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Potential and Effectiveness of Snail Seromucoid and Chitosan as Bioimmunostimulators

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ABSTRACT

The host's cellular immune response plays an important role in the process of eliminating microorganisms that cause infection. Substances that can stimulate an increase in the immune response are called immunostimulators. Snail seromucoid contains bioactive compounds such as glycans, peptides, glycopeptides and chondroitin sulfate. Chitosan as an antimicrobial agent can be used in the biomedical field because chitosan has a number of hydroxyl groups (OH) and amine groups (NH_2). The research objective was to determine the potency and effectiveness of snail seromucoid and chitosan as bioimmunostimulators. The research method is based on laboratory experimental results with the research stages, namely the analysis of the effectiveness of seromucoid and chitosan on lymphocyte proliferation. The results of the one way ANOVA analysis showed a p value of 0.000 so that there was a significant effect between the treatment groups, namely the effect of giving chitosan 65 ug/ml; snail mucus 65 ug/mL and a combination of chitosan (65 ug/mL) and snail mucus (65 ug/mL) ratio of 1:1, can increase lymphocyte proliferation optimally. This shows that snail mucus, chitosan and their combination in a 1:1 ratio are effective as BRM (Biological Response Modifier). It is hoped that the contribution of the results of this study can be further developed in the bioformulation of snail seromucoid preparations and chitosan as therapeutic agents for infectious diseases, including Acute Respiratory Distress Syndrome (ARDS), Tuberculosis and other diseases.

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INTRODUCTION

Inflammation is a complex biological response of vascular tissue to the presence of pathogens, cell damage or irritation. This is an organism's self-protective effort to remove the stimulus that causes injury and initiate the tissue healing process. Currently, many anti-inflammatory drugs have low therapeutic indications, and the maximum dose of drug given only provides minimum effectiveness. The several types of therapy that have been carried out so far, satisfactory results have not been obtained and have adverse side effects, so it is necessary to look for other alternatives in the treatment of various diseases, one of which is by using immunostimulatory substances.

Immunostimulators are compounds that can increase the immune response in various ways, namely increasing the number and activity of T cells, NK cells and macrophages as well as releasing interferons and interleukins to enhance cellular defense. The inflammatory response in the body is characterized by the presence of various mediators, such as pro-inflammatory cytokines in the form of IL-1, Tumor Necrosis Factor (TNF), Interferon (INF), IL-6, IL-12, and IL-18 (Abbas et al, 2014) . In addition, Nitric Oxidase and COX-2 can stimulate the production of pro-inflammatory mediators. Types of anti-inflammatory cytokines such as IL-4, IL-10, IL13, and IFN-a work antagonists against pro-inflammatory cytokines. Th2 cytokines such as IL-4 and IL-13 can inhibit autophagy due to Interferon gamma (IFNG) induction. Diagnostic tools for measuring various types of cytokines produced by lymphoid cells and are preferred in assessing cell function and cell responses to various stimuli include the ELISA method (Harti et al. 2018).

Snail seromucoids contain bioactive compounds such as glycans, peptides, glycopeptides and chondroitin sulfate (Viera et al, 2004). Chondroitin sulfate can function as an immunomodulator and immunosuppressant. Gastropod hemocytes play an important role in cell defensive reactions, namely phagocytosis, encapsulation, nodulation and neutralization of parasites, blood coagulation processes and wound healing. The snail hemolymph bioactive compound has the potential to be a drug derivative that can be used in the medical field, including skin smoothing, treatment of respiratory infections and wound healing (Ulagesan, 2018).

Chitosan is a complex compound of the glycoprotein class derived from chitosan resulting from the deacetylation of chitin which has a 1.4 glucosamine bond. The potential of chitosan as an antimicrobial agent can be used in the biomedical field because chitosan has a number of hydroxyl groups (OH) and amine groups (NH2) (Nakashima, 2014).

The use of bioactive compounds based on natural ingredients as bioimmunostimulators aims to change the activity of the body's immune system by dynamically regulating immune system cells such as cytokines. The results of Harti et al's research (2019a) showed that the bioactive compounds in snail seromucoid and chitosan can increase lymphocyte proliferation so that they have the potential as immunostimulators.

