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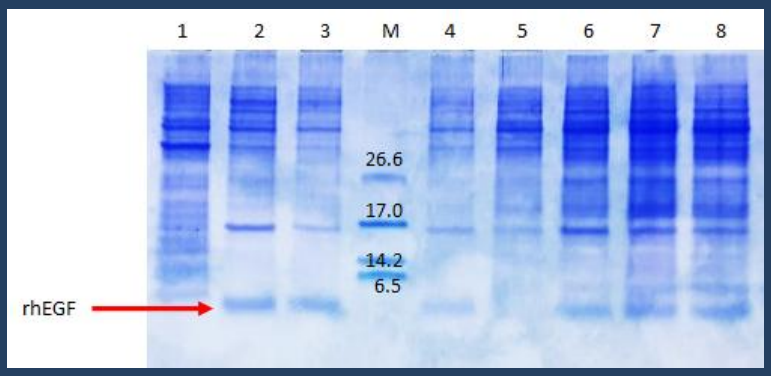

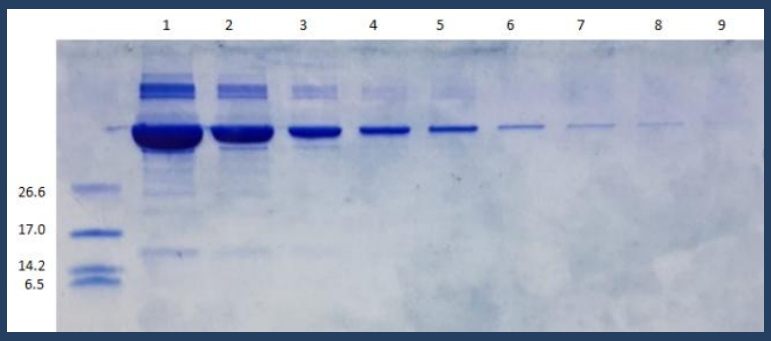

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EDITOR'S PREFACE

Dear Colleagues,

We are delighted to present to you the second number of Annales Bogorienses Volume 25, Year 2021 that contains 1 review paper and 4 original papers.

We would like to take the opportunity to extend our sincere acknowledgement to all the authors and readers of our journal, the Journal of Annales Bogorienses, for extending their kind support and co-operation which truly helped the journal to grow and become as successful as it is today.

December, 2021

The Editor

Mini Review: Prostate Cancer Diagnosis and Therapy

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Abstract

Cancer, a non-communicable illness, is the leading cause of death worldwide. In 2030, cancer is expected to exceed 21 million cases and 13 million cancer deaths globally. One of the most prevalent and significant types is prostate cancer (PCa). It is commonly associated with adenocarcinoma, which develops from the mucous glands within the organ. This review highlights available detection systems, and therapeutic options in PCa management. One prevention of deadly PCa is early diagnoses, such as prostate-specific antigen (PSA) screening or genomic profiling. Further testing like MRI or CT scan may also be needed to detect cancers that have progressed to other body regions. There are several possible treatments for PCa, including watchful cancer waiting, surgery, radiotherapy, hormone therapy, and chemotherapy. Based on current studies, androgen deprivation therapy (ADT) combined with docetaxel therapy enhanced great results to treat advanced PCa. The latest development, called theranostics, is a single entity that can perform both diagnostic and therapeutic functions. It can detect disease borders, track therapy in real-time, and provide prognostic data. The FDA has already authorized two prostate-specific membrane antigen (PSMA) positron emission tomography (PET) devices: including Gallium 68 PSMA-11 (Ga 68 PSMA-11) and Pylarify (piflufolastat F 18).

Keywords: prostate cancer, prostate-specific antigen (PSA), docetaxel, androgen deprivation therapy (ADT), theranostics

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Introduction

Cancer is a non-communicable disease which is expected to rank as the leading cause of death worldwide. In most cases, lifestyle-related factors including tobacco and obesity remain the major contributors of attributable cancer. In addition, environmental factors (e.g., air and water pollution), bacteria, and virus infections, as well as family history can also induce human cancers. It was reported that the cancer cases worldwide in 2020 were 19.29 million incidence and 9.95 million mortality cases (Sung *et al*., 2021), and it is estimated to increase to 21.61 million new cancer cases and 11.36 million cancer deaths in 2025 (Ferlay *et al*., 2020). Furthermore, the global cancer burden is projected to reach 24.04 million cases and 12.9 million deaths from cancer in 2030 with breast, prostate, and lung cancer are remaining as the major cancer diagnoses (Rahib *et al*., 2014; Zarocostas, 2010).

Cancer development is associated with the accumulation of genetic mutations and also epigenetic changes resulting in the loss of control in cellular growth of cancer cells, which could invade normal tissues and organs, and spread throughout the body (Hanahan & Weinberg, 2011; Sharma *et al*., 2010). There are hundreds of different types of cancers, which can have distinct behaviours with various treatment approaches.

Prostate cancer (PCa) is the most common cancer diagnosed in men and a major cause of cancer-related male mortality (Siegel *et al*., 2013). In 2020, it was predicted that the PCa incidence reached 1.4 million new cases with 375,304 mortalities worldwide (Sung *et al*., 2021). The incidence rates are highest among men in developed countries, including North America, Northern and Western Europe, and Australia/New Zealand, as well as in Sub-Saharan Africa, while mortality rates are highest in developing countries. According to recent studies, the differences of incidence and mortality rates of PCa among countries are associated with genetics, environmental factors, general wellness and lifestyles, and access to

early detection and health services (Bray *et al*., 2018; Taitt, 2018). This review will focus on the recent advancements in detection and treatment modalities for PCa management.

What is Prostate Cancer?

PCa is primarily associated with adenocarcinoma, growing from the mucous glands inside the organ. Then, the cancer cells proliferate and spread to the surrounding prostate tissue, starting to form tumor nodules (Jensen *et al*., 1980). Like other solid tumor malignancies, PCa can spread to distant parts of the body, commonly into the bone. A study showed that approximately 80% of PCa deaths were related to bone metastases (Bubendorf *et al*., 2000). In clinical application, cancer staging is used to define the extent of spread of PCa. This clinical stage is generally based on physical examinations, and other detection methods (e.g blood tests, imaging tests or biopsy), commonly using TNM system with stages ranging from 0 to 4, and subclassified in every stage using the letters A, B, and C. The higher the number of a stage, describes the larger the tumor size and the further the extent of spreading from the main tumor (Figure 1) (Cheng *et al*., 2012).

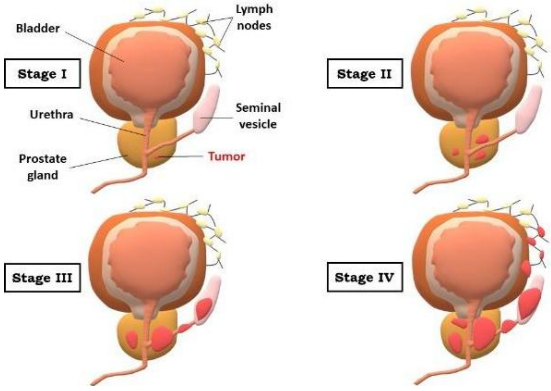

Figure 1. Stages of PCa. Stage I, tumor is only present in one side of the prostate; Stage II, tumor has progressed in one side or both sides of the prostate, but does not spread outside; Stage III, tumor has grown significantly, extends beyond the seminal vesicle; Stage IV, tumor has grown more advanced, invades adjacent tissues other than the seminal vesicle, such as bladder neck, lymph nodes, rectum, or another part of the body.

Prostate Cancer Detection

Prostate epithelial cells, both normal and cancerous, synthesize a certain glycoprotein known as prostate-specific antigen (PSA). This serine protease enzyme has main functions in ejaculation and coagulum hydrolysis. Despite being normally secreted in urine or semen, in some abnormal conditions, elevated levels of PSA also can be detected in blood. Thus, PSA is a common serum biomarker in early detection of prostate cancer (NCI, 2021; Aronson & DeKernion, 2007; David & Leslie, 2021; Gao *et al*., 2019; Sandhu & Schlegel, 2004).

A prostate-specific antigen screening has been widely used for early detection of PCa resulting in a higher number of incidences recorded. Some studies suggest that PSA testing has resulted in improved treatment and has contributed to the modest reduction of mortality from PCa (Welch & Albertsen, 2009). However, raised PSA level in the serum is also found in benign conditions, such as benign prostatic hyperplasia (BPH) and small tumor, which means relying on PSA testing alone in prostatic cancer detection, would potentially cause overdiagnosis (David & Leslie, 2021). Overdiagnosis is defined as detection of tumors, -either benignant or malignant- through screening that are not life-threatening and unlikely to be recognised clinically or symptomatically without the screening (Loeb *et al*., 2014; NCI, 2021). Overdiagnosis could lead to unnecessary further assessments as a final screening, including prostate biopsies and radical prostatectomies (Velonas *et al*., 2013).

These non-essential confirmation tests have resulted in side effects such as urinary bleeding, rectal bleeding, hemoejaculation, and high risk of infectious complications, as well as psychological effects, including depression and anxiety (Minervini *et al*., 2014). Furthermore, the inefficiency of PSA screening also increases health care spending (Fireman *et al*., 1997).

Consequently, to improve the accuracy of PCa detection, researchers have been developing innovative diagnostic tools for effective screening methods, expecting better diagnostic capabilities, including highly sensitive and selective, reliable and accessible, non-invasive procedures resulting in low risk of harm to the patient, and low costs to reduce

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the economic burden. In the past few years, the biological fluids have been used as sources of biomarkers to distinguish between healthy and PCa patients. In general, urine, blood, prostate tissue, and seminal fluid are used to identify tumor-specific compounds, which have increased significantly in PCa patients. However, urine is more favourable as a source for detection since it has several advantages, including inexpensive, non-invasive procedure for collection, easy to handle, and rich in metabolite substances.

Several potential biomarkers exist in the urine, such as DNA (single nucleotide polymorphisms, copy number variations, and methylation), RNA (mRNAs, long non-coding RNAs, and microRNAs) and protein, as well as exosomes and other metabolites which are useful for PCa detection and may improve existing clinical testing methods (Bax *et al*., 2018; Eskra *et al*., 2019). One of DNA-based markers in urine is methylated DNA. Methylated DNA is a prospective biomarker in PCa detection since it frequently occurs during the early stage of tumor development, and is able to be detected in urine. Two examples of PCa biomarkers are methylated *GSTP1* and *APC*, which are tumor suppressor genes, important in preventing DNA damage and cell overgrowth, respectively. Pyrosequencing, droplet digital polymerase chain reaction (ddPCR) and methylation-specific PCR are several methods to quantify DNA methylation, which provide accurate and sensitive assessment (Yoon *et al.*, 2012; Greene *et al.*, 2008; Richiardi *et al.*, 2013).

In term of nucleic acid-based PCa markers in urine, RNAs are the most extensively studied. Among them are PCA3 and TMPRSS2, which are prostate specific and are overexpressed in prostate cancer. Common methods to detect urinary RNA biomarkers are quantitative reverse transcription PCR (RT-qPCR) and transcription-mediated amplification (TMA) (Eskra *et al.*, 2019).

Aside from DNA and RNA, proteins also serve as potential urinary biomarkers which are specific to PCa. Protein markers in urine consist of cellular antigens from exfoliated prostate cells, proteins secreted into prostatic fluid, as well as protein in the enclosed extracellular vesicles, for instance, 5α-reductase, transferrin, and zinc α2-glycoprotein. Even though protein concentration found in urine is relatively lower than in blood, recent technologies are able to overcome the challenge of detecting the small amount of protein. There are diverse methods in PCa-specific protein detection which are high throughput and reproducible, including enzyme-linked immunosorbent assay (ELISA), immunoturbidimetric assay, radioimmunoassay, western blotting, gelatin zymography, and mass spectrometry (Eskra *et al.*, 2019).

Further examination may be performed to detect the spreading tumors in distant parts of the body using several imaging tests, including Magnetic Resonance Imaging (MRI), Computed Tomography (CT) scans or bone scans. Study shows that MRI, using either 1.5 or 3.0 Tesla magnets, and with or without endorectal coil, can improve PCa detection and prevent overdiagnosis (Mayor, 2018; Penzkofer & Tempany-Afdhal, 2014). A bone scan, also known as a radionucleotide scan, is performed by injecting small amounts of radioactive material called tracers into the body. These tracers collect in bones and emit gamma radiation that can be detected and converted into images. The primary purpose of the bone scan is to determine whether prostate cancer has migrated to the bone, which is a very common occurrence. Therefore, a bone scan is highly recommended for patients diagnosed in the late stage of cancer to confirm bone metastases (Chong *et al*., 2014; Lin *et al*., 2017).

Another examination method to prove the spread of PCa in distant organs is by using a monoclonal antibody with radiolabeled tag. Indium-111 capromab pendetide scan (ProstaScint®; Cytogen Corporation, Princeton, NJ) approved by FDA for imaging PCa to detect metastases, particularly in the lymph nodes. This radiolabelled monoclonal antibody binds to prostate-specific membrane antigen (PSMA), the best-known PCa biomarker. The roles of ProstaScint® are basically to improve the accuracy of PCa staging and to detect the recurrent disease (Taneja, 2004). However, a study reports that this imaging agent is not sufficient for detection of bone metastases as the most common metastatic site of PCa, since the ProstaScint cannot identify most sites of abnormalities in bone uptake on bone scan (Bander *et al*., 2003).

Prostate Cancer Treatments

History of cancer treatments has been recorded since ancient Egyptian and Greek where healthy diets and surgical strategies were used for the main cancer treatments. In the late 19th century, when X-rays were discovered, radiotherapy was used for cancer treatment. Then, the revolution of pharmacological approaches resulted in the use of chemotherapeutic drugs, such as doxorubicin which had a cytotoxicity effect for numerous cancer types. Furthermore, the revolution of anti-cancer therapy discovered monoclonal antibodies with the first clinical trial was in 1992, and according to a report in 2021, there are at least 45 different monoclonal antibodies that have been approved and marketed for the treatment of various types of cancers (Arias-Pinilla & Modjtahedi, 2021). Finally, in recent years, comprehensive genomic profiling is being developed to be used in routine clinical testing, providing more accurate risk assessment methods. The genomic profiling is able to identify localized and aggressive PCa. This method is used to guide selection for targeted therapy and enables personalized medicine to optimize the management of PCa therapy (Chung *et al*., 2019; Grasso *et al*., 2015).

Several PCa treatments are available, including cancer watchful waiting, surgery, radiotherapy, hormone therapy and chemotherapy (Goldstraw, 2006). The treatments will depend on the cancer stage. For localized PCa, where the cancer growth is only in the prostate organ without spreading into nearby tissues or distant metastases, the treatment options include cancer watchful waiting and active surveillance, surgery, and radiation. Cancer watchful waiting and active surveillance involve a series of tests to monitor the progression, to prevent significant development of the disease, and to maintain the quality of life. In general, watchful waiting is appropriate for men whose cancer is unlikely to create problems during their lifespan. However, in certain circumstances, cancer may progress more rapidly than predicted and cause symptoms; in these cases, treatment can be administered to control the malignancy and manage any associated symptoms. Active surveillance, by contrast, is for men who have slow-growing cancer that has not moved outside the prostate (localized cancer). It involves more regular hospital tests than watchful waiting, such as MRI scans and prostate biopsies. Moreover, if some therapies are needed, they will usually aim to cure the cancer.

Surgery and radiation are more effective for patients who suffer a more significant PCa, with higher PSA level and palpable tumor under physical examinations. These treatments can reduce the risks of PCa progression and metastases. However, these treatments may have several disadvantages. Side effects related to urinary control and sexual functions are commonly experienced by most post-surgery patients. For patients who have radiation, several side effects are observed, including bowel dysfunction and toxicity, nocturia, and urinary problems (Litwin & Tan, 2017).

In an advanced PCa disease, initial treatment is conducted by using androgen deprivation therapy (ADT), a hormonal therapy to slow the production of testosterone to keep the PCa under control. However, this treatment has several adverse reactions, such as fatigue, sexual dysfunction, hot flashes, osteoporosis, and increase the risks of heart diseases and dementia (Nead *et al*., 2017; Nguyen *et al*., 2011, 2014). Furthermore, in general, ADT can only control the disease for 1 to 1.5 year and the majority of patients develop progressive PCa after this treatment (Seidenfeld *et al*., 2000).

In order to improve advanced PCa treatment, ADT administration along with chemotherapy drugs has been recommended by various health care organizations. Study shows that a combination of ADT and chemotherapy docetaxel have resulted in better outcomes including a decrease in PSA level, lower level of mortality, and prolong overall survival in PCa patients (Sweeney *et al*., 2015). Docetaxel belongs to the taxane class which has been used in standard management treatment of metastatic PCa for many years. Combination therapy of docetaxel with prednisone is used as a gold standard for first-line chemotherapy in men with metastatic castration-resistant prostate cancer (CRPC) (Basch *et al*., 2014; Saad & Hotte, 2010). However, clinical data shows that chemotherapy using a combination of docetaxel with estramustine and prednisolone caused PCa patients to suffer from severe toxicity (Kuramoto *et al*., 2013). In addition,

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around 47% PCa patients relapse after chemotherapy treatment with combination of docetaxel and mitoxantrone followed by surgery (Garzotto *et al.*, 2010). Furthermore, a significant number of patients develop resistance to docetaxel and do not respond to this therapy. Thus, improvement of treatment for this group of patients is highly important, since limited treatment options are available (Hwang, 2012). A study shows that a novel tubulin-binding taxane, called cabazitaxel, is effective for docetaxel-resistant metastatic CRPC patients. Treatment using a combination of cabazitaxel and prednisone also improved overall survival. However, this therapy is also highly toxic with common adverse effects such as diarrhea and neutropenia (de Bono *et al*., 2010).

In recent years, therapy management for patients with metastatic CRPC has been expanded to increase survival rates, reduce pain, and improve quality of life using several novel targeting agents as new second-line drugs, including enzalutamide, abiraterone acetate in combination with prednisone, and immunotherapy using sipuleucel T (Cornford *et al*., 2017). Enzalutamide, a second generation of androgen receptor inhibitor, was originally designed for the treatment of patients with non-metastatic CRPC. However, it can be an option as the first-line therapy in patients with metastatic CRPC. In general, this therapy is well tolerated in both non-metastatic and metastatic CRPC with common adverse events, including hypertension, headache, and hot flashes (Scott, 2018). Abiraterone acetate, a pro-drug of abiraterone, is a CYP17 inhibitor which is able to block androgen biosynthesis preventing prostate tumor growth. A study shows that abiraterone acetate in combination with prednisone has significantly improved the efficacy and prolonged survival rates. The main adverse events are commonly related to cardiac and liver-function disorders, as well as increased mineralocorticoid level including hypertension and hypokalemia (Yang, 2011). Initially, abiraterone acetate plus prednisone was approved as the second-line therapy after docetaxel. However, after a phase III clinical study in 2013, this combination therapy has been approved as the first-line therapy for metastatic CRPC (Ryan *et al*., 2013).

Another new therapy for metastatic CRPC is utilizing immunotherapy, redirecting patients' immune systems to diagnose and eliminate cancer cells. Sipuleucel-T, therapeutic cancer vaccine approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA), effective for patients with low PSA level, with minimal symptoms or without any symptoms of metastatic CRPC. This immunotherapy prolongs overall survival and reduces the risk of death by metastatic CRPC. Low grade of adverse events were reported such as chill, fatigue, nausea, back pain and muscular weakness (Kantoff *et al*., 2010; Pieczonka *et al*., 2015). Denosumab, a monoclonal antibody targeting receptor activator of NF-kappaB ligand (RANK-L), is also reported as a metastatic CRPC immunotherapy approved by US FDA, which aim to prevent bone osteoclastogenesis and bone turnover (El-Amm & Aragon-Ching, 2016; Helo et al., 2012).

