. *4*, 184–191.

xxiii. Wijianto, B.; Ritmaleni; Purnomo, H.; Nurrochmad, A. 2020. Curcumin Mono-Carbonyl Analogs as Potent Antibacterial Compounds: Synthesis, Biological Evaluation and Docking Simulation Study. *RJC*. *13*, 1153–1165. https://doi.org/10.31788/RJC.2020.1325554.

xxiv. Wolcott, R. 2015. Disrupting the Biofilm Matrix Improves Wound Healing Outcomes. *J Wound Care*. *24*, 366–371. https://doi.org/10.12968/jowc.2015.24.8.366.