The research objective was to determine the potential and effectiveness of snail seromucoid and chitosan as bioimmunostimulators. The results of the research are expected to be able to further develop the bioformulation of snail seromucoid preparations and chitosan as therapeutic agents for infectious diseases, including ARDS, tuberculosis and other diseases.

MATERIALS AND METHODS

The research was conducted at the Integrated Research and Testing Laboratory, Gajah Mada University, Yogyakarta in January - June 2021. This type of research is an experimental research. The research variables used consisted of independent variables, namely the formulation of snail seromucoid and chitosan. The dependent variable is the assessment of lymphocyte proliferation test results.

Materials and Equipment

Materials: local snail, Biorechsindo chitosan, Balb/c mice, ELISA reagent kit for IgM and IgG (Sigma), MTT reagent (E.Merck), RPMI medium (Sigma), penicillin, streptomycin, fungizone , fetal bovine serum (FBS). Equipment: electric shock, milipore filter, tissue culture plastic, inverted microscope (Olympus), 5% CO_2 incubator (Heraeus), microplate 96 (Costar), microplate reader (Bio-Rad), micropipette (Gibson), eppendorf tube, vortex, laminar air flow (Nua ire), white tip, yellow tip, blue tip (Brand).

Ethics Statement

This study was approved by the Dr. Moewardi General Hospital Health Research Ethics Committee, Surakarta Indonesia with letter number: 789/VI/HREC/2021.

Research design and design

This study with Quasi-experimental research design:

Intervention (X1) → Lymphocyte proliferation (increased bsorbance)

Control (X2, X3) → Lymphocyte proliferation (increased bsorbance)

Note:
X1: Dosage of chitosan, snail mucus and their combinations
X2: Positive control
X3: Negative control

Freeze drying Seromucoid snail

Local snail samples (*Achantina fulica*) with an average weight of 19 g and a height/width of 25/43 mm obtained from local cultures were placed in a plastic container at room temperature 20 -22^{o} C. The seromucoid fluid obtained was then put in a sealed container or tightly closed. then the freeze drying process was carried out at -48^{o}C for 24 hours using a freeze drying tool. Freeze drying results were weighed. The seromucoid freeze drying process was carried out at the Pharmaceutical Laboratory of the Muhammadiyah University of Surakarta (Figure 1).

Preparation of 2% Chitosan Solution

Pure chitosan (medical grade) was obtained from PT. Biotechsurindo Cirebon Indonesia. Chitosan was weighed accurately and accurately 2 grams and then dissolved in 2% acetic acid solution ad 100 ml.

Lymphocyte Cell Isolation

Isolation of lymphocyte cells obtained from the spleen of Balb/C strain mice. In the first stage, the mice were sacrificed using ether, then the mice were dissected and the spleen tissue was taken. The splenic tissue was isolated aseptically and placed in a 50 mm diameter petri dish containing 10 mL of RPMI medium.