Radium 223 dichloride (Ra-223), an isotop of radium, is a specific radiopharmaceutical agent that uses high-energy, short-range (<100 μm; 2-10 cell diameters) alpha particles preferentially to target bone metastases. The alpha-emitter generates primarily double-stranded DNA breaks, resulting in a powerful and highly localized cytotoxic effect in the target locations while having a negligible effect on nearby healthy tissues, making it a favorable and safe treatment. Ra-223 has been approved in the US and Europe for metastatic CRPC patients who have bone metastases, acting as calcium mimetic which selectively binds to bone metastases sites. It has been reported that Ra-223 has overall survival benefits and exhibits overall improvement with less adverse events (Parker *et al*., 2013). Other bone-targeting agents in metastatic CRPC therapy approved by US FDA, including zoledronic acid, which is a bisphosphonate therapy that potently inhibits osteoclast-mediated bone resorption and denosumab, which is a monoclonal antibody targeting Receptor activator of NF-kappaB ligand (RANK-L) (El-Amm & Aragon-Ching, 2016).

It has been reported that all emergence treatment options for metastatic CRPC have shown a significant impact on survival rates, which can improve the quality of life of PCa patients. However, metastatic CRPC is still incurable. Several new therapeutic agents are under clinical evaluation for metastatic CRPC therapy based on biomarker-selected patients, such as PD-1 and CTLA-4 based checkpoint

inhibitors, PARP inhibitor, tyrosine-kinase inhibitor and PSMA-targeted therapy. Along with the increasing therapeutic options for metastatic CRPC, it is anticipated to identify the best treatment strategy for every single patient to improve the clinical outcomes, including achieving the longest survival rate, preventing the resistance and side effects, and minimizing the cost of treatment (Nuhn *et al*., 2019).

Development of Theranostics in PCa

Theranostics is a term used to describe the combination of diagnostic and therapeutic capabilities within one single entity. This platform offers the opportunity to delineate disease boundaries, monitors therapy in real time and offers prognostic options (Fuchs *et al*., 2015; Pearce *et al*., 2014; Peng *et al*., 2015). History of theranostics in PCa is back to the use of imaging agent ProstaScint®, the radio-labelled anti-PSMA antibody targeting metastatic PCa. Currently, numerous PSMA ligands have been studied for both detection and treatment of PCa, including antibodies, aptamers and small molecule ligands. These PSMA targeting agents are labelled with radionucleotide such as Lutetium (Lu)-177 and actinium (Ac)-225.

Lutetium (Lu)-177 is a radionuclide which emits both β- and γ-radiation and could act as a theranostic isotope (Figure 2). In pharmaceutical application, Lu-177 is chelated to a peptide to effectively deliver and localise cytotoxic radiation to relatively small volume of tumors and destroy them with minimal damage to the neighboring normal tissues. Valuable characteristic of Lu-177 is that it has strong binding affinity to PSMA and highly efficient internalization into prostate cancer cells. Moderate-energy beta particles and low-energy gamma photons provide Lu-177 with a beneficial aspect in low radiation dose. Another advantage of Lu-177 in cancer therapy is its long half-life which not only minimize decay loss, but also required in purifying procedure, performing quality control and administration (Kim & Kim, 2018; Dash *et al*., 2015; Khreish *et al*., 2022).

Actinium (Ac)-225 is an alpha-emitting radioisotope which is administered to patients resistant to Lu-177 PSMA therapy. Similar to Lu-177, Ac-225 needs to be attached to a molecule which would selectively target cancerous tissue and deliver the radiation to the cancer area with minimum harm to the surrounding healthy cells. Actinium-225 (Ac-225) causes higher rates of double-strand DNA damage in prostate cancer cells with less tissue penetration and minimal side effects in normal cells. Thus, Ac-225 is considered to be more efficacious than Lu-177. However, its relatively short half-life (10 days), and its lack of availability limit its promising application (Muthukrishnan, 2021; Doelen *et al*., 2018).

These radionucleotides combined with PSMA (radioligand therapies) are used as a theranostic concept for personalized medication, showing significant clinical results improvement in metastatic catastration-resistant prostatte cancer management (Kratochwil *et al*., 2016; Machulkin *et al*., 2016; Rahbar *et al*., 2018; Virgolini *et al*., 2017).

As of today, there are two PSMA positron emission tomography (PET) which have been approved by FDA; Gallium 68 (Ga 68) PSMA-11 and Pylarify (piflufolastat F 18) (FDA, 2020, 2021). Ga 68 is advantageous for routine clinical exams since commercial germanium-68 generators enable on-site generation of Ga 68 without using a cyclotron. However, Ga 68 has a physical half-life of only 68 minutes. As a result, at big centers with a high number of patients, the requirement for many generators concurrently doubles the expenditures. Due to the limitation, the F 18-labeled PSMA (half-life: 110 min) tracers can be used to increase the capacity at relatively moderate cost. Moreover, F 18 has lower positron energy than GA 68 (0.65 MeV vs. 1.90 MeV), resulting in a theoretically higher image resolution (Sanchez-Crespo, 2013; Dietlein *et al*., 2015).

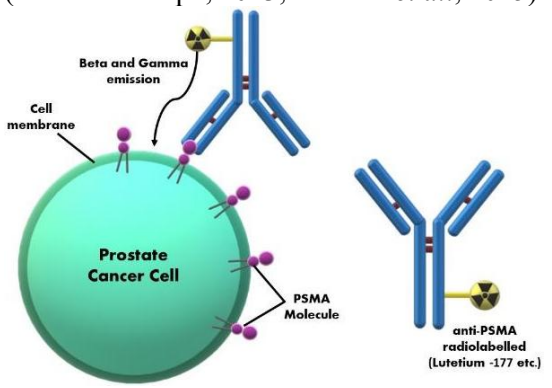

Figure 2. The targeted ligand binds PSMA on PCa cells. Once linked to the neoplastic cell, the 177Lu atom releases strong beta and gamma rays. As a result, radiation that damages DNA is generated.

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Conclusion

Development of PCa testing and therapy are being researched throughout the world. Early diagnosis using PSA is not considered as a perfect method as the first screening of PCa. Therefore, several approaches are being developed and applied to get better precision in PCa diagnosis. As well as detection, the improvements in therapy are being made among standard methods for treating PCa. Finally, the combination of diagnostic and therapeutic, called theranostics are being implemented for better delineate and monitor therapy in real time and offer prognostic options.

References

Aragon-Ching, J. B., & El-Amm, J. (2016). Targeting Bone Metastases in Metastatic Castration-Resistant Prostate Cancer. *Clinical Medicine Insights: Oncology*, *10*, 11-19. https://doi.org/10.4137/cmo.s30751.

Arias-Pinilla, G. A., & Modjtahedi, H. (2021). Therapeutic Application of Monoclonal Antibodies in Pancreatic Cancer: Advances, Challenges and Future Opportunities. *Cancers*, *13*(8), 1781. https://doi.org/10.3390/cancers13081781.

Aronson, W. J., & DeKernion, J. B. (2007). Prostate. Encyclopedia of Gerontology Second Edition, 391-399. Amsterdam: Elsevier. https://doi.org/10.1016/B0-12-370870-2/00028-7.

Bander, N. H., Trabulsi, E. J., Kostakoglu, L., Yao, D., Vallabhajosula, S., Smith-Jones, P., Joyce, M. A., Milowsky, M., Nanus, D. M., & Goldsmith, S. J. (2003). Targeting Metastatic Prostate Cancer with Radiolabeled Monoclonal Antibody J591 to the Extracellular Domain of Prostate Specific Membrane Antigen. *The Journal of Urology*, *170*(5), 1717-1721. https://doi.org/10.1097/01.ju.0000091655.77601.0c.

Basch, E., Loblaw, D. A., & Rumble, R. B. (2014). Systemic Therapy in Men with Metastatic Castration-Resistant Prostate Cancer: American Society of Clinical Oncology and Cancer Care Ontario Clinical Practice Guideline Summary. *Journal of Oncology Practice*, *10*(6), e418-e420. https://doi.org/10.1200/jop.2014.001701.

Bax, C., Taverna, G., Eusebio, L., Sironi, S., Grizzi, F., Guazzoni, G., & Capelli, L. (2018). Innovative Diagnostic Methods for Early Prostate Cancer Detection through Urine Analysis: A Review. *Cancers, 10*(4), 123. https://doi.org/10.3390/cancers10040123.

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians, 68*(6), 394-424. https://doi.org/10.3322/caac.21492.

Bubendorf, L., Schöpfer, A., Wagner, U., Sauter, G., Moch, H., Willi, N., Gasser, T. C., & Mihatsch, M. J. (2000). Metastatic patterns of prostate cancer: An autopsy study of 1,589 patients. *Human Pathology*, *31*(5), 578-583. https://doi.org/10.1053/hp.2000.6698.

Cheng, L., Montironi, R., Bostwick, D. G., Lopez-Beltran, A., & Berney, D. M. (2012). Staging of prostate cancer. *Histopathology*, *60*(1), 87-117. https://doi.org/10.1111/j.1365-2559.2011.04025.x.

Chong, A., Hwang, I., Ha, J., Yu, S. H., Hwang, E. C., Yu, H. S., Kim, S. O., Jung, S.-I., Kang, T. W., Kwon, D. D., & Park, K. (2014). Application of bone scans for prostate cancer staging: Which guideline shows better result? *Canadian Urological Association Journal, 8*(7-8), E515-519. https://doi.org/10.5489/cuaj.2054.

Chung, J. H., Dewal, N., Sokol, E., Mathew, P., Whitehead, R., Millis, S. Z., Frampton, G. M., Bratslavsky, G., Pal, S. K., Lee, R. J., Necchi, A., Gregg, J. P., Lara, P., Antonarakis, E. S., Miller, V. A., Ross, J. S., Ali, S. M., & Agarwal, N. (2019). Prospective Comprehensive Genomic Profiling of Primary and Metastatic Prostate Tumors. *JCO Precision Oncology, 3*, 1-23. https://doi.org/10.1200/po.18.00283.

Cornford, P., Bellmunt, J., Bolla, M., Briers, E., Santis, M. De, Gross, T., Henry, A. M., Joniau, S., Lam, T. B., Mason, M. D., van der Poel, H. G., van der Kwast, T. H., Rouvière, O., Wiegel, T., & Mottet, N. (2017). EAU-ESTRO-SIOG Guidelines on Prostate Cancer. Part II: Treatment of Relapsing, Metastatic, and Castration-Resistant Prostate Cancer. *European Urology*, *71*(4), 630-642. https://doi.org/10.1016/j.eururo.2016.08.002.

Dash, A., Pillai, M. R. A., & Knapp, F. F. (2015). Production of ^{177}Lu for Targeted Radionuclide Therapy: Available Options. *Nuclear Medicine and Molecular Imaging*, *49*(2), 85-107. https://doi.org/10.1007/s13139-014-0315-z.

David, M. K., & Leslie, S. W. (2021). Prostate Specific Antigen. Treasure Island (FL): StatPearls Publishing.

de Bono, J. S., Oudard, S., Ozguroglu, M., Hansen, S., Machiels, J.-P., Kocak, I., Gravis, G., Bodrogi, I., Mackenzie, M. J., Shen, L., Roessner, M., Gupta, S., & Sartor, A. O. (2010). Prednisone plus cabazitaxel or mitoxantrone for metastatic castration-resistant prostate cancer progressing after docetaxel treatment: A

randomised open-label trial. *Lancet*, *376*(9747), 1147-1154. https://doi.org/10.1016/s0140-6736(10)61389-x.

Dietlein, M., Kobe, C., Kuhnert, G., Stockter, S., Fischer, T., Schomacker, L., Schimdt, M., Ditlein, F., Zlatopolskiy, B. D., Krapf, P., Richarz, R., Neubauer, S., Drzezga, A., Neumaier, B. (2015). Comparison of [18F]DCFPyL and [68Ga]GaPSMA-HBED-CC for PSMA-PET Imaging in Patients with Relapsed Prostate Cancer. *Molecular Imaging and Biology*, *17*(4), 575-584. https://doi.org/10.1007/s11307-015-0866-0.

Doelen, M. J. van der, Mehra, N., Smits, M., Oort, I. M. van, Janssen, M. J. R., Haberkorn, U., Kratochwil, C., & Gerritsen, W. (2018). Clinical experience with PSMA-Actinium-225 (Ac-225) radioligand therapy (RLT) in end-stage metastatic castration-resistant prostate cancer (mCRPC) patients. *Journal of Clinical Oncology, 36,* 344-344. https://doi.org/10.1200/JCO.2018.36.6_suppl.344.

Eskra, J. N., Rabizadeh, D., Pavlovich, C. P., Catalona, W. J., & Luo, J. (2019). Approaches to urinary detection of prostate cancer. *Prostate Cancer and Prostatic Diseases, 22*, 362-381. https://doi.org/10.1038/s41391-019-0127-4.

Ferlay J, Laversanne M, Ervik M, Lam F, Colombet M, Mery L, Piñeros M, Znaor A, Soerjomataram I, B. F. (2020). *Global Cancer Observatory: Cancer Tomorrow.* Lyon, France: International Agency for Research on Cancer. Available from https://gco.iarc.fr/tomorrow, accessed 24 January 2022.

Fireman, B. H., Quesenberry, C. P., Somkin, C. P., Jacobson, A. S., Baer, D., West, D., Potosky, A. L., & Brown, M. L. (1997). Cost of care for cancer in a health maintenance organization. *Health Care Financing Review*, *18*(4), 51-76.

Fuchs, A. V, Gemmell, A. C., & Thurecht, K. J. (2015). Utilising polymers to understand diseases: Advanced molecular imaging agents. *Polymer Chemistry*, *6*(6), 868-880. https://doi.org/10.1039/c4py01311e.

Gao, Y., Zhang, M., Li, X., Zeng, P., Wang, P., & Zhang, L. (2019). Serum PSA levels in patients with prostate cancer and other 33 different types of diseases. *Progress in Molecular Biology and Translational Science*, *162*, 377-390. https://doi.org/10.1016/bs.pmbts.2018.12.013.

Garzotto, M., Higano, C. S., O'Brien, C., Rademacher, B. L., Janeba, N., Fazli, L., Lange, P. H., Lieberman, S., & Beer, T. M. (2010). Phase 1/2 study of preoperative docetaxel and mitoxantrone for high-risk prostate cancer. *Cancer*, *116*(7), 1699-1708. https://doi.org/10.1002/cncr.24960.

Goldstraw, M. A. (2006). Prostate cancer treatment. The case for radical prostatectomy. *Annals of the Royal College of Surgeons of England*, *88*(5), 439-442. https://doi.org/10.1308/003588406X117133.

Grasso, C. S., Cani, A. K., Hovelson, D. H., Quist, M. J., Douville, N. J., Yadati, V., Amin, A. M., Nelson, P. S., Betz, B. L., Liu, C.-J., Knudsen, K. E., Cooney, K. A., Feng, F. Y., McDaniel, A. S., & Tomlins, S. A. (2015). Integrative molecular profiling of routine clinical prostate cancer specimens. *Annals of Oncology*, *26*(6), 1110-1118. https://doi.org/10.1093/annonc/mdv134.

Greene, K. L., Li, L. C., Okino, S. T., & Carroll, P. R. (2008). Molecular Basis of Prostate Cancer. *The Molecular Basis of Cancer Third Edition*, 431-440. Philadelphia: W.B. Saunders. https://doi.org/10.1016/B978-141603703-3.10033-0.

Hanahan, D., & Weinberg, R. (2011). Hallmarks of cancer: the next generation. *Cell*, *144*(5), 646-674. https://doi.org/10.1016/j.cell.2011.02.013.

Helo, S., Manger, J. P., & Krupski, T. L. (2012). Role of denosumab in prostate cancer. *Prostate Cancer and Prostatic Diseases*, *15*(3), 231-236. https://doi.org/10.1038/pcan.2012.2.

Hwang, C. (2012). Overcoming docetaxel resistance in prostate cancer: A perspective review. *Therapeutic Advances in Medical Oncology, 4*(6), 329-340. https://doi.org/10.1177/1758834012449685.

Jensen, P. E., Gardner, W. A., & Piserchia, P. V. (1980). Prostatic crystalloids: Association with adenocarcinoma. *The Prostate*, *1*(1), 25-30. https://doi.org/10.1002/pros.2990010105.

Kantoff, P. W., Higano, C. S., Shore, N. D., Berger, E. R., Small, E. J., Penson, D. F., Redfern, C. H., Ferrari, A. C., Dreicer, R., Sims, R. B., Xu, Y., Frohlich, M. W., & Schellhammer, P. F. (2010). Sipuleucel-T Immunotherapy for Castration-Resistant Prostate Cancer. *The New England Journal of Medicine*, *363*(5), 411-422. https://doi.org/10.1056/nejmoa1001294.

Khreish, F., Ghazal, Z., Marlowe, R. J., Rosar, F., Sabet, A., Maus, S., Linxweiler, J., Bartholomä, M., & Ezziddin, S. (2022). 177 Lu-PSMA-617 radioligand therapy of metastatic castration-resistant prostate cancer: Initial 254-patient results from a prospective registry (REALITY Study). *European Journal of Nuclear Medicine and Molecular Imaging*, *49*(3), 1075-1085. https://doi.org/10.1007/S00259-021-05525-7.

Kim, K., & Kim, S. J. (2018). Lu-177-Based Peptide Receptor Radionuclide Therapy for Advanced Neuroendocrine Tumors. *Nuclear Medicine and Molecular Imaging*, *52*(3), 208-215. https://doi.org/10.1007/S13139-017-0505-6.

Kratochwil, C., Bruchertseifer, F., Giesel, F. L., Weis, M., Verburg, F. A., Mottaghy, F., Kopka, K., Apostolidis, C., Haberkorn, U., &

Morgenstern, A. (2016). 225Ac-PSMA-617 for PSMA-Targeted α-Radiation Therapy of Metastatic Castration-Resistant Prostate Cancer. *Journal of Nuclear Medicine*, *57*(12), 1941-1944. https://doi.org/10.2967/jnumed.116.178673

Kuramoto, T., Inagaki, T., Fujii, R., Sasaki, Y., Nishizawa, S., Nanpo, Y., Matusmura, N., Kohjimoto, Y., & Hara, I. (2013). Docetaxel in combination with estramustine and prednisolone for castration-resistant prostate cancer. *International Journal of Clinical Oncology*, *18*(5), 890-897. https://doi.org/10.1007/s10147-012-0463-z.

Lin, Y., Mao, Q., Chen, B., Wang, L., Liu, B., Zheng, X., & Xie, L. (2017). When to perform bone scintigraphy in patients with newly diagnosed prostate cancer? a retrospective study. *BMC Urology*, *17*(1), 41. https://doi.org/10.1186/s12894-017-0229-z.

Litwin, M. S., & Tan, H.-J. (2017). The Diagnosis and Treatment of Prostate Cancer: A Review. *Journal of the American Medical Association*, *317*(24), 2532-2542. https://doi.org/10.1001/jama.2017.7248.

Loeb, S., Bjurlin, M. A., Nicholson, J., Tammela, T. L., Penson, D. F., Carter, H. B., Carroll, P., & Etzioni, R. (2014). Overdiagnosis and overtreatment of prostate cancer. *European Urology*, *65*(6), 1046-1055. https://doi.org/10.1016/j.eururo.2013.12.062.

Machulkin, A. E., Ivanenkov, Y. A., Aladinskaya, A. V, Veselov, M. S., Aladinskiy, V. A., Beloglazkina, E. K., Koteliansky, V. E., Shakhbazyan, A. G., Sandulenko, Y. B., & Majouga, A. G. (2016). Small-molecule PSMA ligands. Current state, SAR and perspectives. *Journal of Drug Targeting*, *24*(8), 679-693. https://doi.org/10.3109/1061186x.2016.1154564.