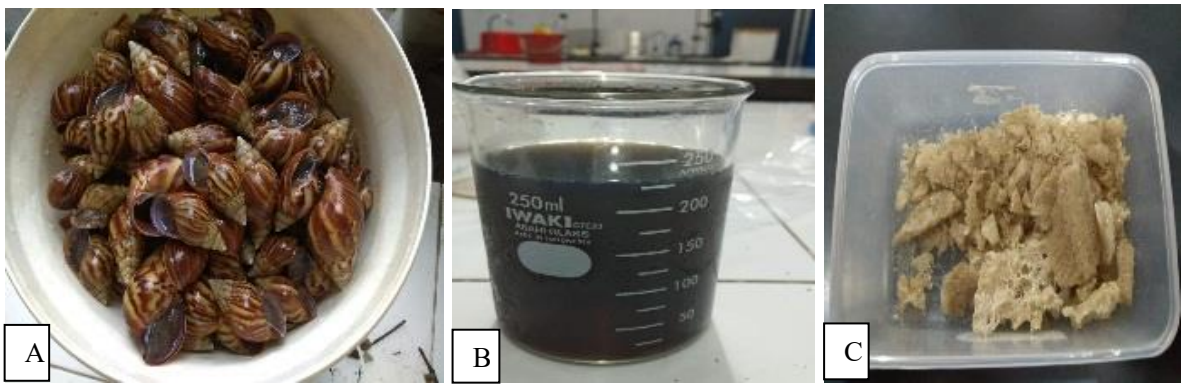

Figure 1. (A) Snail, (B) Seromucoid, (C) Freeze drying

Spleen tissue is lysed by means of RPMI media being pumped into the spleen so that lymphocytes come out with the media. The cell suspension was put in a 10 mL centrifugation tube and centrifuged at 3000 rpm 4^{o}C for 5 minutes. The pellet obtained was suspended in 5 mL of ammonium chloride tris buffer to lyse the erythrocytes. The cells were mixed until homogeneous and left at room temperature for 15 minutes or until the color changed to slightly yellowish. Then 10 mL of RPMI ad was added, centrifuged at 3000 rpm 4^{o}C for 5 minutes and the supernatant was discarded. The pellets obtained were washed 2 times with complete RPMI containing 10% Fetal Bovine Serum (FBS), 0.5% fungizone, 2% Penicillin-Streptomycin, homogenized, then cell count was performed using a hemocytometer.

Lymphocyte Cell Count Determination Test and Optimum Con A Concentration

To find out the number of lymphocyte cells and the proper concentration of Cocanavalin A (Con A) to be used in the lymphocyte cell proliferation activity test, a preliminary test was carried out. This test uses lymphocyte cell cultures of 1x10^{4}cell/ml, 2x10^{4}cell/ml, 4x10^{4}cell/ml, and 10x10^{4}cell/ml. While the concentration of Con A used 10 µg/ml, 25 µg/ml, 50 µg/ml, 75 µg/ml and 100 µg/ml. Assay using complete RPMI medium and incubated on cell culture plates for 48 hours. The results that give the highest average number of lymphocytes will be used in future studies.

Proliferation test with MTT reduction

Cell proliferation was detected by the color change method using methylthiazoltetrazolium solution. The principle of proliferating cells is that their mitochondria will absorb MTT (Microtetrazolium) so that these cells will turn dark purple due to the formation of tetrazolium crystals. Based on this principle, 4 hours before the 72-hour incubation period, the micro-culture plate is removed from the CO2 incubator. Each well was given 20 µl (100 µg) MTT solution, then the cell culture was incubated in an incubator. A total of 200 µl of medium from each well at the end of a total incubation period of 72 hours was carefully aspirated using a micropipette. Next, 100 µl of isopropanol containing 0.04 N HCl was added to each of the wells to resuspend the tetrazolium crystals that formed. After the crystals dissolve, a purple solution is formed with an intensity proportional to the level of cell proliferation. Color intensity was measured using an ELISA photoreader at a wavelength of 570 nm. The number of lymphocyte cells used was 2 x 10^{5} cells in each well. Determination of the percentage of lymphocyte proliferation is carried out using the formula:

$$\text{Lymphocyte proliferation} = \frac{\text{Abs of sampel}}{\text{Abs of control}} \times 100\%$$

Data analysis

Data were analyzed statistically using the SPSS program. Test the normality of the distribution with the Kolmogorov-Smirnonov Test of Homogeneity of Varience. Parametric test with the One Way Anova test followed by the LSD test.

RESULTS AND DISCUSSION

The results of the research on the effect of the dose of snail mucus, chitosan and a combination of snail mucus and chitosan ratio of 1:1 on lymphocyte proliferation based on the MTT method are as listed in table 1.

The results of the one way ANOVA analysis showed a p-value of 0.000 so that there was a significant effect between the treatment groups, namely the administration of chitosan, snail slime and a combination of snail mucus and chitosan ratio of 1:1 on increasing lymphocyte proliferation. Chitosan 65 ug/ml; snail mucus 65 ug/mL and a combination of chitosan (65ug/mL) and snail mucus (65ug/mL) ratio of 1:1, can increase lymphocyte proliferation optimally. This

shows that snail mucus, chitosan and their combination in a 1:1 ratio are effective as BRM (Biological Response Modifier).