Mayor, S. (2018). MRI increases detection of prostate cancers and reduces overdiagnosis, finds study. *British Medical Journal*, *360*, k1268. https://doi.org/10.1136/bmj.k1268

Minervini, A., Siena, G., Vittori, G., & Carini, M. (2014). Morbidity and psychological impact of prostate biopsy: The future calls for a change. *Asian Journal of Andrology*, *16*(3), 415-417. https://doi.org/10.4103/1008-682x.126388.

Muthukrishnan, A. (2021). PSMA Theranostics Is Poised to Change Prostate Cancer Landscape. *Targeted Therapies in Oncology*, *10*(7). https://www.targetedonc.com/view/psma-theranostics-is-poised-to-change-prostate-cancer-landscape.

NCI. (2021). *Prostate-Specific Antigen (PSA) Test.* National Cancer Institute. Available from https://www.cancer.gov/types/prostate/psa-fact-sheet, accessed 24 January 2022.

Nead, K. T., Gaskin, G., Chester, C., Swisher-McClure, S., Leeper, N. J., & Shah, N. H. (2017). Association Between Androgen Deprivation Therapy and Risk of Dementia. *JAMA Oncology*, *3*(1), 49-55. https://doi.org/10.1001/jamaoncol.2016.3662.

Nguyen, P. L., Alibhai, S. M. H., Basaria, S., D'Amico, A. V, Kantoff, P. W., Keating, N. L., Penson, D. F., Rosario, D. J., Tombal, B., & Smith, M. R. (2014). Adverse effects of androgen deprivation therapy and strategies to mitigate them. *European Urology*, *67*(5), 825-836. https://doi.org/10.1016/j.eururo.2014.07.010

Nguyen, P. L., Je, Y., Schutz, F. A. B., Hoffman, K. E., Hu, J. C., Parekh, A., Beckman, J. A., & Choueiri, T. K. (2011). Association of androgen deprivation therapy with cardiovascular death in patients with prostate cancer: A meta-analysis of randomized trials. *Journal of the American Medical Association*, *306*(21), 2359-2366. https://doi.org/10.1001/jama.2011.1745.

Nuhn, P., Bono, J. S. De, Fizazi, K., Freedland, S. J., Grilli, M., Kantoff, P. W., Sonpavde, G., Sternberg, C. N., Yegnasubramanian, S., & Antonarakis, E. S. (2019). Update on Systemic Prostate Cancer Therapies: Management of Metastatic Castration-resistant Prostate Cancer in the Era of Precision Oncology. *European Urology*, *75*(1), 88-99. https://doi.org/10.1016/j.eururo.2018.03.028.

Parker, C., Nilsson, S., Heinrich, D., Helle, S. I., O'Sullivan, J. M., Fosså, S. D., Chodacki, A., Wiechno, P., Logue, J., Seke, M., Widmark, A., Johannessen, D. C., Hoskin, P., Bottomley, D., James, N. D., Solberg, A., Syndikus, I., Kliment, J., Wedel, S., Boehmer S., Dall'Oglio M., Franzén L., Coleman R., Vogelzang N. J., O'Bryan-Tear C. G., Staudacher K., Garcia-Vargas J., Shan M., Bruland Ø. S., & Sartor O. for the ALSYMPCA (2013). Alpha emitter radium-223 and survival in metastatic prostate cancer. *The New England Journal of Medicine*, *369*(3), 213-223. https://doi.org/10.1056/NEJMoa1213755.

Pearce, A. K., Rolfe, B. E., Russell, P. J., Tse, B. W.-C., Whittaker, A. K., Fuchs, A. V, & Thurecht, K. J. (2014). Development of a polymer theranostic for prostate cancer. *Polymer Chemistry*, *5*(24), 6932-6942. https://doi.org/10.1039/c4py00999a.

Peng, H., Liu, X., Wang, G., Li, M., Bratlie, K. M., Cochran, E., & Wang, Q. (2015). Polymeric multifunctional nanomaterials for theranostics. *Journal of Materials Chemistry B*, *3*(34), 6856-6870. https://doi.org/10.1039/c5tb00617a.

Penzkofer,T., & Tempany-Afdhal, C. M. (2014). Prostate Cancer Detection and Diagnosis: The Role of MR and its Comparison to other Diagnostic Modalities - A Radiologist's Perspective. *NMR in Biomedicine*, *27*(1), 3-15. https://doi.org/10.1002/NBM.3002.

Pieczonka, C. M., Telonis, D., Mouraviev, V., & Albala, D. (2015). Sipuleucel-T for the Treatment of Patients With Metastatic Castrate-resistant Prostate Cancer: Considerations for Clinical Practice. *Reviews in Urology*, *17*(4), 203-210.

Rahbar, K., Afshar-Oromieh, A., Jadvar, H., & Ahmadzadehfar, H. (2018). PSMA Theranostics: Current Status and Future Directions. *Molecular Imaging*, *17*, 153601211877606. https://doi.org/10.1177/1536012118776068.

Rahib, L., Smith, B. D., Aizenberg, R., Rosenzweig, A. B., Fleshman, J. M., & Matrisian, L. M. (2014). Projecting cancer incidence and deaths to 2030: The unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Research*, *74*(11), 2913-2921. https://doi.org/10.1158/0008-5472.can-14-0155.

Richiardi, L., Fiano, V., Grasso, C., Zugna, D., Delsedime, L., Gillio-Tos, A., & Merletti, F. (2013). Methylation of *APC* and *GSTP1* in Non-Neoplastic Tissue Adjacent to Prostate Tumour and Mortality from Prostate Cancer. *PLOS ONE*, *8*(7), e68162. https://doi.org/10.1371/journal.pone.0068162.

Ryan, C. J., Smith, M. R., de Bono, J. S., Molina, A., Logothetis, C. J., de Souza, P., Fizazi, K., Mainwaring, P., Piulats, J. M., Ng, S., Carles, J., Mulders, P. F. A., Basch, E., Small, E. J., Saad, F., Schrijvers, D., Van Poppel, H., Mukherjee, S. D., Suttmann, H., … Rathkopf, D. E. (2013). Abiraterone in Metastatic Prostate Cancer without Previous Chemotherapy. *New England Journal of Medicine*, *368*(2), 138-148. https://doi.org/10.1056/NEJMoa1209096.

Saad, F., & Hotte, S. J. (2010). Guidelines for the management of castrate-resistant prostate cancer. *Canadian Urological Association Journal*, *4*(6), 380-384. https://doi.org/10.5489/cuaj.10167.

Sanchez-Crespo A. (2013). Comparison of Gallium-68 and Fluorine-18 imaging characteristics in positron emission tomography. *Applied Radiation and Isotopes*, *76*, 55-62.

Sandhu, J. S., & Schlegel, P. N. (2004). Prostate Cancer. *Encyclopedia of Endocrine Diseases*, 125-130. Massachusetts: Academic Press.

Scott, L. J. (2018). Enzalutamide: A Review in Castration-Resistant Prostate Cancer. *Drugs*, *78*(18), 1913-1924. https://doi.org/10.1007/s40265-018-1029-9.

Seidenfeld, J., Samson, D. J., Hasselblad, V., Aronson, N., Albertsen, P. C., Bennett, C. L., & Wilt, T. J. (2000). Single-Therapy Androgen Suppression in Men with Advanced Prostate Cancer: A Systematic Review and Meta-Analysis. *Annals of Internal Medicine*, *132*(7), 566-577. https://doi.org/10.7326/0003-4819-132-7-200004040-00009.

Sharma, S., Kelly, T. K., & Jones, P. A. (2010). Epigenetics in cancer. *Carcinogenesis*, *31*(1), 27-36. https://doi.org/10.1093/carcin/bgp220

Siegel, R., Naishadham, D., & Jemal, A. (2013). Cancer statistics, 2013. *CA: A Cancer Journal for Clinicians*, *63*(1), 11-30. https://doi.org/10.3322/caac.21166.

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA: A Cancer Journal for Clinicians*, *71*(3), 209-249. https://doi.org/10.3322/caac.21660.

Sweeney, C. J., Chen, Y.-H., Carducci, M., Liu, G., Jarrard, D. F., Eisenberger, M., Wong, Y.-N., Hahn, N., Kohli, M., Cooney, M. M., Dreicer, R., Vogelzang, N. J., Picus, J., Shevrin, D., Hussain, M., Garcia, J. A., & DiPaola, R. S. (2015). Chemohormonal Therapy in Metastatic Hormone-Sensitive Prostate Cancer. *The New England Journal of Medicine*, *373*(8), 737-746. https://doi.org/10.1056/nejmoa1503747.

Taitt, H. E. (2018). Global Trends and Prostate Cancer: A Review of Incidence, Detection, and Mortality as Influenced by Race, Ethnicity, and Geographic Location. *American Journal of Men's Health*, *12*(6), 1807-1823. https://doi.org/10.1177/1557988318798279.

Taneja, S. S. (2004). ProstaScint(R) Scan: Contemporary Use in Clinical Practice. *Reviews in Urology*, *6 Suppl 10*, S19-28.

FDA. (2020). *FDA Approves First PSMA-Targeted PET Imaging Drug for Men with Prostate Cancer*. The United States Food and Drug Administration. Available from https://www.fda.gov/news-events/press-announcements/fda-approves-first-psma-targeted-pet-imaging-drug-men-prostate-cancer, accessed 24 January 2022.

FDA. (2021). *FDA approves second PSMA-targeted PET imaging drug for men with prostate cancer*. The United States Food and Drug Administration. Available from https://www.fda.gov/drugs/drug-safety-and-availability/fda-approves-second-psma-targeted-pet-imaging-drug-men-prostate-cancer, accessed 24 January 2022.

Velonas, V., Woo, H., Remedios, C., & Assinder, S. (2013). Current Status of Biomarkers for Prostate Cancer. *International Journal of Molecular Sciences*, *14*(6), 11034-11060. https://doi.org/10.3390/ijms140611034.

Virgolini, I., Decristoforo, C., Haug, A., Fanti, S., & Uprimny, C. (2017). Current status of theranostics in prostate cancer. *European Journal of Nuclear Medicine and Molecular Imaging*, *45*(3), 471-495. https://doi.org/10.1007/s00259-017-3882-2.

Welch, H. G., & Albertsen, P. C. (2009). Prostate Cancer Diagnosis and Treatment After the Introduction of Prostate-Specific Antigen

Screening: 1986-2005. *Journal of the National Cancer Institute*, *101*(19), 1325-1329. https://doi.org/10.1093/jnci/djp278.

Yang, L. P. H. (2011). Abiraterone Acetate. *Drugs*, *71*(15), 2067-2077. https://doi.org/10.2165/11208080-000000000-00000.

Yoon, H. Y., Kim, S. K., Kim, Y. W., Kang, H. W., Lee, S. C., Ryu, K. H., Shon, H. S., Kim, W. J., & Kim, Y. J. (2012). Combined hypermethylation of *APC* and *GSTP1* as a molecular marker for prostate cancer: quantitative pyrosequencing analysis. *Journal of Biomolecular Screening*, *17*(7), 987-992. https://doi.org/10.1177/1087057112444445.

Zarocostas, J. (2010). Global cancer cases and deaths are set to rise by 70% in next 20 years. *British Medical Journal*, *340*, c3041. https://doi.org/10.1136/bmj.c3041.

Factors Affecting Expression Level of Recombinant Human Epidermal Growth Factor in *Escherichia coli* BL21(DE3) and Size Exclusion Purification Thereof

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Abstract

Recombinant human epidermal growth factor (rhEGF) has been developed to provide the protein for therapeutic uses. Isopropyl β-d-1-thiogalactopyranoside (IPTG)-induced expression in *Escherichia coli* BL21(DE3) has shown the most effective system among other inducers systems in a similar host. However, suitable conditions related to IPTG concentration, incubation time, and incubation temperature are different depending on the amino acid content of the recombinant protein. This study aimed to evaluate the effects of various IPTG concentration, incubation time, and incubation temperature on rhEGF concentration. According to each analysis of those factors, induction with 0.05 mM IPTG for 2 h at 23°C was the most appropriate condition to obtain the highest concentration of rhEGF. The rhEGF was positively confirmed with a monoclonal anti-hEGF antibody and purified in high purity reaching 95.2%, yet recovery was low (1.44%) due to loss in fractions containing endogenous proteins. Therefore, further studies related to type of matrix, column length, and sample concentration in applying size exclusion chromatography are requested for higher recovery.

Keywords: IPTG concentration, incubation time, incubation temperature, recombinant human epidermal growth factor, *Escherichia coli* BL21(DE3)

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Introduction

Human epidermal growth factor (hEGF) is a 6.2 kD polypeptide containing 53 amino acid residues and three disulphide bonds. It plays an important role in regenerating epithelial and endothelial cells and stimulating tissue recovery (Eissazadeh *et al.*, 2017). Conventionally, hEGF was purified from animal urine, but it yields low efficiency and needs complex processes. Therefore, Wong *et al.* (2018) reported developing of protein by genetic engineering as a recombinant hEGF (rhEGF).

Escherichia coli BL21(DE3) strain is commonly used as a host for recombinant protein production. FDA has approved the BL21(DE3) strain for therapeutic purposes. The chromosome of DE3 prophase in BL21(DE3) expresses T7 polymerase RNA under the control of the lacUV5 promotor. This strain lacks of Lon and OmpT protease leading to stabilize expression of recombinant protein (Joseph *et al.*, 2015).

In the other studies, rhEGF has been expressed in *E. coli* using solubilizing tagging, such as GB1, SUMO, and thioredoxine (Trx) that cost expensive proteases to purify the rhEGF with the tagging proteins (Zheng *et al*., 2016; Ma *et al*., 2016; Shams *et al*., 2019). In this study, the rhEGF was designed without any solubilizing tagging, which generates challenges in protein purification and refolding process.

Expression of rhEGF in this study was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG). IPTG has been known to offer the highest yield compared

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with other inducers, such as L-rhamnose and L-arabinose (Gellisen, 2005). IPTG works under lac operon control (Mühlmann *et al.*, 2017). However, several optimization-related to IPTG concentration, incubation time, and incubation temperature are requested to obtain an optimized result with IPTG induction.

Numbers of protein purification methods are available. Even so, for the rhEGF, only size exclusion chromatography is applicable, because it had been designed without any tagging and contaminated with massive endogenous proteins. This leads to other methods like affinity and ion exchange chromatography to be unsuitable (Figure 1). Moreover, size exclusion chromatography is considered the most efficient because no incubation is needed and relatively faster than other methods (Hagel & Haneskog, 2010).

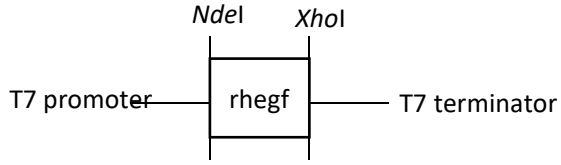

Figure 1. rhegf construction in vector pET21. The gene was inserted at multiple cloning sites using *Nde*I and *Xho*I restriction enzymes (Rodiansyah *et al.,* 2019).

This study aimed to investigate induction conditions for the highest result and to purify rhEGF using size exclusion chromatography.

Materials and Methods

Expression of Recombinant Human Epidermal Growth Factor (rhEGF) in *E. coli* BL21(DE3) with Variation of IPTG Concentration and Incubation Conditions (Temperature and Time). A single colony of BL21(DE3) harboring recombinant plasmid pET21-rhEGF was obtained from the previous study on site-directed mutagenesis of rhEGF (Rodiansyah *et al.*, 2019). The cells were precultured in 1 mL Luria Bertani (LB) (Caisson) medium added with 1 µg/mL ampicillin (Sigma), and then incubated at 37°C, 200 rpm overnight.

On the following day, the preculture was refreshed in 10 mL LB medium with 10 µg/mL ampicillin (Sigma) and incubated at 37°C, 200 rpm for 1.5-2 h or until reaching optical density (OD) 0.7-0.8. Afterward, the cultures were incubated at 23°C, 30°C, or 37°C, induced with IPTG (Thermo Scientific) (0.01, 0.5, and 1 mM), and re-incubated with varied incubation times (2 h, 4 h, and 18 h) (Table 1). Duplo experiment set was performed.

Table 1. Experimental design of the rhEGF expression with IPTG induction at various temperatures and incubation time.

Treatment		
Incubation temperature at 23°C	Incubation time for 2 h	0.01 mM IPTG
		0.1 mM IPTG
		0.5 mM IPTG
		Uninduced
	Incubation time for 4 h	0.01 mM IPTG
		0.1 mM IPTG
		0.5 mM IPTG
		Uninduced
	Incubation time for 18 h	0.01 mM IPTG
		0.1 mM IPTG
		0.5 mM IPTG
		Uninduced
Incubation temperature at 30°C	Incubation time for 2 h	0.01 mM IPTG
		0.1 mM IPTG
		0.5 mM IPTG
		Uninduced
	Incubation time for 4 h	0.01 mM IPTG
		0.1 mM IPTG
		0.5 mM IPTG
		Uninduced
	Incubation time for 18 h	0.01 mM IPTG
		0.1 mM IPTG
		0.5 mM IPTG
		Uninduced
Incubation temperature at 37°C	Incubation time for 2 h	0.01 mM IPTG
		0.1 mM IPTG
		0.5 mM IPTG
		Uninduced
	Incubation time for 4 h	0.01 mM IPTG
		0.1 mM IPTG
		0.5 mM IPTG
		Uninduced
	Incubation time for 18 h	0.01 mM IPTG
		0.1 mM IPTG
		0.5 mM IPTG
		Uninduced

Note: The experiment was conducted in duplication in which each replication consisted of two batches. For the data analysis, all samples of uninduced groups were calculated as one group.

The induced cells were harvested by centrifugation at 6,000 rpm for 15 min at 4°C. Subsequently, the cell pellet was solubilized in 2 mL solubilization buffer containing 8 M urea, 80 mM β-mercaptoethanol, and 50 mM glycine (Biobasic and Merck), without pre-

isolation step that had been optimized in our previous study (unpublished data). For solubilization, the cell suspension was incubated for 3 days at cold temperature of 4-8°C. Solubilized protein and cell debris were separated by centrifugation at high speed (12,000 rpm) for 15 min at 4°C.

The solubilized protein in the supernatant was analyzed by tricine (Sigma) SDS PAGE. The tricine SDS PAGE samples were treated with 2×loading dye (Sigma) and boiled at 95-100°C for 5 min. A 15 µL boiled sample was used. Tricine SDS PAGE was conducted using 15% acrylamide-bisacrylamide gel (Biorad) at 70 V for 2.5-3 h.

Characterization of rhEGF with Western Blotting. The rhEGF was characterized using western blotting with a monoclonal anti-hEGF antibody (Santa Cruz) and visualized with alkaline phosphatase (Thermo Scientific). After transferring the protein into a nitrocellulose membrane (Biorad), the membrane was blocked with 10% skim milk in 1×TBS (Merck and Biobasic) for 2 h. Prior to applying the antibody, the membrane was washed with 0.01% Tween 20 (Merck) in 1×TBS (15 min, 15 min, and 5 min, respectively). In order to visualize the blotted band, the membrane was added with 1-2 mL alkaline phosphatase (Biorad). The band appeared less than 5 min after incubation in a dark room at room temperature.

Semiquantification of rhEGF Concentration Using ImageJ Software. The area of the rhGEF band was measured with ImageJ Software ver 1.5b (National Institute of Health, USA). The measured area was plotted to linear regression obtained from the BSA standard (2, 1, 0.5, 0.25, 0.125, 0.0625, and 0.0313 mg/mL).

Data Analysis. Data were grouped according to each parameter (IPTG concentration, incubation time, and incubation temperature). The variance of groups was analyzed with One Way ANOVA ($\alpha = 0.05$) followed by post hoc test LSD method ($\alpha = 0.05$) using IBM SPSS Statistics 26 (IBM®).