The immunostimulatory effect found in snail seromucoid and chitosan can be used to increase the body's immunity against intracellular facultative pathogenic bacterial infections. The content of bioactive compounds in serromucoid and chitosan can stimulate the function of cellular immunity, namely lymphocyte proliferation and production of reactive oxygen intermediated macrophages. Testing the bioactivity of snail seromucoid on lymphocyte proliferation can be carried out on lymphocyte cells which are treated as normal human cells. If an agent is not toxic to lymphocyte cells, then it can be hypothesized that the agent is also not toxic to normal cells. Lymphocyte proliferation activity against agent exposure is influenced by cell quality and quantity factors as well as inducing agents including the type and number of lymphocytes, the active compound of an agent. The mechanism of an anti-inflammatory agent is capable of inducing apoptosis by inhibiting the targets of several cell-signaling pathways including transcription factors, oncogenes and signaling proteins (Abbas *et al*, 2014). The three mechanisms of proliferation are mitosis, amitosis and cytoplasmic fragmentation (Dolashka, 2015).

Table 1. The results of the optical density of lymphocyte proliferation with MTT method

Parameter	Mean O.D of lymphocyte proliferation
Dosage of chitosan (ug/mL)	
30	0.097
35	0.142
40	0.166
45	0.206
50	0.243
55	0.261
60	0.466
65	0.491
Dosage of snail mucus (ug/mL)	
30	0.180
35	0.191
40	0.228
45	0.210
50	0.329
55	0.358
60	0.396
65	0.462
Dosage combination of snail mucus and chitosan ratio of 1:1 (ug/mL)	
30 : 30	0.271
35 : 35	0.471
40 : 40	0.524
45 : 45	0.656
50 : 50	0.630
55 : 55	0.644
60 : 60	0.758
65 : 65	1.103
Control cells negative without ConA	0.330
Control cells positive with ConA	0.661
Media control	0.059

Based on the results of Harti *et al*'s research (2019 a) on the characterization of the snail seromucoid protein profile using the SDS-PAGE method, it shows that there are 3 protein subunits, namely the range of 55 – 72 kDa and 1 specific 43 kDa protein subunit which is suspected as an adhesion protein and functions as an immunostimulatory BRM. . The effect of snail mucus as an anti-inflammatory agent will further accelerate the inflammatory phase so that the lymphocyte proliferation phase will also be faster in wound healing. Snail seromucoid concentration of 100% is antibacterial against *Staphylococcus aureus, Candida albicans* and *Pseudomonas aeruginosa* (Harti et al, 2016 a). Based on the research results of Harti et al (2018) showed that the optimum effectiveness of the 5% chitosan mixture; 100% snail mucus and 5% snail mucus cream on lymphocyte proliferation in vitro.

A number of lectin proteins in snail mucus, namely selectin, galectin, C-type lectin, and fibrinogen-related protein (FREPs) function in the pathogen agglutination process, cytotoxicity and phagocytosis (Rosanto *et al,* 2021). Various types of proteins or known as achasin proteins in snails have important biological functions, including as bacterial protein (enzyme) binding receptors (Dang, 2015). In addition, the presence of aldolase and myosin was identified as a protein that plays a role in regulating hemocyte migration and has an impact on the process of killing pathogens through cytotoxic reactions and phagocytosis (Suwannatri, 2016). Snail mucus is 100% effective in inhibiting the growth of Gram positive bacteria (*Staphylococcus aureus*) and Gram negative bacteria (*Salmonella typhosa*) (Huda, 2016). The inhibition and antibacterial potency of snail mucus on wound isolates of *Staphylococcus sp, Streptococcus sp* and *Pseudomonas sp* were varied (Etim, 2015). Snail mucus protein (*Achatina fulica*) with a molecular weight of 50.81 kDa, 15 kDa, 11.45 kDa as achacin protein (Zhuang, 2015) has antimicrobial activity against *Streptococcus mutans* and *Actinobacillus actinomycetemcomitans* (Greistorfer, 2017). The lectin compound or *Helix pomatia* agglutinin (HPA) in the *Helix pomatia* snail species can be used as a prognostic indicator in several cancer cases, namely breast, gastric and intestinal cancer, namely the presence of HPA in the fixation of tissue preparations as a glycoprotein which is associated with cancer metastasis (Bonnemain, 2005). Various types of proteins known as Achasin proteins in snails have important biological functions, including as bacterial protein (enzyme) binding receptors (Dolaskha, 2015). Snail mucus is capable of being antibacterial against *Streptococcus mutans* and *Escherichia coli* (Berniyanti and Suwarno, 2007) and inhibiting the growth of Methicillin Resistant *Staphylococcus aureus* (MRSA) (Anggraini, 2018).

Giant African snail slime contains substances that are antimicrobial peptides, namely mytimacin-like antimicrobial and glycolic acid (El Mubarak *et al*, 2013). Acharan sulfate as a glycosaminoglycan in African giant snails, structurally similar to heparin and heparan sulfate; widely used in medical preparations (Sallam, 2019). Glycoprotein achacin as an antibacterial factor in *Lissachatina fulica* is known as the African giant snail and *Pomacea canaliculata* as the golden snail which has anti-bacterial properties in the cell membrane against Gram-positive and Gram-negative bacteria, namely against *Staphylococcus aureus, S. epidermidis*, Methicillin-Resistant Staphylococcus Aureus (MRSA), *Staphylococcus epidermidis* and *Corynebacterium sp* (Nantarat *et al*., 2019). The type of protein resulting from genetic expression for each different snail strain (Bismili, 2013). The results of the research by Sutanto et al (2020) and (2021 a and b) showed that snail seromucoid was not effective as an antimicrobial against MTB isolates in vitro.

Chitosan is a β-(1.4)-2 amino-2deoxy D-glucopyranose compound, as a product of deacetylation of chitin. Chitosan has been used extensively in the biomedical and pharmaceutical fields because it is biodegradable, non-toxic, non-immunogenic, and biocompatible with animal

tissues (Kazami, 2005). The effectiveness of chitosan as an antimicrobial is related to the role of the Chito-Oligosaccharide (COS) compound, which is a group of glycan-binding protein complex compounds that have 1,4-b-glucosamine which is a deacetylated chitosan derivative of chitin (Ibrahim, 2016). The uniqueness of COS is polycationic which can suppress the growth rate of diarrheagenic *Escherichia coli* in vitro. (Harti et al. 2016 a; Harti et al. 2016 b; Harti et al. 2018). Chitosan has biocompatible, biodegradable, non-toxic, antimicrobial and hydrating properties. Chitosan also affects the blood clotting process so that it can be used as a hemostatic and positive effect on wound healing.

The content of bioactive compounds in seromucoid and snail chitosan can stimulate cellular immune function, namely lymphocyte proliferation and production of reactive oxygen intermediate macrophages. Seromucoid forms of freeze drying and chitosan showed significant activity on lymphocyte proliferation in vitro compared to non freeze drying snail mucus (Harti et al. 2019).

The essence of the research is the need for further research related to the development of bioformulation of snail seromucoid preparations and their combination with chitosan which is the most effective as an anti-inflammatory drug candidate.

CONCLUSION

The addition of chitosan at a dose of 65 ug/mL and snail mucus at a dose of 65 ug/mL and a mixture of chitosan (65ug/mL): snail mucus (65ug/mL) = 1:1 can significantly increase lymphocyte proliferation. There were significant differences between the treatment groups. A mixture of snail mucus and chitosan is effective as BRM and has potential as a drug candidate for anti-inflammatory cell therapy, so further research is needed.

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CONFLICT OF INTEREST

We have no conflict of interest related to this work.

REFERENCES

Abbas, A.K., Lichman, A.H., Andrew, H. and Shiv, P.(2014). *Effector Mechanisms of T Cell Mediated Immunity Functions of T Cells in Host Defense. Basic Immunology : Functions and Disorders of the Immune System.* Philadelphia USA: Elsevier Saunders.