Purification of rhEGF Using Size Exclusion Chromatography. Sephadex G-50 matrix (Sigma) was set up in a chromatography column with 1 cm in diameter and 30 cm in length. Before usage, the matrix was equilibrated with Tris-Cl pH 6.0 to remove the ethanol used during storage. Protein crude was diluted with Tris-Cl pH 6.0 (1:1 or 10 mL of total volume) and then applied to the column. Twenty-five fractions were collected. Each fraction (1 mL) was collected in a 1.5 mL microtube. Several representing fractions were analyzed with SDS PAGE and Western blotting.

Results

Influence of IPTG Concentration, Incubation time, and Incubation Temperature for Expression Level of rhEGF.

Experiment to investigate the effect of IPTG concentration, incubation time, and incubation temperature for the expression level of rhEGF was carried out simultaneously using a factorial design. Twenty-seven groups of treatment were conducted in duplicate. Figure 2 represents samples in one set experiment. Protein of interest was found at size approximately 6 kD, and no target band was observed from uninduced treatment on the stained SDS PAGE.

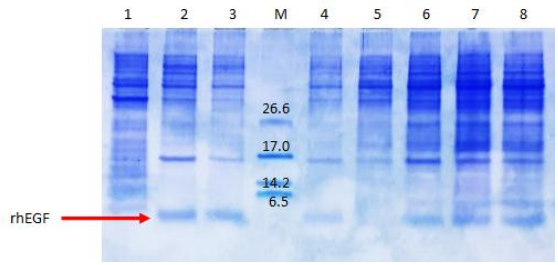

Figure 2. SDS PAGE of rhEGF expressed at 37ºC, various IPTG concentrations, and various incubation times. Lane 1: uninduced, 2 h; 2: 0.01 mM IPTG, 2 h; 3: 0.05 mM IPTG, 2 h; M: ultra-low protein marker; 4: 1 mM IPTG, 2 h; 5: uninduced, 4 h; 6: 0.01 mM IPTG, 4 h; 7: 0.05 mM IPTG, 4 h; 8: 1 mM IPTG, 4 h.

Protein concentration was measured semiquantitatively by identifying the area of the rhEGF band using ImageJ software. The band area was plotted to a linear equation of the BSA standard (Figure 3).

Protein concentration was calculated per optical density value. Figures 4, 5, and 6 depict the effect of each parameter. IPTG induction at 0.05 mM resulted in the highest protein concentration, yet the other two concentrations

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allow statistically similar protein expression levels. All IPTG induction proved a significant effect compared with the uninduced group (Figure 4). In uninduced group, a small area under curve (AUC) was detected and calculated as value of protein concentration. However, this area could be ignored and considered as noise in the densitometry using ImageJ software. Moreover, this finding is strongly supported by the statistical analysis.

IPTG is known to be toxic to the bacteria; however, since used in low concentration (below 0.1 mM), no negative outcome was found in bacterial growth. Either induced or uninduced group showed almost similar optical density.

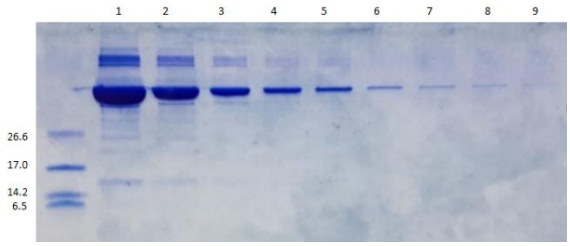

Figure 3. SDS PAGE of a serial concentration of BSA standard.

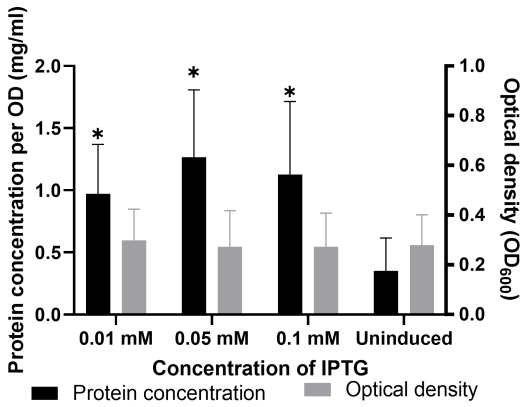

Figure 4. The effect of concentration of IPTG to protein concentration and optical density at all temperatures and incubation times (* shows a significant difference with α = 0.05).

Incubation time revealed a contrary influence on protein concentration and optical density (the longer incubation, the higher optical density). However, the highest protein concentration was found in the group incubated for 2 h after IPTG induction (Figure 5).

Figure 6 illustrates the influence of incubation temperature on protein concentration and optical density. Surprisingly, the incubation time did not show linear trend related to both parameters. The highest protein concentration was obtained by incubation at 23ºC, then followed by 37ºC and 30ºC. Meanwhile, the lowest to the highest optical density was found in group that incubated at 37ºC, 23ºC, and 30ºC.

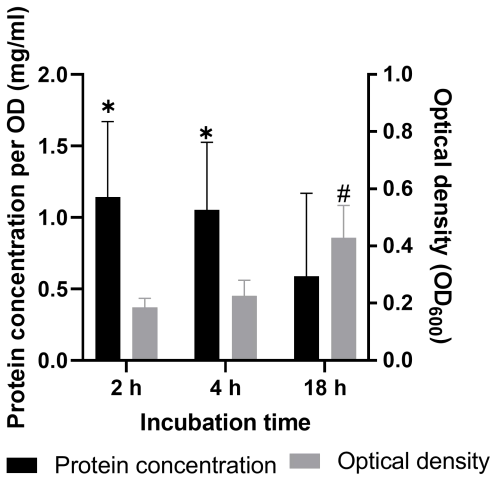

Figure 5. The effect of incubation time on protein concentration and optical density at all IPTG concentrations and temperatures. (*shows a significant difference of protein concentration groups with α = 0.05; # a significant difference of OD groups with α = 0.05).

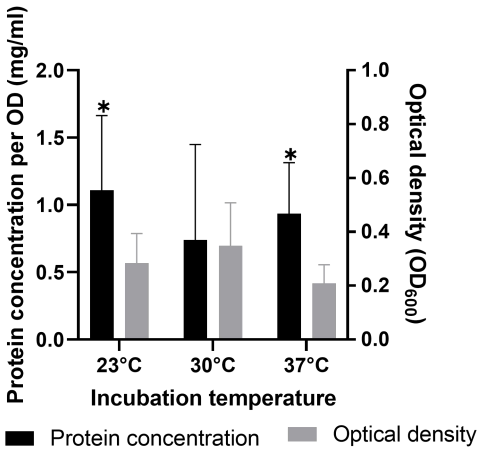

Figure 6. Effect of incubation temperature to protein concentration and optical density at all IPTG concentrations and incubation times (* shows a significant difference with α = 0.05).

Purification of rhEGF Using Size Exclusion Chromatography and Protein Confirmation with Western Blotting.

Figure 7 shows fractions collected in the purification process. Proteins started to appear in the fifth fraction (Figure 7 lane 6).

Unfortunately, the protein of interest was also released in early elutions, which also contained host endogenous proteins (lanes 6-10). Starting from lanes 11-13, the contaminant protein significantly decreased. Purified rhEGF was obtained in lanes 14-15. In lanes 16-18, all proteins have been released from the column. Recovery of purified protein was 1.44% with purity 95.2%. Protein confirmation was carried out using a monoclonal anti-hEGF antibody (Figure 8). A single band at ~6 kD was blotted in crude protein and elution fractions containing rhEGF.

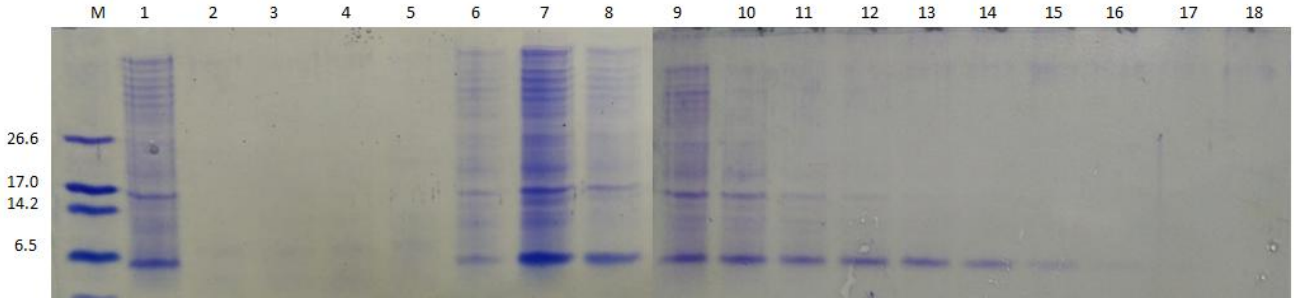

Figure 7. SDS PAGE of protein purification using size exclusion chromatography. Lane M: protein marker, 1: crude protein, 2-18: elution fractions

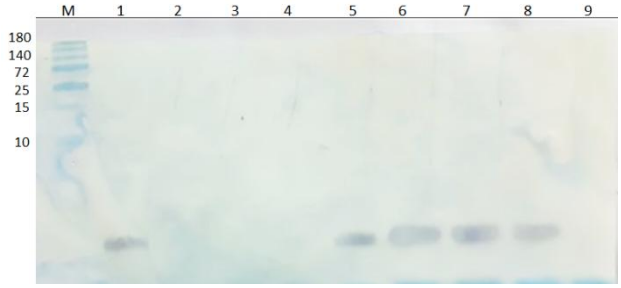

Figure 8. Western blotting using a monoclonal anti-hEGF antibody. Lane M: protein marker, 1: crude protein, 2-4: elution fraction No. 2-4, 5-6: elution fractions No. 6-7, 7-8: elution fractions No. 14-15, 9: elution fraction No. 18

Discussion

IPTG induces recombinant protein to be overexpressed under lac operon control to distinguish the protein of interest from endogenous protein (Mühlmann *et al.*, 2017). Unlike other inducers such as lactose and galactose, IPTG is unmetabolized in the cell; thus, the concentration remains constant during incubation. However, the IPTG concentration needs to be optimized to avoid extreme over-expression which eventually leads to inclusion bodies (Rizkia *et al.*, 2015). The amount of inducer depends on promotor potency, availability of repressor, cellular expression location of recombinant protein, the solubility of recombinant protein, and characteristics of the recombinant protein (Babeipour *et al.*, 2013). Ranjbari *et al.* (2015) mentioned that IPTG concentration is determined not only based on the characteristics of recombinant protein, but also expression of host and incubation conditions. Insufficient IPTG concentration causes ineffective recombinant protein expression; while excessive IPTG concentration is toxic to the expression host (Huang *et al.*, 2017; Ashengroph *et al.*, 2013).

In correlation to those other studies, rhEGF concentration obtained with 0.01 mM IPTG was the lowest among other IPTG-treated groups. This finding proves that 0.01 mM IPTG is deficient for rhEGF expression in the current expression system. The highest concentration of rhEGF was resulted after induced with 0.05 mM IPTG; meanwhile, the rhEGF concentration slightly decreased with the higher IPTG concentration (0.1 mM). However, up to 0.1 mM, no toxic effect was indicated because all groups showed similar optical density (Figure 4). All IPTG-induced groups were significantly different with uninduced groups (Figure 4). Figure 2 revealed that no rhEGF was expressed without IPTG induction.

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Incubation time is related to bacterial growth. Figure 5 shows a significant increasing of optical density found after incubation for 18 h, yet the rhEGF concentration per optical density was notably the lowest among the others. Overall, incubation time and optical density are inversely correlated to rhEGF concentration.

Incubation temperature corresponds to *E. coli* growth. It has minimum, maximum, and optimum growth temperature of *E. coli* (Knob *et al.*, 2008). *E. coli* optimized growth temperature is at 37°C (Sulistiyoningrum *et al.*, 2013). Nevertheless, regarding recombinant protein expression induced with IPTG, the optimum temperature and other conditions differed for each recombinant protein depending on amino acid content (Gutiérrez-González *et al.*, 2019). Cysteines in the hEGF sequence showed positive correlation with IPTG, meanwhile lysine and arginine which influence solubility are not found in the hEGF amino acid sequence (Gutiérrez-González *et al.*, 2019).

High temperature tends to form inclusion bodies. On the other hand, low temperature causes ineffective growth of *E. coli*. In this study, among 23°C, 30°C, and 37°C, the incubation temperature at 30°C showed the highest optical density, yet the lowest rhEGF concentration. No significant difference was found in terms of optical density caused by variation of incubation temperature. Interestingly, rhEGF concentration was significantly higher in group 23°C and 37°C compared with that of in group 30°C. The highest rhEGF concentration was observed by incubation at 23°C resulting the second highest of optical density (Figure 6).

The rhEGF was designed without any tagging to facilitate purification to obtain a recombinant protein as similar to the native one. Besides, it is known difficult to separate a small protein such hEGF from its tagging protein after being cleaved with proteases. Therefore, the suitable method to purify the protein of interest is by using size exclusion chromatography. This method separates proteins based on hydrodynamic size in which the proteins with large hydrodynamic size are eluted first from chromatography matrix (Brusotti *et al*., 2017). The rhEGF was the smallest protein in the protein crude, so this protein was eluted after all endogenous contaminants were completely released.

In our preliminary study, ion exchange and hydrophobic interaction chromatography has been applied to purify the rhEGF. However, the rhEGF purity was low due to abundant endogenous protein having similar pI and hydrophobicity-related physical characteristics (Agustina, 2019).

Using the size exclusion chromatography, the rhEGF was obtained in high purity, but percentage of recovery was low. The rhEGF was also released in the several first elutions along with other endogenous contaminant proteins. For further study, selecting other types of matrices, longer columns, and optimizing the sample concentration loaded into the matrix of size exclusion chromatography are needed to avoid preliminary release of rhEGF in the earlier fractions which eventually increase the purification efficiency.

Conclusion

Vary of IPTG concentration, incubation time, and incubation temperature have been evaluated for rhEGF expression. The rhEGF was purified using size exclusion chromatography. High purity was obtained, yet further studies are requested for higher percentage of recovery. The rhEGF was confirmed with a monoclonal anti-hEGF antibody.

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References

Agustina, N. (2019). Purifikasi Protein Recombinant Human Epidermal Growth Factor (rhEGF) dengan Menggunakan Ion Exchange Chromatography dan Size Exclusion Chromatography. *Laporan Kerja Praktik*, Departemen Biologi, Fakultas Sains, Institut Teknologi Sepuluh November, Surabaya.

Ashengroph, M., Nahvi, I., & Amini, J. (2013). Application of Taguchi design and response surface methodology for improving conversion of isoeugenol into vanillin by resting cells of *Psychrobacter* sp. CSW4. *Iranian Journal of Pharmaceutical Research: IJPR, 12*(3), 411.

Brusotti, G., Calleri, E., Colombo, R., Massolini, G., Rinaldi, F., & Temporini, C. (2018). Advances on size exclusion chromatography and applications on the analysis of protein biopharmaceuticals and protein aggregates: a mini review.

Chromatographia, 81(1), 3-23. DOI: 10.1007/s10337-017-3380-5.

Eissazadeh, S., Moeini, H., Dezfouli, M. G., Heidary, S., Nelofer, R., & Abdullah, M. P. (2017). Production of recombinant human epidermal growth factor in *Pichia pastoris*. *Brazilian Journal of Microbiology, 48*, 286-293. DOI: 10.1016/j.bjm.2016.10.017

Gellissen, G. (Ed.). (2006). *Production of recombinant proteins: Novel microbial and eukaryotic expression systems.* John Wiley & Sons.

Gutiérrez-González, M., Farías, C., Tello, S., Pérez-Etcheverry, D., Romero, A., Zúñiga, R., ... & Molina, M. C. (2019). Optimization of culture conditions for the expression of three different insoluble proteins in *Escherichia coli*. *Scientific Reports, 9*(1), 1-11. DOI: 10.1038/s41598-019-53200-7.

Hagel, L. & Lars, H. (2010). *Size Exclusion Chromatography: Encyclopedia of Life Science*, Chichester, UK, John Wiley and Sons, Ltd.

Huang, L., Xu, Z., Zhong, Z., Peng, L., Chen, H., & Cen, P. (2007). Enhanced expression and primary purification of soluble HBD3 fusion protein in *Escherichia coli*. *Applied Biochemistry and Biotechnology, 142*(2), 139-147. DOI: 10.1007/s12010-007-0009-4.

Joseph, B. C., Pichaimuthu, S., Srimeenakshi, S., Murthy, M., Selvakumar, K., Ganesan, M., & Manjunath, S. R. (2015). An overview of the parameters for recombinant protein expression in *Escherichia coli*. *Journal of Cell Science and Therapy, 6*(5), 221. (5), 1-7. DOI: 10.4172/2157-7013.1000221.

Knob, A., & Carmona, E. C. (2008). Xylanase production by *Penicillium sclerotiorum* and its characterization. *World Applied Sciences Journal, 4*(2), 277-283.

Ma, Y., Yu, J., Lin, J., Wu, S., Li, S., & Wang, J. (2016). High efficient expression, purification, and functional characterization of native human epidermal growth factor in *Escherichia coli*. *BioMed research international*, 3758941. DOI: 10.1156/2016/3758941.

Mühlmann, M., Forsten, E., Noack, S., & Büchs, J. (2017). Optimizing recombinant protein expression via automated induction profiling in microtiter plates at different temperatures. *Microbial Cell Factories, 16*(1), 1-12. DOI: 10.1186/s12934-017-0832-4.

Ranjbari, J., Babaeipour, V., Vahidi, H., Moghimi, H., Mofid, M. R., Namvaran, M. M., & Jafari, S. (2015). Enhanced production of insulin-like growth factor I protein in *Escherichia coli* by optimization of five key factors. *Iranian Journal of Pharmaceutical Research: IJPR, 14*(3), 907.

Rizkia, P. R., Silaban, S., Hasan, K., Kamara, D. S., Subroto, T., Soemitro, S., & Maksum, I. P. (2015). Effect of Isopropyl-β-D-thiogalactopyranoside concentration on prethrombin-2 recombinan gene expression in *Escherichia coli* ER2566. *Procedia Chemistry, 17*, 118-124. DOI: 10.1016/j.proche.2015.12.121.

Rodiansyah, A., Pratiwi, R. D., Zanjabila, S., & Fuad, A. M. (2019). Modification of recombinant human epidermal growth factor (rh-EGF) expression vector by site-directed mutagenesis for therapeutic protein production. *Indonesian Journal of Biotechnology, 24*(1), 8-16.

Shams, D., Alizadeh, M., Azari, S., Hosseini, S., Yasami-Khiabani, S., Samani, S., & Shokrgozar, M. A. (2019). High expression level of human epidermal growth factor (hEGF) using a well-designed fusion protein-tagged construct in *E. coli. Bratislavske Lekarske Listy, 120*(10), 757-763.

Sulistiyoningrum, R. S., Suprijanto, J., & Sabdono, A. (2013). Aktivitas anti bakteri kitosan dari cangkang kerang simping pada kondisi lingkungan yang berbeda: kajian pemanfaatan limbah kerang simping (*Amusium* sp.). *Journal of Marine Research, 2*(4), 111-117.

Wong, W. K., Ng, K. L., & Hu, X. (2018). Authentic human epidermal growth factor: a panacea for wound healing. *EC Endocrinology and Metabolic Research, 3*(4), 138.

Zheng, X., Wu, X., Fu, X., Dai, D., & Wang, F. (2016). Expression and purification of human epidermal growth factor (hEGF) fused with GB1. Biotechnology & Biotechnological Equipment, 30(4), 813-818. DOI: 10.1080/13102818.2016.1166984.