Anggraini D, Sri Darmawati, Endang T.W. M., (2018). Protein profile and anti-microbial power of snail slime (Achantina fulica) against Methicillin Resistant Staphylococcus aureus (MRSA). *Proceedings of the Unimus Student National Seminar* 1, pp. 73 - 77

Berniyanti T. and Suwarno,S. (2007). Characterization of local isolate snail slime (achasin) protein as an antibacterial factor. *Journal of Veterinary Media*, 23 (3) pp. 139-144

Benkendorff, K., Rudd, D., Nongmaithem, B. D., Liu, L., Young, F., Edwards, V., Avila, C., & Abbott, C. A. (2015). Are the traditional medical uses of muricidae molluscs substantiated by their pharmacological properties and bioactive compounds?. *Marine drugs*, *13*(8), 5237–5275. https://doi.org/10.3390/md13085237

Bislimi, K.., Halili, J., Mazreku, I., Osmani, F., Halili, F. (2013). Comparative analysis of some biochemical parameters in emolymph of garden snail (*Helix pomatia* L.) of the Kastriot and Ferizaj Regions, Kosovo. *International Journal of Engineering and Applied Sciences* 4 (6) pp 11-18 www.eaas-journal.org

Bonnemain B. (2005). Helix and Drugs: snails for western health care from antiquity to the present. *Evidence-based complementary and alternative medicine : eCAM*, *2*(1), 25–28. https://doi.org/10.1093/ecam/neh057

Dang, V. T., Benkendorff, K., Green, T., & Speck, P. (2015). Marine snails and slugs: a great place to look for antiviral drugs. *Journal of virology*, 89(16), 8114–8118. https://doi.org/10.1128/JVI.00287-15

Dolashka, P., Dolashki, A., Van Beeumen, J., Floetenmeyer, M., Velkova, L., Stevanovic, S., & Voelter, W. (2016). Antimicrobial activity of molluscan hemocyanins from helix and rapana snails. *Current pharmaceutical biotechnology*, *17*(3), 263–270. https://doi.org/10.2174/1389201016666150907113435

El Mubarak, M. A., Lamari, F. N., & Kontoyannis, C. (2013). Simultaneous determination of allantoin and glycolic acid in snail mucus and cosmetic creams with high performance liquid chromatography and ultraviolet detection. *Journal of chromatography. A*, *1322*, 49–53. https://doi.org/10.1016/j.chroma.2013.10.086

Etim, L.B., Aleruchi, C., & Obande, G.A. (2016). Antibacterial properties of snail mucus on bacteria isolated from patients with wound infection. *British microbiology research journal, 11*, 1-9. https://doi.org/10.9734/BMRJ/2016/21731

Harti, A.S, Puspawati, N., Putriningrum, R. (2019). Antimicrobial bioactive compounds of snail seromucoid as biological response modifier immunostimulator. *Journal of Microbiology Indonesia*, 13 (2), pp 56-63. https://doi.org/10.5454/mi.13.2

Harti, A.S., Estuningsih, E., Kusumawati, H.N., Siswiyanti, S., and Setyaningtyas, A., (2016 a). In vitro synergistic effect of snail slime and chitosan against Staphylococcus aureus. *International Journal of Pharma Medicine and Biological Science*, 5 (2), pp. 137-141. https://doi.org/10.18178/ijpmbs.5.2.137-141

Harti, A.S., Sulisetyawati, D.S, Murharyati, A., Oktariani, M., Wijayanti, I.B. (2016b). The effectiveness of snail slime and chitosan in wound healing. *International Journal of Pharma Medicine and Biological Science* 5 (1), pp 76-80. https://doi.org/10.18178/ijpmbs.5.1.7680

Harti, A.S., Murharyati, A., Sulisetyawati, D.S., Meri Oktariani, M. (2018). The effectiveness of snail mucus (achantina fulica) and chitosan towards lymphocyte proliferation in vitro. *Asian Journal Pharmaceutical and Clinical Research;* 11 (3), pp. 85-88. https://doi.org/10.22159/ajpcr.2018.v11s3.30041

Huda M., and Marhamah, M. (2016). Effect of snail mucus (*Achantina fulica*) on the growth of Gram-positive and Gram-negative bacteria. *Journal of Health Analyst* . 5 (2), pp. 547 - 555 http://dx.doi.org/10.26630/jak.v5i2.461

Ibrahim, K. A., El-Eswed, B. I., Abu-Sbeih, K. A., Arafat, T. A., Al Omari, M. M., Darras, F. H., & Badwan, A. A. (2016). Preparation of chito-oligomers by hydrolysis of chitosan in the presence of zeolite as adsorbent. *Marine drugs*, *14*(8), 43. https://doi.org/10.3390/md14080043

Greistorfer, S., Klepal, W., Cyran, N., Gugumuck, A., Rudoll, L., Suppan, J., & von Byern, J. (2017). Snail mucus - glandular origin and composition in Helix pomatia. *Zoology (Jena, Germany)*, *122*, 126–138. https://doi.org/10.1016/j.zool.2017.05.001

Naoshi, K., Yasusato, S., Masakichi, S., and Masao,K. (2005) Preparation of chito-oligosaccharides by two step hydrolysis. *Chitin and Chitosan Research Journal* . 11 (2). pp. 170-171.