Screening of Active Compounds and LC_{50} Toxicity Assay of Sunda Porcupine's (*Hystrix javanica* F. Cuvier 1823) Quills Crude Extract

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Abstract

Sunda porcupine (*Hystrix javanica* F. Cuvier 1823) is an endemic fauna from Indonesia that its quills are believed to have medical benefits by local people in some regions of Indonesia. However, the benefits have never been well recorded nor proven scientifically. Local people believe the Sunda porcupine's quills have efficacy to relieve property for treating toothache. There is limited research on Sunda porcupine's quills, especially the active compounds, which may affect toothache. This research aims to perform basic pharmacological experiments on Sunda porcupine's quill samples, which includes screening for the active compounds and determining the LC_{50} toxicity using brine shrimp lethality test (BSLT) method. Sunda porcupine's quills were first prepared into simplicia powder (60 mesh in size) and then extracted with 70% ethanol by maceration to produce crude extract. We found that the crude extract of Sunda porcupine's quills contains some active compounds, including alkaloids, flavonoids, saponins, triterpenoids, steroids, and peptides. The LC_{50} value of the crude extract was 2,683.19 ppm; thus, categorized as non-toxic. These findings can be used to identify the active compounds in Sunda porcupine's quills and can be used as a background for further research.

Keywords: *Hystrix javanica*, quills, crude extract, active compound, toxicity

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Introduction

The Sunda porcupine (*Hystrix javanica* F. Cuvier 1823) is a terrestrial rodent-mammal fauna native to Indonesia. It can be found in some regions of Indonesia, such as Java, Bali, Sumbawa, Flores, Lombok, Madura, and Tonahdjampea. The porcupine is active at night (nocturnal) and lives in groups. It is an abundant species and categorized as the least concern (LC) species based on IUCN Red List (van Weers, 1979; Van Weers, 1983; Woods & Kilpatrick, 2005; Aplin, 2016) (Figure 1).

The Sunda porcupine is known as an agricultural pest by the local people where the Sunda porcupine can be found (Farida, 2013). Local people hunt the porcupine to decrease the damage to crops since the porcupine eats various types of crops like tubers and corn. Some local people hunt the porcupine for its meat or medicine. They believe that the porcupine has medical benefits; however, this ethnomedicine is not well recorded. Anita *et al*. (2018) reported that the tail meat of Sunda porcupine has aphrodisiac potency. On the other hand, its family, the American porcupine quills (*Erethizon dorsatum*) has been reported to have antibiotic properties (Roze *et al*., 1990).

Exploration of active compounds from bioresources remains interesting. The research of active compounds from Sunda porcupine is still needs to be expanded despite local people believing much in its ethnomedicine. One of them is that the local people in some regions of Indonesia believe that Sunda porcupine's quills can be used for treating toothache. A basic pharmacological study should be performed to initiate more advanced research so that this ethnomedicine can be both well recorded and proven scientifically. This

research aims to perform basic pharmacological experiments to identify the active compounds in Sunda porcupine's quills, which includes screening of the active compounds and LC_{50} toxicity assay using brine shrimp lethality test (BSLT) method. The findings of this research could identify the active compounds in Sunda porcupine's quills and also can be used as background for further research.

Figure 1. Sunda porcupine (*Hystrix javanica* F. Cuvier 1823)

Materials and Methods

Simplicia Preparation. The Sunda porcupine's quills were the collection of Nutrition Laboratory, Zoology Division, Research Center for Biology, Indonesian Institute of Sciences taken from the remains of physiological research samples which has been approved by ethical commissions with the number B-15897/IPH/KS.02.04/XII/2019 signed by Deputy of Life Sciences, Indonesian Institute of Sciences. The fresh samples of quills were cleaned under running water to eliminate dirt and put under light at room temperature. The clean and dry sample was then weighed to obtain the wet weight. After that, the samples were dried in an oven at 50°C for 3 days to be simplicia. The simplicia was ready after the drying process and weighed to obtain the dry weight. The weight shrinkage percentage was calculated using the Formula 1.

$$\text{weight shrinkage (\%)} = \frac{\text{wet weight-dry weight}}{\text{wet weight}} \text{x100\% (1)}$$

The simplicia was mashed and sifted to obtain simplicia powder with the size of 60 mesh before being stored. The non specific character (water content) of simplicia powder was analyzed using AOAC method (2003) to ensure that the quality of simplicia powder was good enough before being stored and used for further purposes. The water content of simplicia should be less than 10% to minimize the risk of simplicia damage from enzymatic and microbial activities (Manoi, 2006). The amount of water inside the simplicia powder was eliminated by heating the simplicia powder at 105°C for 3 hours or until a constant final weight was reached. The weight before heating (Wo) and the weight after heating (Wt) were measured to determine the water content. The water content of the simplicia was calculated by the Formula 2.

$$\text{water content (\%)} = \frac{\text{Wo-Wt}}{\text{Wo}} \text{x100\% (2)}$$

The specific characteristics of simplicia powder were also determined based on organoleptic test. The test was performed to get the character of color, odor, taste, and texture of the simplicia powder.

Extraction. The obtained simplicia powder was extracted by maceration using 70% ethanol as the solvent. The ratio between the powder and the solvent was 1:10. Extraction was performed by shaking at 120 rpm for 3 days. The extraction process was repeated three times using the same simplicia powder. After the process, the filtrate was separated using filter paper. The filtrate was then dried to obtain crude extract. The drying process of filtrate was conducted using a rotary evaporator with temperature of 50°C at 120 rpm. The yield of crude extract was then calculated using the Formula 3.

$$\text{yield extract (\%)} = \frac{\text{weight of crude extract}}{\text{weight of simplicia powder}} \text{x100\% (3)}$$

The specific characteristics of the crude extract were also determined by an organoleptic test similar to that performed on simplicia powder. The characteristics tested were color, odor, taste, and texture of the crude extract. The characters can be used as identity of the extract, which distinguish it with other crude extract.

Screening of Active Compounds. The crude extract was screened qualitatively for the content of its active compounds. The qualitative analyses conducted in this research

were alkaloid, steroid-triterpenoid, flavonoid, saponin, tannin, and peptide assays.

Alkaloid assay. The assay was performed by the reagents of Meyer, Wagner, and Dragendorf. About 40 mg of crude extract was stirred firmly with ammonia 25%. Next, 20 mL of chloroform was added and crushed intently. The mixture was then filtered with filter paper to obtain the filtrate. The filtrate was mixed with 10% HCl while shaking to form several fractions. The top layer fraction solution was divided into three test tubes. Meyer's reagent was added to the first tube, drop by drop. A positive result is indicated by a white precipitate. Wagner's reagent was added to the second tube. A positive result is indicated by a brownish-red precipitate. Dragendorff's reagent is added to the third tube. A positive result is an orange-red brick precipitate (Aziz, 2015; Bintang, 2010; Harborne, 1996).

*Steroid-triterpenoid assay***.** The crude extract was macerated with 10 mL of ether for 2 hours in an evaporating dish and covered with aluminum foil. After that, it was filtered using filter paper and a separating funnel. Then the filtrate was taken and heated at 50°C until the residue was obtained. Then, sulfuric acid was added drop by drop to the residue until the green color was formed, indicating the presence of steroid, while the formation of red color indicates a positive triterpenoid (Balamurugan *et al*., 2019; Harborne, 1996).

Flavonoid assay. The crude extract was added with 100 mL of distilled water. Then, the mixture was heated for 5 minutes and filtered. The filtrate in the test tube was added with magnesium powder, concentrated HCl, and amyl alcohol. Next, the test tube was shaken quickly and was allowed to form several layers. A positive indicator of the presence of flavonoid compounds is the formation of orange color on the amyl alcohol layer (Nea *et al*., 2021).

Saponin assay. Flavonoid test result filtrates was added with 5 mL of 0.5 M alcoholic KOH in a tube reaction. The filtrates were shaken for 10 minutes and were allowed to stand for a few minutes. The formation of stable foam that does not disappear with the addition of one drop of 1% HCl in the test tube indicates the presence of a saponin group (Harbone,1996).

Tannin assay. The crude extract was added with 25 mL of distilled water, boiled for 15 minutes, cooled and filtered. The filtration results were divided into two parts. The first filtrate was added with 1% iron (III) chloride solution drop-wise until a dark blue or blackish green color formed as a positive indicator of the tannin group. The second filtrate was added with Stiasny's reagent and heated in a water bath. The formation of a pink precipitate indicated condensed tannins. Next, the pink precipitate was filtered using filter paper and a separating funnel, then the filtrate was saturated with sodium acetate, and a few drops of 1% $FeCl_3$ were added. The dark blue color indicates the presence of false-positive tannins (Benzidia *et al*., 2019; Djamil & Anelia. T, 2009).

Peptide assay. The crude extract was reacted using Bradford's reagent. The positive reaction is signed by the bluish-purple solution at the end of the reaction (Bradford, 1976; Bintang, 2010).

Toxicity of LC_{50}. The toxicity was assayed using the method of brine shrimp lethality test (BSLT). The shrimp *Artemia salina* was used for the assay. The shrimp eggs were put into an aquarium containing seawater and aerated for 48 hours under light at room temperature. After 48 hours, the eggs will hatch into nauplii. A series of solutions of the Sunda porcupine's quills crude extract was prepared in a concentration of 1,500 pp;, 1,000 ppm; 500 ppm; 250 ppm; 125 ppm; 62.5 ppm; 31.25 ppm; and 0 ppm (placebo). Each concentration was placed on a vial test and was performed triplo (Jelita *et al*., 2020; Meyer *et al*., 1982; Zuraida, 2018). The assay was conducted by placing 10 nauplii in each vial of concentration. After 24 hours, the nauplii which were still alive were counted and the percentage of mortality was calculated using the Formula 4.

$$\text{Mortality (\%)} = \frac{\text{number of dead nauplii}}{\text{number of tested nauplii}} \text{x}100 \quad (4)$$

The percentage of mortality was then analyzed by probit using SPSS 25. The value of LC_{50} was then determined.

Results

Organoleptic Characteristics of Simplicia Powder and Crude Extract.

The porcupine's quills were prepared into small cuts of simplicia, simplicia powder, and crude extract (Figure 2). Both the simplicia powder and the crude extract were analyzed through an organoleptic test and the results are listed in Table 1. The colors of simplicia powder and crude extract were totally different. The color of simplisia powder was gainsboro gray into light gray while the crude extract was golden yellow. The characteristics of simplicia powder were relatively weak (had no taste and odor), while the characteristics of the crude extract were strong (had specific odor and taste). The weak characters of the simplicia powder occurred because the compounds that gave the characters did not concentrate, while the compounds in the crude extract were well concentrated.

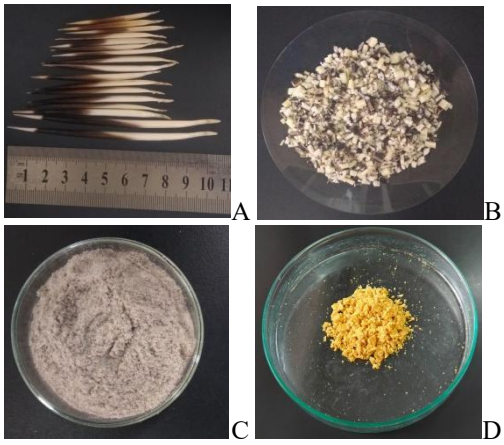

Figure 2. A. Sunda porcupine's quills; B. small cuts of simplicia of Sunda porcupine's quills; C. simplicia powder of Sunda porcupine's quills; D. crude extract of Sunda porcupine's quills.

The Shrinkage of Simplicia Preparation and The Yield of Crude Extract.

The Sunda porcupine's quills were well prepared as simplicia powder and crude extract. The parameters we used in this study were the percentage of weight shrinkage, the water content of simplicia, and the percentage of yield extract. The results of these parameters are shown in Table 2. The weight shrinkage of the quills was relatively lower than samples from plant resources. As a consequence, the yield extract of the quills was also relatively lower than plant resources. The water content was less than 10%, indicating that the drying process has been well performed and the quality of the simplicia produced was good for both to be saved and extracted.

Table 1. Results of organoleptic test on simplicia powder and crude extract of Sunda porcupine's quills

Organoleptic Characters	Simplicia Powder	Crude Extract
Color	gainsboro gray into ligh tgray	Golden yellow
Odor	odorless, bit musty, bit dusty	pungent, acidic, unpleasant smell
Taste	tasteless, light dusty	salty and bitter
Texture	lightweight, easy to fly, smooth bit rough	sticky, paste

Table 2. Extraction parameters of Sunda porcupine's quills

Parameters	Result
Weight shrinkage (%)	12.32 ±1.51
The water content of simplicia (%)	9.1 ± 0.13
Yield extract (%)	3.32%

Screening of Active Compound.

The screening results of active compounds of the crude extract of Sunda porcupine's quills is shown in Table 3. The crude extract of Sunda porcupine's quills gave a positive result for several groups of active compounds, including alkaloid, flavonoid, saponin, steroid/ triterpenoid, and peptide. The crude extract did not give a positive result on tannin assay as it did not form dark blue solution after the reaction.

Toxicity of LC_{50}.

The toxicity test was performed using the brine shrimp lethality test (BSLT), which used *Artemia salina* as an animal model. The extract is considered as non-cytotoxic if LC_{50} > 1,000 ppm, low cytotoxicity if LC_{50} between 500 and 1,000 ppm, moderate cytotoxicity if LC_{50} between 100 and 500 ppm, and high cytotoxicity if LC_{50} < 100 ppm (Meyer, 1982). The result is presented in Table 4. The data showed that the percentage of mortality increased with the increasing concentration of the extract. The extract exposured at 500 ppm and 1,000 ppm has the same mortality percentage. The extract would exert the same effect between 500 ppm and 1,000 ppm, may be due to the concentration of 500 ppm being the lower limit and 1,000 ppm being the upper limit, which could give effect 26.67%

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mortality. The crude extract of Sunda porcupine's quills is categorized as non-toxic since the value of LC_{50} is 2,683.19 ppm, which is higher than 1,000 ppm.

Table 3. Screening results of active compounds in the Sunda porcupine's quills

Group of Active Compound	Type of Assay	Observations	Result
Alkaloid	Meyer	white precipitate	+
	Wagner	brown precipitate	
	Dragendorf	orange precipitate	
Flavonoid	Bate Smith & Metcalf	pale-transparent orange	+
Saponin	Froth	stable foam	+
Steroid/ triterpenoid	Lieberman-Burchard/ Keller Killiani	red (initial-center)/ green (late-side)	+
Tannin	Stiasny		
$FeCl_3$	orange precipitate (doesn't form dark blue)	-	
Peptide	Brad-ford	dark blue	+

Table 4. The toxicity of LC_{50}

Concentration (ppm)	Mortality (%)	LC50 (ppm)	Toxic Category
0	0	2,683.19	non-toxic
31.25	0		
62.50	0		
125.00	6.67		
250.00	10.00		
500.00	26.67		
1,000.00	26.67		
1,500.00	33.33		

Discussion

Simplicia preparation of Sunda porcupine's quills is the first procedure performed before extracting the active compounds of the quills. The fresh quills must be cleaned up from any dirt to minimize any contaminants that might be carried along. The drying process was performed by using an oven at temperature 50°C, which aimed to minimize the risk of damage to the active compounds. The percentage of shrinkage of Sunda porcupine's quills was about 12.32%. It was much lower compared to plant resources. The water content of Sunda porcupine's quills was 10% as reported by Inayah *et al*. (2020), while the water content of plant resources like vegetables and fruits is about 80-95% (Khan *et al*., 2017). Beside that, Inayah *et al.* (2020) reported that the major component of Sunda porcupine's quills was protein that reaches more than 90%, different to other tissue from bioresources that the major component is water, fills up the cell and the extracellular environment.

The organoleptic characters of simplicia powder were gainsboro gray into light gray in color, odorless-bit, musty-bit, dusty odor, tasteless-light, dusty taste, and lightweight-smooth bit with a rough texture. Meanwhile, the crude extract characters was golden yellow in color, had pungent-acidic-unpleasant smell, salty-bitter taste, and sticky like paste texture.

The structure of the porcupine's quills was hard and sharp, so we needed to prepare it into small pieces before mashing them into powder. The hardness of the quills may be caused by the high protein content, which is thought to be keratin protein (Inayah *et al*., 2020). The small cuts of the dry quills were then called simplicia and then mashed into powder. The simplicia powder had a water content of about 9.1%, less than 10%, which indicated that the simplicia is ready to be extracted or stored since the low water content of simplicia inhibits microbial growth.

The extraction was conducted using the maceration technique with 70% ethanol. The yield of the crude extract was about 3.32%. Compared to plant resources, the crude extract of porcupine's quills was much lower than that obtained from plant resources, which is up to 10-30% (Dinata *et al*., 2015; Rahman *et al*., 2017; Wirnawati *et al*., 2020). It may be caused by the difference in the major the components that fill up the tissue matrix since the Sunda porcupine's quills are filled with protein while plant resources are filled up with water. The protein, which acts as the major component in the Sunda porcupine's quills, cannot be removed from the quills by the drying process and still contributes to the weight of the simplicia during the extraction process. In contrast, water, a major component in plant resources, can be removed in high quantities during the drying process. Consequently, the active compounds inside the simplicia of plant resources are more

concentrated than the simplicia of Sunda porcupine's quills.

The number of active compounds from animals found in various sources is predicted about 50,000-100,000. The group of active compounds in animals generally functions as antibiotic (Berdy, 2005). The qualitative test found that the Sunda porcupine's quills contain alkaloids, flavonoids, saponins, triterpenoids, and peptides. These compounds may affect human health and interact with various receptors to induce metabolic effects.

Alkaloids are nitrogen compounds with unique molecular structures found in nature (Srivastava & Singh, 2020). The types of alkaloids include caffeine, morphine, codeine, reserpine, and so on. One of the functions of alkaloids, especially in animals, is as a defense system against pathogens. Therefore, alkaloids are potential as antibacterial, antioxidant, and anti-inflammatory (Atanasov *et al*., 2015; Azam *et al*., 2003). Based on the results of this study, the Sunda porcupine's quills contain alkaloids that may have the potential as antibacterial, antioxidant, or anti-inflammatory properties.

Flavonoids are phenolic compounds known as natural products. The source of flavonoids in animals is mainly from plant-based diet, and a small part is biosynthesized *in situ* (Kumar & Pandey, 2013; Yao *et al*., 2004). Many studies found that flavonoid compounds act as an anti-inflammatory in several disease cases, e.g., pulpitis. Flavonoids play a role in suppressing several cytokine pro-inflammation and endogenous enzymes that induce an inflammation response, such as cyclooxygenase (COX) (Choy *et al*., 2019; Maleki *et al*., 2019). The crude extract of Sunda porcupine's quills contains flavonoids and may be potential as anti-inflammation.

Saponins are organic compounds primarily found in plants and a small part in animals (Podolak *et al*., 2010). Types of saponins include triterpenoid saponin, steroidal saponin, and saponin alkaloids in the presence of sugar bonds (Ashour *et al*., 2019). One of the functions of saponins is antibacterial with various mechanism pathways. This study showed that Sunda porcupine's quills contain saponins that may cause cell lysis in bacteria by damaging the membrane permeability. Not only saponins but also steroids/triterpenoids compounds were found in Sunda porcupine's quills. Positive identification of steroids/triterpenoids was shown by the formation of red color (initial center) using Lieberman-Burchard method and green color (late-side) using the Keller Killian method. In general, the biological activity of steroids/triterpenoids as analgesics is relieving pain due to inflammation (Del Grossi Moura *et al*., 2018; Howes, 2018). It is worth further discovering the function of active compounds in Sunda porcupine's quills for treating toothache with more attractive methods.