Nakashima, M., & Iohara, K. (2014). Mobilized dental pulp stem cells for pulp regeneration: initiation of clinical trial. *Journal of endodontics*, *40*(4 Suppl), S26–S32. https://doi.org/10.1016/j.joen.2014.01.020

Nantarat, N., Tragoolpua, Y., & Gunama, P. (2019). Antibacterial activity of the mucus extract from the giant african snail (*Lissachatina fulica*) and golden apple snail (*Pomacea canaliculata*) against pathogenic bacteria causing skin diseases. *Tropical Natural History*, *19*(2), 103–112. Retrieved from https://li01.tci-thaijo.org/index.php/tnh/article/view/150872

Rosanto,Y.B., Hasan, C.Y., Rahardjo R. and Pangestiningsih, T.W. (2021). Effect of snail mucus on angiogenesis during wound healing *F1000Research* 2021, **10**:181, pp. 1-17 https://doi.org/10.12688/f1000research.51297.2

Sallam, A.A.A, El-Massry, S.A., and I.N. Nasr, I.N. (2009). Chemical analysis of mucus from certain land snails under Egyptian conditions. *Archives of Phytopathology and Plant Protection*. 42(9) pp.874-881. DOI: 10.1080/03235400701494448.

Suwannatri, K., Aiporn, S., Paitra. T., Jariya,U.W., Sirikachorn ,T., Cinzia,C. et al. (2016) Differential protein expression in the hemolymph of bithynia siamensis goniomphalos infected with Opisthorchis viverrini. *PLOS Neglected Tropical Disease Journal* . 10 (11), pp. 1-20. https://doi.org/10.1371/journal.pntd.0005104

Sutanto, Y. S., Sutanto, M., Harti, A. S., & Puspawati, N., 2020. The sensitivity test of *Mycobacterium tuberculosis* isolates from suspect tuberculosis patients to the seromucous of snail and chitosan as an alternative anti-tuberculosis drug. *Asian Journal of Pharmaceutical and Clinical Research*, 13(10) pp. 44-49. https://doi.org/10.22159/ajpcr.22020.v22113i22110.38266

Sutanto, Y.S. Sutanto, M., Harti, A.S., 2021. Anti-tuberculosis drugs against the resistance level of *Mycobacterium tuberculosis* isolates. KEMAS, 17 (1) 14-23. https://doi.org/10.15294/kemas.v17i1.24158

Sutanto, Y. S., Sutanto, M., Harti, A. S., & Puspawati, N. (2021). The Sensitivity test of *Mycobacterium tuberculosis* to snail seromucoid and chitosan in vitro. *Biomedika* 14 (1), pp 36-46. https://doi.org/10.31001/biomedika.v14i1.1128

Ulagesan, S.; Kim, H.J. Antibacterial and antifungal activities of proteins extracted from seven different snails. *Appl. Sci.* **2018**, *8*, 1362. https://doi.org/10.3390/app8081362

Vieira, T. C., Costa-Filho, A., Salgado, N. C., Allodi, S., Valente, A. P., Nasciutti, L. E., & Silva, L. C. (2004). Acharan sulfate, the new glycosaminoglycan from Achatina fulica Bowdich 1822. Structural heterogeneity, metabolic labeling and localization in the body, mucus and the organic shell matrix. *European journal of biochemistry*, *271*(4), 845–854. https://doi.org/10.1111/j.1432-1033.2004.03989.x

Zhuang, J., Coates, C. J., Zhu, H., Zhu, P., Wu, Z., & Xie, L. (2015). Identification of candidate antimicrobial peptides derived from abalone hemocyanin. *Developmental and comparative immunology*, *49*(1), 96–102. https://doi.org/10.1016/j.dci.2014.11.008