Peptides are commonly the primary metabolites since peptides can be found as structural or functional components inside living organisms. Recent studies showed that peptides can perform as secondary metabolites since they had antimicrobial activity, hence, are grouped as antimicrobial peptides (AMPs). The AMPs are recently known as host defense peptides that can be found in all forms of life. The molecules of AMPs are commonly short and negatively charged. The AMPs may have the potential to be antimicrobial, antiviral, antifungal, immunomodulator, and wound healer (Kumar *et al*., 2018; Mahlapuu *et al*., 2016). The AMPs can be found in the outer part of vertebrate body in the skin case of the frog *Xenopus laevis* containing AMPs of margainins (Zasloff, 1987). The quills are the outer part of Sunda porcupine's body and act as a defense system. The qualitative result showed that the crude extract of Sunda porcupine's quills was positive for peptides based on Bradford reaction. The peptides inside the crude extract may be AMPs; therefore, may have potential function like AMPs.

The crude extract of Sunda porcupine's quills was categorized as non-toxic since the value of LC_{50} is more than 1,000 ppm. We used brine shrimp (*Artemia salina* Leach) since they are sensitive to toxic compounds and are easier to handle than mice (Awaludin *et al*., 2020). The result showed that the crude extract of Sunda porcupine's quills had a high LC_{50} value (>1,000 ppm), and low mortality (<50%) indicated that the extract was non-toxic. Other studies had different results from herbs or botanicals with significant toxic potential and high bioactivity (Charen & Harbord, 2020). Toxicity is directly proportional to bioactivity. Therefore, the crude extract of Sunda porcupine's quills has low bioactivity against brine shrimp, but has not been tested on other types of organisms

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such as bacteria, fungi, or other pathogens. Further investigation using other animal models to find out the potential and characteristics of pharmacology issues of Sunda porcupine's quills is needed.

We have shown that the crude extract of Sunda porcupine's quills contains active compounds of alkaloids, flavonoids, saponins, steroids, triterpenoids, and peptides group. These results suggest that the extract may have potential as antibacterial, antiviral, antioxidant, antifungal, immunomodulator, wound healer, anti-inflammatory, and analgesics. As reported by Roze *et al*. (1990), the quills of New World porcupines are coated in fatty acids from exocrine secretions that prevent bacterial growth and are likely to prevent infection from self-inflicted wounds. However, the potentials need deeper investigation through further research using *in vitro*, *in vivo*, and *in silico* approaches.

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References

Anita, S., Agusta, A., Farida, W. R., Nugroho, H. A., & Wulansari, D. (2018). A preliminary study of aphrodisiac property from porcupine tail meat ethanol extract in male mice. *Zoo Indonesia*, *26*(1).

AOAC. (2003). Official Methods of analysis. Vol.I. 17th ed. Association of Analytical Washington, DC, USA.

Aplin, K. (2016). *Hystrix javanica*. The IUCN Red List of Threatened Species.

Ashour, A. S., El Aziz, M. M. A., & Gomha Melad, A. S. (2019). A review on saponins from medicinal plants: chemistry, isolation, and determination. *Journal of Nanomedicine Research*, *7*(4). https://doi.org/10.15406/jnmr.2019.07.00199.

Atanasov, A. G., Waltenberger, B., Pferschy-Wenzig, E. M., Linder, T., Wawrosch, C., Uhrin, P., Temml, V., Wang, L., Schwaiger, S., Heiss, E. H., Rollinger, J. M., Schuster, D., Breuss, J. M., Bochkov, V., Mihovilovic, M. D., Kopp, B., Bauer, R., Dirsch, V. M., & Stuppner, H. (2015). Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotechnology Advances*, *33*(8), 1582-1614. https://doi.org/10.1016/j.biotechadv.2015.08.001.

Awaludin, Kartina, Maulianawati, D., Manalu, W., Andriyanto, Septiana, R., Arfandi, A., & Lalang, Y. (2020). Phytochemical screening and toxicity of ethanol extract of *Sauropus androgynus*. *Biodiversitas*, *21*(7). https://doi.org/10.13057/biodiv/d210712.

Azam, S., Hadi, N., Khan, N. U., & Hadi, S. M. (2003). Antioxidant and prooxidant properties of caffeine, theobromine and xanthine. *Medical Science Monitor*, *9*(9).

Aziz, M. A. (2015). Qualitative phytochemical screening and evaluation of anti-inflammatory, analgesic and antipyretic activities of *Microcos paniculata* barks and fruits. *Journal of Integrative Medicine*, *13*(3). https://doi.org/10.1016/S2095-4964(15)60179-0.

Balamurugan, V., Fatima, S., & Velurajan, S. (2019). A guide to phytochemical analysis. *International Journal of Advance Research and Innovative Ideas in Education*, *5*(1), 236-45.

Benzidia, B., Barbouchi, M., Hammouch, H., Belahbib, N., Zouarhi, M., Erramli, H., Ait Daoud, N., Badrane, N., & Hajjaji, N. (2019). Chemical composition and antioxidant activity of tannins extract from green rind of *Aloe vera* (L.) Burm. F. *Journal of King Saud University - Science*, *31*(4), 1175-1181. https://doi.org/https://doi.org/10.1016/j.jksus.2018.05.022.

Berdy, J. (2005). Bioactive microbial metabolites. *The Journal of Antibiotics*, *58*(1), 1-26.

Bintang, M. (2010). *Biokimia: Teknik Penelitian*. Penerbit Erlangga.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*(1-2), 248-254.

Choy, K. W., Murugan, D., Leong, X. F., Abas, R., Alias, A., & Mustafa, M. R. (2019). Flavonoids as natural anti-inflammatory agents targeting nuclear factor-kappa B (NFκB) signaling in cardiovascular diseases: A mini review. *Frontiers in Pharmacology*, 10,1295. https://doi.org/10.3389/fphar.2019.01295.

Charen, E., & Harbord, N. (2020). Toxicity of herbs, vitamins, and supplements. *Advances in Chronic Kidney Disease*, *27*(1), 67-71. https://doi.org/10.1053/j.ackd.2019.08.003.

Del Grossi Moura, M., Lopes, L. C., Silva, M. T., Barberato-Filho, S., Motta, R. H. L., & De Cássia Bergamaschi, C. (2018). Use of steroid

and nonsteroidal anti-inflammatories in the treatment of rheumatoid arthritis: Systematic review protocol. *PloS One*, *16*(4), e0248866. https://doi.org/10.1097/MD.0000000000012658.

Dinata, D. I., Supriyadi, D., Djafar, G., Syerliana, S., Wijayanti, W., & Suherman, S. E. (2015). Effect of adding granul basil (*Ocimum americanum*) as antioxidants in fried foods. *Indonesian Journal of Pharmaceutical Science and Technology*, *2*(1). https://doi.org/10.15416/ijpst.v2i1.7807.

Djamil, R., & Anelia. T. (2009). Penapisan Fitokimia Uji BSLT dan uji antioksidan ekstrak metanol beberapa spesies papilionaceae. *Jurnal Ilmu Kefarmasian Indonesia*, *7*(2).

Farida, W. R. (2013). The physical and chemical characteristics of Sunda porcupine meat (*Hystrix javanica* F. Cuvier, 1823) given additional concentrate feed. *Jurnal Biologi Indonesia*, *9*(2), 311-325.

Fardiansyah, (2020). *Mengenal lebih dekat landak jawa, durinya bisa obati sakit gigi*. [Online]. Retrieved from https://techno.okezone.com/read/2020/06/11/56/2228231/mengenal-lebih-dekat-landak-jawa-durinya-bisa-obati-sakit-gigi.

Harborne, J. B (1996). *Metode Fitokimia : Penuntun Cara Modern Menganalisa Tumbuhan*. Bandung, ITB Press.

Howes, M. J. R. (2018). Phytochemicals as anti-inflammatory nutraceuticals and phytopharmaceuticals. *Immunity and Inflammation in Health and Disease* (pp. 363-388). Academic Press.

Inayah, N., Farida, W. R., & Purwaningsih, E. (2020). Microstructure of quills in Sunda porcupine *Hystrix javanica* (F. Cuvier, 1823). *Jurnal Biologi Indonesia*, *16*(1), 81-88. https://doi.org/10.47349/jbi/16012020/81

Jelita, S. F., Setyowati, G. W., & Ferdinand, M. (2020). Uji toksisitas infusa *Acalypha Siamensis* dengan metode Brine Shrimp Lethality Test (BSLT). *Farmaka*, *18*(1), 14-22.

Khan, M. I. H., Kumar, C., Joardder, M. U. H., & Karim, M. A. (2017). Determination of appropriate effective diffusivity for different food materials. *Drying Technology*, *35*(3), 335-346.

Kumar, P., Kizhakkedathu, J. N., & Straus, S. K. (2018). Antimicrobial peptides: diversity, mechanism of action and strategies to improve the activity and biocompatibility in vivo. *Biomolecules*, *8*(1), 4.
https://doi.org/10.3390/biom8010004

Kumar, S., & Pandey, A. K. (2013). Chemistry and biological activities of flavonoids: An Overview. *The Scientific World Journal*, *2013*, 162750. https://doi.org/10.1155/2013/162750

Mahlapuu, M., Håkansson, J., Ringstad, L., & Björn, C. (2016). Antimicrobial peptides: An emerging category of therapeutic agents. *Frontiers in Cellular and Infection Microbiology*, *6*, 194. https://doi.org/10.3389/fcimb.2016.00194.

Maleki, S. J., Crespo, J. F., & Cabanillas, B. (2019). Anti-inflammatory effects of flavonoids. *Food Chemistry*, *299*, 125124.

Manoi, F. (2006). Pengaruh cara pengeringan terhadap mutu simplisia sambiloto. *Buletin Penelitian Tanaman Rempah dan Obat, 17*(1), 1-5.

Meyer, B. N., Ferrigni, N. R., Putnam, J. E., Jacobsen, L. B., Nichols, D. E. j, & McLaughlin, J. L. (1982). Brine shrimp: A convenient general bioassay for active plant constituents. *Planta Medica*, *45*(05), 31-34.

Nea, F., Bitchi, M. B., Genva, M., Ledoux, A., Tchinda, A. T., Damblon, C., Frederich, M., Tonzibo, Z. F., & Fauconnier, M. L. (2021). Phytochemical investigation and biological activities of *Lantana rhodesiensis*. *Molecules*, *26*(4).

Podolak, I., Galanty, A., & Sobolewska, D. (2010). Saponins as cytotoxic agents: A review. *Phytochemistry reviews : Proceedings of the Phytochemical Society of Europe*, *9*(3), 425-474.

Rahman, S. M., Shohael, A. M., Ahamed, T., & Rahman, S. K. M. (2017). Thin layer chromatographic profiling and phytochemical screening of six medicinal plants in Bangladesh. *International Journal of Biosciences (IJB)*, *11*(1), 131-140. https://doi.org/10.12692/ijb/11.1.131-140.

Roze, U., Locke, D. C., & Vatakis, N. (1990). Antibiotic properties of porcupine quills. *Journal of Chemical Ecology*, *16*(3), 725-734. https://doi.org/10.1007/BF01016483.

Srivastava, A., & Singh, V. (2020). Physiological role of some alkaloids*, Journal of Medicine and Surgical Sciences*, *2*(3).

Van Weers, D. J. (1979). Notes on Southeast Asian Porcupines (Hystricidae, Rodentia). IV. On the taxonomy of the subgenus Acanthion F. Cuvier, 1823 with notes on the other taxa of the family. *Beaufortia*, *29*(356), 215-272.

Van Weers, D. J. (1983). Specific distinction in Old World porcupines. *Der Zoologische Garten, Jena*, *53*, 226-232.

Wirnawati, W., Mentari, I., & Pernando, N. (2020). The characterization of simplicia and extract of clove leaves (*Syzigium aromaticum* Merr. Et Perry L) for herbal mouthwash active ingredients. *Jurnal Ilmu Kesehatan*, *8*, 26-33. https://doi.org/10.30650/jik.v8i1.1377.

Woods, & Kilpatrick. (2005). Infraorder Hystricognathi Brandt, 1855. *Mammal Species of the World. A Taxonomic and Geographic Reference*, *2*, 1538-1600.

Yao, L. H., Jiang, Y. M., Shi, J., Tomás-Barberán, F. A., Datta, N., Singanusong, R., & Chen, S. S.

(2004). Flavonoids in food and their health benefits. *Plant foods for human nutrition (Dordrecht, Netherlands)*, *59*(3), 113-122. https://doi.org/10.1007/s11130-004-0049-7.

Zasloff M. (1987). Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proceedings of the National Academy of Sciences of the United States of America*, *84*(15), 5449-5453. https://doi.org/10.1073/pnas.84.15.5449.

Zuraida, Z. (2018). Analisis toksisitas beberapa tumbuhan hutan dengan metode Brine Shrimp Lethality Test (BSLT). *Jurnal Penelitian Hasil Hutan*, *36*(3). https://doi.org/10.20886/jphh.2018.36.3.239-246

Antioxidant, Antibacterial, and Antidiabetic Activities of Roselle (*Hibiscus sabdariffa*) Extracts

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Abstract

Hibiscus sabdariffa L., also referred to as roselle, is commonly utilized in the pharmaceutical and food industries. Roselle contains bioactive compounds such as phenolics, alkaloids, tannins, flavonoids, saponins, and organic acids, which have pharmacological properties, such as antioxidant, antibacterial, immune booster, antidiabetic, anti-inflammatory, and anti-hypertensive properties. There are many studies regarding the pharmacological activities of roselle extract and its applications. However, there has been no research to study the effectiveness of the solvent in testing roselle petal extracts against antibacterial, antioxidant, and antidiabetic activities, simultaneously. This research used two kinds of polar solvents, dH_2O and ethanol, with various concentrations for antibacterial activity test by five pathogenic bacteria, for antioxidant test by the DPPH method, and for antidiabetic test by the alpha-glucosidase inhibition method. The result showed that the ethanol extract of roselle had higher antibacterial activity compare to the roselle water extract. Antioxidant activity of roselle ethanol extract at 20% concentration had the highest activity 69.75 ± 0.002%; while, the 100% concentration of roselle water extract had the highest antioxidant activity 138.73 ± 0.013%. antidiabetic activity of roselle ethanol and water extract at 100% concentration had the highest activity 1,195.44 ± 0.007% and 1,552.49 ± 0.069%, respectively.

Keywords: antibacterial activity, antioxidant activity, antidiabetic activity, roselle, pharmacological bioprospection

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Introduction

Hibiscus sabdariffa L (roselle) is commonly utilized in the pharmaceutical and food industries. Roselle seeds are often used as an oil producer for cosmetics, powdered seeds and leaves are often used as animal feed, and roselle flower petals are used as herbal beverage products (Da-Costa-Rocha *et al*., 2014). Roselle petal contains phytochemical compounds, such as phenolics, alkaloids, tannins, flavonoids, saponins, and organic acids that have pharmacological activities including antioxidant, antibacterial, immune booster, antidiabetic, anti-inflammatory, and antihypertensive (Alaa, 2012; Brown *et al*., 2019; Herdiani & Wikurendra, 2020; Wang *et al*., 2011). Many studies regarding the pharmacological activity of roselle extract and its applications have been conducted (Izquierdo-Vega *et al*., 2020; Shruthi *et al*., 2016; Qi *et al*., 2005). However, there has been no research on the effectiveness of the solvent extract of roselle for antimicrobial, antioxidant, and antidiabetic activities, simultaneously.

Márquez-Rodríguez *et al*. (2020) reported that the roselle ethanol extract inhibited the expansion of food spoilage pathogenic bacteria, including *Escherichia coli, Salmonella*

typhimurium, Staphylococcus aureus, Listeria monocytogenes, and *Bacillus cereus*. Additionally, Abou-Arab *et al*. (2011) stated that the ethanol extract of roselle with the addition of 1% citric acid showed higher antioxidant activity than using other solvents. The high levels of polyphenolic compounds in the roselle petal extract are known to be effective in reducing blood glucose levels in diabetes mellitus rats (Rosemary & Haro, 2014; Herdiani & Wikurendra, 2020). The aim of this study was to analyze the pharmacological profile of roselle extract on an antibacterial, an antioxidant, and an antidiabetic test simultaneously by extracting roselle petals using various concentrations of polar solvents (water and ethanol). The results of this study are expected to be as scientific information regarding the pharmacological bioprospection of roselle petal extract as a source of antibacterials, antioxidants, and antidiabetics.

Materials and Methods

Extraction of Roselle Petals. The 100 g of roselle flower petals were collected and then extracted by the maceration method using distilled water or 96% ethanol as solvent with a concentration of 100% (100 g roselle petals: 100 mL solvent). The 100% extracts were poured into dilution bottles of 200 µL, 400 µL, 600 µL, and 800 µL, respectively, and subsequently added with sterile distilled water until each solution amounted to 1 mL. So, the extracts obtained had respective concentrations of 20%, 40%, 60%, and 80%. The extracts obtained were stored at room temperature.

Antibacterial Activity. The antibacterial activity test was carried out using the disk diffusion method (Manguntungi *et al*., 2020). Roselle petal extracts in water or ethanol are used in concentrations of 20%, 40%, 60%, 80%, and 100%. Five pathogenic bacteria used in this test were *E. coli* ATCC 25922, PIDT *Proteus* obtained from IPB University, *Salmonella tyhposa* obtained from Research Center for Chemistry, Indonesian Institute of Sciences*, S. aureus* ATCC 25923*,* and *Listeria monocytogenes* obtained from Universitas Gadjah Mada. The area of the inhibition zone was then measured in mm to determine the antibacterial activity of the roselle petal extracts.

Antioxidant Activity. The antioxidant test against DPPH free radicals was used with a slight modification (Zahratunnisa *et al*., 2017). Roselle petal extracts in water or ethanol were used in concentrations of 20%, 40%, 60%, 80%, and 100%. Vitamin C with a concentration of 50 ppm was used as a positive control. All samples that had been incubated for 30 minutes at 27^{o}C were measured for absorbance values using a UV-Vis spectrophotometer at a wavelength of 517 nm.

Antidiabetic Activity. An antidiabetic test using the alpha-glucosidase inhibition method was conducted (Yuniarto & Selifiana, 2018). Roselle petal extracts in water or ethanol were used in concentrations of 20%, 40%, 60%, 80%, and 100%. Acarbose with a concentration of 100 ppm was used as a positive control. The extracts were measured with ELISA Reader at a wavelength of 200 nm.

Analysis Data. At the initial stage, the data are analyzed for normality. If the results are normal and homogeneous, then a comparative analysis can be carried out between groups using a one-way ANOVA test with a 5% confidence interval.

Results

Pharmacological Activity of the Roselle Petals Extract using Ethanol Solvent.

In this study, Gram-positive (*S. thyposa, S. aureus,* and *L. monocytogenes*) and Gram-negative bacteria (*E. coli* and PIDT *proteus*) were used as test bacteria for the antibacterial activity of the roselle flower petals' ethanol extracts. The various concentrations of the roselle petal ethanol extracts against the five pathogenic bacteria showed different zones of inhibition. The results of the antibacterialactivity test of the ethanolic extracts of roselle petals are presented in Table 1. As a positive control, the antibiotic ampicillin with a concentration 0.5 µg/µL was used. As a negative control, 96% ethanol was used.

Based on the results of the data analysis in Table 1, compared to a positive control, the roselle ethanol extract at 80% concentration had the highest antibacterialactivity against *S. aureus* and *L. monocytogenes* with inhibition zones of 12.67±1.15 mm and 23.00±0.00 mm,

respectively. The 100% concentration of roselle petal ethanol extracts were able to inhibit *E. coli* and PIDT *Proteus* with inhibition zones of 17.00±0.00 mm and 14.00±0.00 mm, respectively. While, the highest value of antibacterialactivity against *S. tyhposa* was found in the control (+) with an area of inhibition zone of 21.00±0.00 mm.

Table 1. The results of the antibacterial test of roselle petal extracts with ethanol as a solvent

Concentration of roselle flower petal extracts	Antibacterial activity of roselle petal extracts with ethanol as a solvent (mm)				
	E. coli	PIDT *Proteus*	*S. tyhposa*	*S. aureus*	*L. monocytogenes*
20%	7.00 ± 1.00^{b}	7.00 ± 0.00^{c}	3.33 ± 1.15^{b}	3.33 ± 1.15^{bc}	7.67 ± 0.00^{b}
40%	12.67 ± 2.88^{c}	4.67 ± 1.15^{b}	7.00 ± 0.00^{c}	6.33 ± 0.57^{d}	9.00 ± 0.00^{b}
60%	12.00 ± 1.73^{c}	8.33 ± 0.57^{d}	12.00 ± 0.00^{d}	12.00 ± 1.73^{e}	12.00 ± 0.00^{c}
80%	16.00 ± 0.00^{d}	11.00 ± 0.00^{e}	12.67 ± 1.15^{d}	**12.67 ± 1.15^{e}**	**23.00 ± 0.00f**
100%	**17.00 ± 0.00^{d}**	**14.00 ± 0.00^{f}**	18.00 ± 0.00^{e}	2.00 ± 0.00^{b}	21.00 ± 0.00^{e}
C (+) Ampicillin	14.67 ± 1.15^{cd}	9.00 ± 0.00^{d}	**21.00 ± 0.00^{f}**	4.00 ± 0.00^{c}	15.00 ± 0.00^{d}
C (−) ethanol 96%	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

Note: Numbers followed by the same letters in the same column show no significant difference in the one-way ANOVA test.

An antioxidant test of roselle petal ethanol extracts as a solvent is shown in Table 2. The antioxidant activity of roselle petal extracts using ethanol as a solvent with varying concentrations in inhibiting DPPH free radicals showed significantly different results among experimental groups.

Based on the results of the data analysis in Table 2, compared to vitamin C with a concentration of 50 ppm as a positive control, all the extracts have stronger antioxidant activities than vitamin C and ethanol as a negative control. The 20% concentration of roselle ethanol extract has the highest antioxidant activity 69.75±0.002% no significant difference with the 40% concentration compared to concentrations of 60%, 80%, 100%, and a positive control (vitamin C).

Table 2. Antioxidant test results of roselle petals extracts with ethanol as a solvent

Concentration of roselle flower petal extract	Antioxidant activity of roselle petal extract with ethanol as a solvent (%)
20%	**69.75 ± 0.002^{f}**
40%	68.66 ± 0.001^{f}
60%	66.67 ± 0.000^{e}
80%	59.10 ± 0.001^{d}
100%	54.13 ± 0.001^{c}
C (＋) Vitamin C	49.00 ± 0.001^{b}
C (−) Ethanol 96%	0.00 ± 0.00^{a}

Note: Numbers followed by the same letters in the same column show no significant difference in the one-way ANOVA test.

Roselle petal extracts using ethanol as a solvent showed antidiabetic activity by inhibiting alpha-glucosidase in 30 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside. The results of the antidiabetic test of the ethanol extracts of roselle petal are shown in Table 3.

Based on the result of the data analysis in Table 3, it is shown that, compared to acarbose with a 100 ppm concentration as a positive control except for a 20% concentration of roselle petal ethanol extracts, all treatments showed strong antidiabetic activities . The 100% concentration of roselle petal ethanol extracts had the highest antidiabetic activity, 1,195.44±0.007% compared to other treatment groups. All treatment groups showed significant differences.

Table 3. antidiabetic test results of roselle petal extracts with ethanol as a solvent

Concentration of roselle flower petal extract	Antidiabetic activity of roselle petal extracts with ethanol as a solvent (%)
20%	251.56 ± 0.005[b]
40%	526.14 ± 0.020[d]
60%	798.08 ± 0.005[e]
80%	1,019.42 ± 0.013[f]
100%	**1,195.44 ± 0.007[g]**
C (+) Acarbose	490.59 ± 0.074[c]
C (−) Ethanol	0.00 ± 0.00[a]

Note: Numbers followed by the same letters in the same column show no significant difference in the one-way ANOVA test.

Pharmacological Activity of Roselle Extract using Water Solvent.

Roselle petals extracted using water were tested on *E. coli*, PITD *proteus*, *S. tyhposa*, *S. aureus*, and *L. monocytogenes* showed an antibacterial activity (Table 4). As a positive control and negative control, the antibiotic ampicillin with a concentration of 0.5 µg/µL and water were used, respectively.

Based on the results of the data analysis in Table 4, the roselle petal water extract at 80% and 100% concentrations were able to inhibit *E. coli* with the highest inhibition zone area of 7.00±0.00 mm. While, the roselle petal water extracts at 40% and 60% concentrations had the highest antibacterial activity against PIDT *Proteus* with an inhibition zone 5.33±0.57 mm. The highest value of microbial activity against *S. tyhposa* and *S. aureus* was found in the 100% treatment, 6.00±1.73 mm and 9.00±0.00 mm, respectively. The positive control treatment had the highest antibacterial activity with an inhibitory zone of 6.00±0.00 mm against *L. monocytogenes*.

Table 4. The results of the antibacterial test of roselle petal extracts with water as a solvent

Concentration of roselle flower petal extract	Antibacterial activity of roselle petal extracts with water as a solvent (mm)				
	E. coli	**PIDT *Proteus***	***S. tyhposa***	***S. aureus***	***L. monocytogenes***
20%	5.67 ± 1.15[bc]	3.33 ± 1.15[b]	3.33 ± 0.57[b]	4.00 ± 0.00[b]	2.00 ± 0.00[b]
40%	6.00 ± 0.00[bc]	**5.33 ± 0.57[d]**	3.67 ± 1.15[b]	5.67 ± 0.57[cd]	2.33 ± 0.57[b]
60%	5.33 ± 2.88[bc]	**5.33 ± 0.57[d]**	4.33 ± 0.57[bc]	6.33 ± 0.57[d]	3.67 ± 0.57[c]
80%	**7.00 ± 0.00[c]**	4.00 ± 0.00[b]	4.67 ± 0.57[bc]	7.67 ± 1.15[e]	4.33 ± 0.57[c]
100%	**7.00 ± 0.00[c]**	4.33 ± 0.57[bcd]	**6.00 ± 1.73[c]**	**9.00 ± 0.00[f]**	5.33 ± 0.57[d]
C (+) Ampicillin	4.00 ± 0.00[b]	5.00 ± 0.00[bc]	5.00 ± 0.00[bc]	5.00 ± 0.00[bc]	**6.00 ± 0.00[d]**
C (−) water	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]

Note: Numbers followed by the same letters in the same column show no significant difference in the one-way ANOVA test.

Table 5. The results of the antioxidant test of roselle petal extracts with water as a solvent

Concentration of roselle flower petal extract	Antioxidant activity of roselle petal extract with water as a solvent (%)
20%	39.77 ± 0.128[c]
40%	12.88 ± 0.041[b]
60%	12.05 ± 0.006[b]
80%	52.02 ± 0.006[e]
100%	**138.73 ± 0.013[f]**
C (+) Vitamin C	49.00 ± 0.001[d]
C (−) Water	0.00 ± 0.00[a]

Note: Numbers followed by the same letters in the same column show no significant difference in the one-way ANOVA test.

The antioxidant activity of roselle extract using water as solvent with various concentrations in inhibiting DPPH free radicals showed significantly different results between experimental groups (Table 5).

Based on the results of the data analysis in Table 5, the 100% concentration of roselle water extract had the highest antioxidant activity, 138.73 ± 0.013% compared to concentrations of 20%, 40%, 60%, 80%, positive (vitamin C), and negative control (water).

Roselle petals extracted using water showed antidiabetic activity by inhibiting alpha-glucosidase in 30 µl of 5 mM p-nitrophenyl-α-D-glucopyranoside. The results of the antidiabetic test of roselle petal water extracts is shown in Table 6.

Based on the results of data analysis in Table 6, the 100% concentration of roselle petals water extract had the highest antidiabetic activity, 1,552.49±0.069% compared to other treatment groups. All treatment groups showed significant differences.

Table 6. Antidiabetic test results of roselle petal extracts with water as a solvent

Concentration of roselle flower petal extract	Antidiabetic activity of roselle petal extracts with water as a solvent (%)
20%	317.91 ± 0.005[b]
40%	657.46 ± 0.015[b]
60%	969.65 ± 0.008[e]
80%	1,243.03 ± 0.023[f]
100%	**1,552.49 ± 0.069[g]**
C (+) Acarbose	437.06 ± 0.176[c]
C (−) Water	0.00 ± 0.00[a]

Note: Numbers followed by the same letters in the same column show no significant difference in the one-way ANOVA test.

Discussion

Polar substances or compounds will dissolve in polar solvents, so in this study two types of polar solvents (distilled water and ethanol) with different concentrations showed a different effect on each test performed.

The antibacterial activity of the ethanol extracts and the distilled water extracts of roselle petals against each tested bacterial pathogen has different effectiveness (Tables 1 and 4). This difference in effectiveness is due to differences in the optimization of the types of bioactive compounds that can be extracted from roselle petals. Haeriah *et al*. (2018) reported the different levels of each bioactive compound contained in roselle petals was depend on variations of concentration, time of extraction, and the type and age of the plant, so that it will have a different effect on antibacterial activity. According to Márquez-Rodríguez *et al*. (2020) the high content of phenolic acids in the phase 1 fraction of roselle extract showed the most effective antibacterial activity in inhibiting food spoilage bacteria such as *E. coli*, *S. tyhposa*, *S. aureus*, *L. monocytogenes*, and *B. cereus*.

The roselle petal ethanol extracts had higher antibacterial activity in comparison to the roselle petal water extracts. Similarly, Alaa (2012) discovered that the antibacterial activity of ethanol extracts was greater than that of roselle water extracts against *Streptococcu*s mutant and *E. coli*, indicating that alcohol solvent was the most effective solvent in extracting roselle phenolic compounds that act as antibacterial. Bioactive compounds release antibacterial activity through various mechanisms, such as oxidative stress on cell membranes, inhibiting cellular division, and bacterial cell metabolic enzymes.

The antioxidant test of the ethanol extract of roselle petals showed that the 20% concentration of DPPH free radicals indicated a higher value than other concentrations; while, the 100% concentration of roselle petal water extracts had higher antioxidant activity than other concentrations. In addition, the antioxidant activity of the roselle petals water extracts were higher than the roselle ethanol extracts (Tables 2 and 5). This is indicated that water extracts is more effective as an antioxidant. Yang *et al*. (2012) reported the result of roselle petals water

extract have higher antioxidanrt activity compared to 30% and 60% roselle ethanol extract. Alaa (2012) stated that roselle petals contain phenolic compounds related to their ability as antioxidants. In addition, roselle extract also contains steroid compounds, alkaloids, tannins, terpenoids, flavonoids, and saponins (Aryati *et al*., 2020). Phenol and flavonoid compounds contained in roselle water extract have the most effective ability to chelate Fe^{2+} metal, which acts as a highly reactive free radical (Alaa, 2012).

Ethanol and water of roselle petals extract at 100% concentration (Tables 3 and 6) showed better antidiabetic activity than acarbose. Acarbose is used as a synthetic drug in diabetics by inhibiting the alpha-glucosidase enzyme, which plays a role in the absorption and digestion of glucose (Yuniarto & Selifiana, 2018). The content of bioactive compounds in roselle extract acts as an antioxidant to ward off free radicals, which are also associated with the cause of diabetes mellitus and diabetes complications. The content of polyphenolic compounds in roselle water extract can reduce the effect of insulin resistance and hyperglycemia (Ajiboye *et al*., 2015). Furthermore, Wang *et al*. (2011) discovered that roselle extracts had a potential effect in improving diabetic nephropathy by increasing antioxidants and regulating the Akt/bad/14-3-3γ signal associated with cell apoptosis caused by hyperglycemia.

Based on the results of the study, it concluded that the ethanol and water extracts with varying concentrations inhibited the pathogenic bacteria. Each treatment concentration of ethanol and water extracts of roselle petals had a different antibacterial activity on each bacterium. The roselle petal ethanol extracts showed higher antibacterial activity than the roselle petals water extracts.

The antioxidant test on water extract or roselle petals against DPPH free radicals was stronger than the ethanol extract of roselle petals; while, the antidiabetic test of water extracts was more effective than the ethanol extracts of roselle petals. The higher the concentration of roselle petals water extract, the higher the antioxidant and an antidiabetic activity.

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References

Abou-Arab, A. A., Abu-Salem, F. M., & Abou-Arab, E. A. (2011). Physico-chemical properties of natural pigments (anthocyanin) extracted from Roselle calyces (*Hibiscus subdariffa*). *Journal of American Science, 7*(7), 445-456.

Alaa, G. A. H. (2012). Antioxidant and antibacterial activities of *Hibiscus sabdariffa* L. extracts. African Journal of Food Science, 6(21), 506-511. https://doi.org/10.5897/AJFS12.099.

Aryati, D. L., Rohadi, & Pratiwi, E. (2020). Aktivitas Antioksidan Ekstrak Kelopak Bunga Rosela (*H. sabdariffa* L.) Merah Pada Berbagai Suhu Pemanasan. *Jurnal Teknologi Pangan Dan Hasil Pertanian*, *15*(1), 1-9. https://doi.org/10.26623/jtphp.v13i1.1845.kodeartikel

Brown, F., González, J., & Monan, M. (2019). Pharmacognostic, Physicochemical and Phytochemical Analysis of Two Cultivars from *Hibiscus sabdariffa* L. in Cuba. *Open Access Library Journal, 6*(12), 1-8. https://doi.org/10.4236/oalib.1105894.

Da-Costa-Rocha, I., Bonnlaender, B., Sievers, H., Pischel, I., & Heinrich, M. (2014). *Hibiscus sabdariffa* L.–A phytochemical and pharmacological review. *Food chemistry, 165*, 424-443. Elsevier Ltd. https://doi.org/10.1016/j.foodchem.2014.05.002

Haeriah, H., Djide, N., Alam, G., & Sartini, S. (2018). Sinergitas Aktivitas Antibakteri dari Kelopak Bunga Rosella dan Kitosan Terhadap Staphylococcus aureus. *Jurnal Farmasi Galenika (Galenika Journal of Pharmacy)(e-Journal), 4*(2), 93-97. https://doi.org/10.22487/j24428744.10590.

Herdiani, N., & Wikurendra, E. A. (2020). Efek Antioksidan Ekstrak Kelopak Rosella Terhadap Glukosa Darah Tikus Diabetes Mellitus Tipe 2. *An-Nadaa: Jurnal Kesehatan Masyarakat (e-Journal), 7*(2), 89-93. https://ojs.uniska-bjm.ac.id/index.php/ANN/article/view/3321.

Izquierdo-Vega, J. A., Arteaga-Badillo, D. A., Sánchez-Gutiérrez, M., Morales-González, J. A., Vargas-Mendoza, N., Gómez-Aldapa, C. A., ... & Madrigal-Santillán, E. (2020). Organic acids from Roselle (*Hibiscus sabdariffa* L.)—A brief review of its pharmacological effects. *Biomedicines, 8*(5), 100.

Manguntungi, B., Saputri, D. S., Mustopa, A. Z., Ekawati, N., Nurfatwa, M., Prastyowati, A., ... & Fidien, K. A. (2020). Antidiabetic, Antioxidants and Antibacterial Activities of Lactic Acid Bacteria (LAB) from Masin (Fermented Sauce from Sumbawa, West Nusa Tenggara, Indonesia). *Annales Bogorienses*, *24*(1), 27-34.

Márquez-Rodríguez, A. S., Nevárez-Baca, S., Lerma-Hernández, J. C., Hernández-Ochoa, L. R., Nevárez-Moorillon, G. V., Gutiérrez-Méndez, N., ... & Salas, E. (2020). In vitro antibacterial activity of *Hibiscus sabdariffa* L. phenolic extract and its in situ application on shelf-life of beef meat. *Foods, 9*(8), 1080.

Qi, Y., Chin, K. L., Malekian, F., Berhane, M., & Gager, J. (2005). Biological characteristics, nutritional and medicinal value of roselle, Hibiscus sabdariffa. *Circular-urban Forestry Natural Resources and Environment, 604*, 1-2.

Ramadhanti, E., Rustini, & Marlina. (2020). A Review: Antibacterial Activity of Roselle (*Hibiscus sabdariffa*) Calyx Extract against Pathogenic Bacteria. *IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)*, 2319-7676. https://doi.org/10.9790/3008-1505075560.

Rosemary, R., & Haro, G. (2014). Antidiabetic effect of roselle calyces extract (*Hibiscus sabdariffa* L.) in streptozotocin induced mice. *International Journal of PharmTech Research, 6*(5), 1703-1711.

Shruthi, V. H., Ramachandra, C. T., Nidoni, U., Hiregoudar, S., Naik, N., & Kurubar, A. R. (2016). Roselle (Hibiscus sabdariffa L.) as a source of natural colour: a review. *Plant Archives, 16*(2), 515-522.

Wang, S. C., Lee, H. J., Lee, S. F., Wang, C. J., Lee, C. H., & Lee, W. C. (2011). Aqueous extract from hibiscus sabdariffa linnaeus ameliorate diabetic nephropathy via regulating oxidative status and Akt/Bad/14-3-3γ in an experimental animal model. *Evidence-Based Complementary and Alternative Medicine*, *2011*. https://doi.org/10.1093/ecam/nep181.

Yang, L., Gou, Y., Zhao, T., Zhao, J., Li, F., Zhang, B., & Wu, X. (2012). Antioxidant capacity of extracts from calyx fruits of roselle (*Hibiscus sabdariffa* L.). *African Journal of Biotechnology*, *11*(17), 4063-4068.

Yuniarto, A., & Selifiana, N. (2018). Aktivitas Inhibisi Enzim Alfa-glukosidase dari Ekstrak Rimpang Bangle (Zingiber cassumunar Roxb.) secara In vitro. *MPI (Media Pharmaceutica Indonesiana)*, *2*(1), 22–25. https://doi.org/10.24123/mpi.v2i1.1299.

Zahratunnisa, N., Elya, B., & Noviani, A. (2017). Inhibition of Alpha-glucosidase and antioxidant test of stem bark extracts of Garcinia fruticosa Lauterb. *Pharmacognosy Journal*, 9(2).

Bacterial Carbonate Precipitation for Biogrouting

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Abstract

Biogrouting is a process that transforms soil or sand into calcarenite or sandstone by calcium carbonate precipitation bacteria. The mechanisms of this process are that the bacteria hydrolyze urea catalyzed by urease, and with the existence of dissolved Ca^{2+}, a solid crystalline calcite or calcium carbonate ($CaCO_3$) will be produced. The main advantage of biogrout is that soil or sand can be strengthened without interfering with the hydraulics of the treated soil or sand and without excavation or replacement. In this study, the isolation and identification of calcium carbonate precipitation bacteria, and characterization of urease produced by bacteria were conducted. In the isolation method, the enrichment method using urea as a carbon source was carried out. The formation of crystalline calcite was observed by a light microscope. The urease enzyme activity was determined by the 3, 5-Dinitrosalicylic acid (DNS) method. The molecular identification of isolates was analyzed by the determination of 16S rRNA gene sequencing. As a result, 19 calcium carbonate precipitation bacteria have been isolated from soils, sands, water, and rocks collected from several areas in Indonesia. They showed calcite formation in a medium with urea. Molecular identification of isolates with high urease enzyme activity revealed that the isolates belong to the group Bacilli. The highest enzyme activity produced by ID10-U004 is 374.94 U/mL. The preliminary biogrouting experiment was also conducted using isolated strain, and the reaction was able to make the sand solid and stiff.

Keywords: calcium carbonate precipitation bacteria, urease, biogrouting, Bacilli

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Introduction

Precipitation of calcite or calcium carbonate ($CaCO_3$) by bacteria is a common natural phenomenon occurring in different environments, from hot springs, marine environments, caves, calcite rocks, soils, geological formations, freshwater biofilms, oceans, and saline lakes (Boquet *et al*., 1973; Hammes *et al*., 2003a). Various types of polymorphic crystalline carbonate (calcite, aragonite, dolomite, and others) with the number, size, and type, depending on the type of bacteria and growth, could form from this natural phenomenon.

Carbonate precipitation by bacteria has paid the attention of scientists in recent years mainly due to its important role in marine systems as carbon sinks to boost the global production of CO_2 and mineralization of stone. Calcite resulting from carbonate precipitation, is a mineral widely distributed on earth and found in rocks as marble and sand stone in the waters or on land (Hammes & Verstraete, 2002).

In geology, biogrouting is a process that transforms soil or sand into calcarenite or sandstone by calcium carbonate precipitation bacteria developed with mechanisms based on the mediation of carbonate precipitation. Carbonate precipitation can theoretically occur in natural environments by increasing the concentration of calcium and carbonate in solution or by lowering the solubility of

calcium and/or carbonate. The carbonate precipitation bacteria can be used to accelerate the process *in situ*. Precipitation (deposition) of calcite is at least determined by 3 parameters: (1) the concentration of calcium, 2) the concentration of carbonate, and (3) the pH environment and the availability of nucleation sites (Hammes & Verstraete, 2002; Hammes *et al*., 2003b). The mechanism of calcite precipitation by bacteria depends on urease enzyme activity. Enzymatic hydrolysis of urea presents a straightforward model for studying bacterial carbonate precipitation. In this reaction, urease (EC 3.5.1.5, urea amidohydrolase) will hydrolyze urea to become ammonia. One mol of urea is hydrolyzed intracellularly to 1 mol of ammonia and 1 mol of carbamate, which spontaneously hydrolyzes to form an additional 1 mol of ammonia and carbonic acid. These products subsequently equilibrate in water to form bicarbonate and 2 mol of ammonium and hydroxide ions. The latter gives rise to pH increase, which in turn can shift the bicarbonate equilibrium, resulting in the formation of carbonate ions, which in the presence of soluble calcium ions precipitate as $CaCO_3$ (Stocks-Fischer *et al*., 1999; Hammes *et al*., 2003b; Rodrigues-Navarro *et al*., 2003).

Biogrouting is also called as biocementation. Ideally the source of microorganisms for biogrouting should be resistant or tolerant to high concentrations of urea and calcium. Urease-producing microorganisms can be classified into 2 groups based on the response to ammonium: (1) urease enzyme activity is suppressed by the presence of ammonium, for example, *Pseudomonas aeruginosa*, *Alcaligenes autrophus*, and *Bacillus megaterium* (Kaltwasser *et al*., 1972) and *Klebsiella aerogenes* (Friedrich & Magasanik, 1997) and (2) urease enzyme activity is not affected by ammonium, for example, *Sporosarcina pasteurii* (*Bacillus pasteurii*), *Proteus vulgaris*, *Helicobacter pylori*. In the carbonate precipitation reaction, the high concentration of urea will hydrolyzed by the group of bacteria that their enzyme activity is not suppressed by ammonium, which is suitable for use. At this time, genus *Sporosarcina* (*Bacillus*) has been applied to biogrouting process because it has a high urease activity and are not pathogenic (Mobley *et al*., 1995; Fujita *et al*., 2000).

Carbonate precipitation has value in technical and industrial applications for preserving and restoring sands or calcareous stone. In this study, the isolation and identification of calcium carbonate precipitation bacteria from Indonesian environments and characterization of urease produced by bacteria were conducted for further uses for the application of biogrouting.

Materials and Methods

Isolation and Purification. Sample were taken from soils, sands, marine water, and rocks in 3 areas in Indonesia. Soil and rock samples were ground before use. The sample was directly plated by serial dilution onto B4 medium with the composition 3 g/L nutrient broth, 20 g/L urea, 2.12 g/L $NaHCO_3$, 10 g/L NH_4Cl, 4.41 g/L $CaCl_2.2H_2O$, and 15 g/L agar if needed. The plates were incubated at room temperature for 5 days. Colonies of bacteria were picked up and purified using four-way streak method to obtain pure bacterial isolates (Cappuccino & Sherman, 2005).

Observation Crystal Using a Light Microscope. Bacterial colonies with crystalline-forming in the medium were observed after 5 and 10 days of cultivation with a light microscope, Olympus CX41. Photographs were made at the time of observation.

Screening of Urease-producing Bacteria. The screening was carried out by growing the isolates in the urea broth medium added with phenol red as pH indicator for qualitative urease test using the method of Hammes *et al.* (2003b). The reaction was incubated at 30ºC for 3 days. Bacterial isolates which urease activity will raise the pH of the medium to alkaline environment and changes the color of the medium from yellow (negative) to fuchsia (positive).

Urease Activity. The quantitative test of urease activity conducted in the following method: isolates were grown in a production medium with the composition of 20 g/L yeast extract, 10 g/L NH_4Cl, and 10 µM of $NiCl_2$ in 1 L distilled water, incubated on shaker incubator at 30ºC for 120 hours. Urease activity was measured using the method of

Weatherburn (1967) with modification as follows, Natrium hypochlorite was used in an alkaline solution instead of NaOH. The time of color formation was changed from 20 minutes to 30 minutes. Reactions were carried out in test tubes containing 100 µL of sample, 500 µL of 50 mM urea, and 500 µL of 100 mM KH_2PO_4 buffer (pH 8.0) so that the total volume was 1.1 mL. The reaction mixture was incubated in a water bath with at 37ºC for 30 minutes. This reaction was stopped by transferring 50 µL of the reaction mixture into tubes containing 500 µL solution of phenol-sodium nitroprusside. An alkaline hypochlorite solution 500 µL was added to the tube and incubated at room temperature for 30 minutes. Then the solution was measured the optical density (OD) with a spectrophotometer (Shimadzu UV 160) at a wavelength of 630 nm and compared with a standard curve $(NH_4)_2SO_4$. One unit of enzyme activity is the amount of enzyme required to liberate 1 µmol NH_3 from urea per minute under standard assay.

DNA Extraction. Two-day-old suspension of bacterial colonies was added to a microcentrifuge tube then centrifuged at 13.000 rpm for 1 min. The supernatant were decanted and the pellets were resuspended. The total DNA of the bacteria was extracted using a Genomic DNA mini kit (Geneaid Biotech Ltd.). The DNA was then stored at -20ºC.

Amplification of 16S rRNA Gene and DNA Sequencing. One µL of DNA template was mixed with 49 µL PCR solution containing 20 µL of ddH_2O, 25 µL of buffer ready (PCR) mix (KAPA), 2 µL of 20 pmol primer 9F, 2 µL of 20 pmol primer 1541R in total final reaction volume of 50 µL. Amplification of 16S rRNA genes was carried out using the universal primer 9F (5'-GAGTTTGATCCTGGCTCAG-3') and primer 1541R (5'-AAGGAGGTGATCCAGCC-3'). The PCR (Applied Biosystems, USA) reaction conditions were the initial denaturation step at 96ºC for 5 min, followed by 30 cycles of 96ºC for 30 s, 55ºC for 30 s, 72ºC for 1 min, and the final extension step at 72ºC for 7 min. The PCR product was purified using a Gel/PCR or DNA fragments extraction kit (Geneaid Biotech Ltd.). Sequencing of 16S rRNA gene was analyzed by sending the PCR product to Geneaid Biotech Ltd. services in Singapore. DNA sequence information from the sequence database was compared to track homology with known strains in GeneBank/DDBJ/EMBL based on BLAST (Altschul *et al.,* 1997).

Preliminary Test of Biogrouting in Sand. To test the feasibility of bacterial-induced carbonate precipitation, a small-scale tray experiment (12×8×12,5 cm^3) was conducted. A bacterial suspension was grown to late exponential phase to a final optical density of 2,880 (OD_{600}). A 270 mL of diluted bacterial suspension was injected into the sand core and then immediately followed by injection of 270 mL of fixation fluid contains 50 mM $CaCl_2$. The cementation fluid contains 1 M $CaCl_2$ and urea was flushed through the tray. After 2 h of reaction time, the cementation fluid was flushed again through the tray. The fluid in the tray was then allowed tp react for 24 h. The next day, the tray was flushed with cementation fluid again (Harkes *et al.,* 2009). The experiment was observed visually.

Results

Isolation and Screening of Calcium Carbonate Precipitation Bacteria.

Various natural habitats samples, including soil, sand, water, and rock were used to isolate bacterial calcium carbonate precipitation. As described in research the samples have been crushed, serially diluted, and directly grown in isolation medium. Then, the bacterial colonies was observed under the microscope. If the observed colony produced crystal, a single colony was picked up, grown in isolation medium, and purified to obtain a pure culture. As a result, a total of 146 isolates are capable crystalline-forming in the medium (Figure 1).

Screening of bacterial biogrouting was conducted to determine the ability of bacterial isolates to produce urease enzyme. This screening was conducted by growing the 146 isolates in a urease test liquid medium. Screening results for urease qualitative with urea broth showed that 19 isolates showed positive reaction on the urease test. The urease test was used for the selection of the bacteria producing urease enzyme. The hydrolysis of urea by the enzyme urease produces ammonia and carbon dioxide, which increases the pH of the medium and causes the phenol red

indicator in medium become fuchsia. This result showed that only 13% of total isolates that could grow on urea medium (B4) expressed urease activity. The bacteria positive of urease test isolated from Papua are ID10-U001, ID10-U002, ID10-U003, and ID10-U004; from Yogyakarta are ID10-U005 until ID10-U014, and from Sulawesi are ID10-U015 until ID10-U019 (Tables 1, 2, 3). Most of isolates have bacilli shape and Gram-positive, and ID10-U001 has coccus shape.

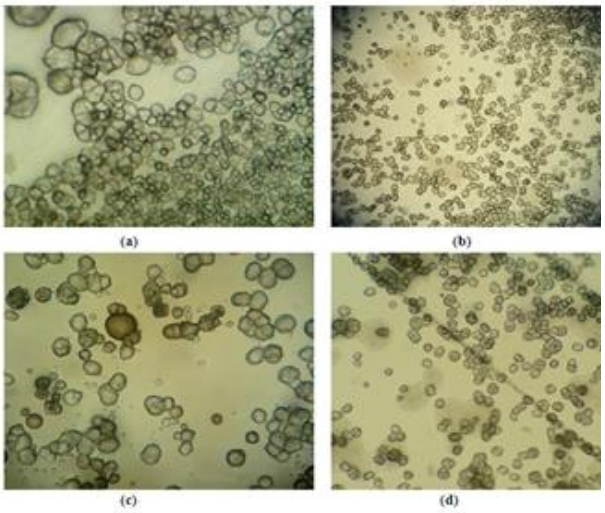

Figure 1. Morphological differences in calcite crystal within bacterial colonies of bacterial carbonate precipitation grown on semisolid medium. The types of crystal, a) spherulite type with fibrous surface texture (ID110-U005), b) rhombohedral type (ID10-U004), c) spherical vaterite type (ID10-U017), and d) trianguler type (ID10-U013) (magnitude, 20×).

Table 1. The number of bacteria with the ability crystalline-forming isolated from Papua

Year	Location	Sample	Number of bacteria
2010	Grasberg	Soil	0
	Grasberg	Soil	0
	Grasberg	Soil	46
	Grasberg	Sands	0
	Grasberg	Sands	19
	Grasberg	Soil	14
	Grasberg	Soil	0
	Total		**79**

Urease Enzyme Activity.

After that, the 19 positive urease test were measured their urease enzyme activity using the method of Weatherburn (1967) with modification. Determination of urease enzyme activity of positive urease test indicated that the isolates had the ability of urease activity (Figure 2). Isolate ID10-U004 has the highest urease activity (374.94 U/mL) (Figure 2). As information, reference strain for biogrouting *Sporosarcina pasteurii* DSMZ 33^T has the urease activity 294.77 U/mL.

Table 2. The number of bacteria with the ability crystalline-forming isolated from Yogyakarta.

Year	Location	Sample	Number of bacteria
	Selarong cave	Rock	0
	Selarong cave	Rock	2
	Parangtritis coast	Sand	1
2010	Parangtritis coast	Sand	10
	Parangtritis coast	Water	9
	Parangtritis coast	Rock	9
	Parangtritis coast	Rock	8
	Total		**39**

Table 3. The number of bacteria with ability crystalline-forming isolated from Southeast Sulawesi.

Year	Location	Sample	Number of bacteria
2010	Batu cave, BNP	Water	0
	Batu cave, BNP	Rock	0
	Batu cave, BNP	Soil	0
	Mimpi cave, BNP	Water	0
	Mimpi cave, BNP	Rock	0
	Mimpi cave, BNP	Soil	0
	Mimpi cave, BNP	Water	0
	Mimpi cave, BNP	Rock	0
	Mimpi cave, BNP	Soil	8
	BNP	Water	0
	Pangkap	Water	0
	Rotterdam castle	Rock	0
	Rotterdam castle	Soil	0
	Lae-Lae island coast	Water	0
	Lae-Lae island coast	Soil	0
	Samalona island coast	Water	14
	Samalona island coast	Soil	0
	Samalona island coast	Rock	0
	Samalona island coast	Soil	0
	Samalona island coast	Rock	6
	Total		**28**

Ammonium was produced variously for each of isolates depending on isolates (Figure 3). Generally, there is a correlation between the enzyme activity and ammonium concentration (Figure 3). In addition, four

isolates from Papua area have higher urease enzyme activity than isolates from another areas. The data of urease activity in terms of U/mg protein have not shown.

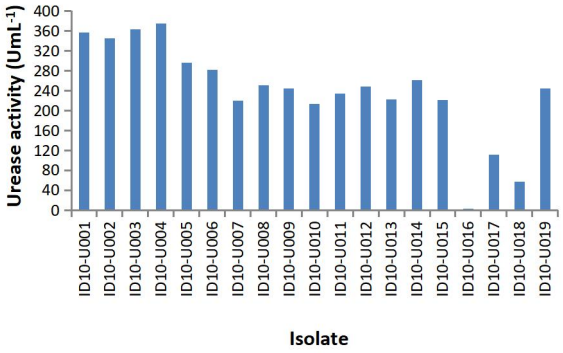

Figure 2. Urease enzyme activity of isolates

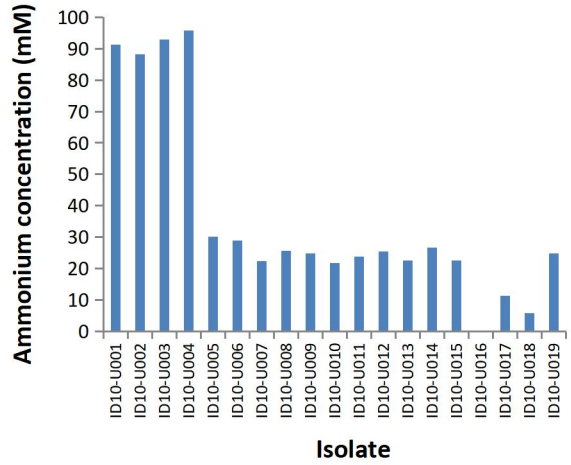

Figure 3. Ammonium concentration of isolates

Molecular Identification of Bacteria.

Almost 1,500 base pairs long of 16S rRNA gene was amplified, purified, and sequenced. All of the 19 bacterial carbonate precipitation were dominated by *Bacillus* genera (8 isolates = 42.1%). Generally, the bacterial carbonate precipitatioon is characterized as an alkalophile bacteria (pH 7-9), bacilli shape, Gram-positive (Lee, 2003). One isolate was identified into the genus *Staphylococcus*, 4 isolates into the genus *Oceanobacillus*, 5 isolates into the genus *Sporosarcina*, and 1 isolate into the genus *Schineria* (Table 4).

Preliminary Test of Biogrouting in Sand.

A small-scale biogrouting experiment for feasibility the capability of the isolate for sand reinforcement have been done. ID10-U004 isolate was used in the experiment through biocementation process. After incubation times for 1 week, the sand particles became compact, strong, and stiff (Figure 4). During reaction, as shown in Figure 5 that the pH was increased for 14-day incubation to near pH 9.

Table 4. Identification of bacterial carbonate precipitation based on 16S rRNA sequencing

Isolat ID	BLAST Similarity with Bacterial strain	Homology (%)
ID10-U001	*Staphylococcus haemolyticus* strain LEH2_2A	98%
ID10-U002	*Oceanobacillus profundus* strain CL-MP28	98%
ID10-U003	*Oceanobacillus* sp. BSi20641	92%
ID10-U004	*Oceanobacillus* sp. R-27401	95%
ID10-U005	*Bacillus pichinotyi* strain RS2	97%
ID10-U006	*Bacillus* sp. strain WCC 4585	96%
ID10-U007	*Bacillus* sp. WB7	98%
ID10-U008	*Sporosarcina* sp. 106	96%
ID10-U009	*Sporosarcina pasteurii* NCCB 48021	97%
ID10-U010	*Schineria* sp. CHNDP40	97%
ID10-U011	*Sporosarcina luteola*	99%
ID10-U012	*Bacillus* sp. VS1	90%
ID10-U013	*Bacillus sp. WB7*	92%
ID10-U014	*Sporosarcina pasteurii* NCCB 4802	98%
ID10-U015	*Sporosarcina soli* strain I80	95%
ID10-U016	*Bacillus* sp. KSM-P358	94%
ID10-U017	*Bacillus lentus* strain UR41	96%
ID10-U018	*Oceanobacillus chironomi* strain T3944D	89%
ID10-U019	*Bacillus lentus* strain NCIMB8773	98%

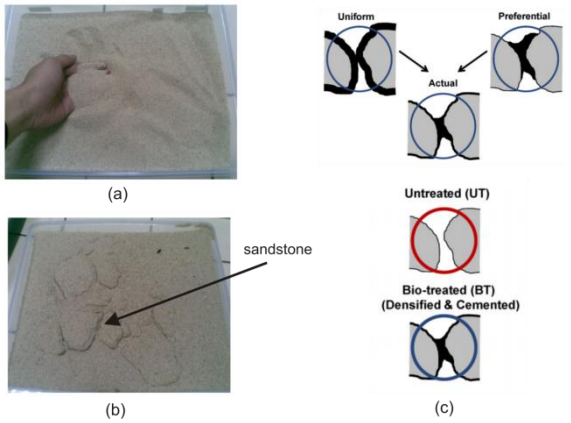

Figure 4. The small scale of biogrouting experiment using ID10-U004 isolate in sands from Pari Island, Indonesia; (a) control negative without adding bacteria and cementation fluid, (b) sand was injected by bacteria and cementation fluid and calcarenite stone resulted by bacteria induce carbonate precipitation activity; and (c) illustration of calcite distribution in biocementation (pick up from DeJong *et al*., 2008).

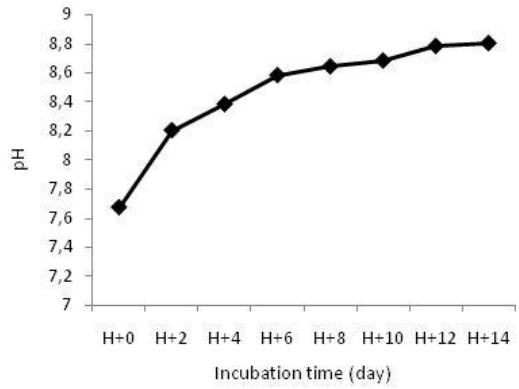

Figure 5. Profile of pH in biogrouting experiment for 14 days.

Discussion

Bacterial-induced carbonate precipitation can be used for several industrial applications, such as restoration of calcareous stone materials (Tiano *et al*., 1999; Castanier *et al*., 2000; Rodriguez-Navarro *et al*., 2003; Joshi *et al*., 2021), bioremediation (Fujita *et al*., 2000; Warren *et al*., 2001; Ivanov *et al*., 2019), wastewater treatment (Hammes *et al*., 2003b), and strengthening of concrete (Ramachandran *et al*., 2001). The advantage of isolation bacterial-induced carbonate precipitation from several areas in Indonesia was to collect isolates producing novel urease activity. Urease produced from these isolates can be used for many purposes.

This study has shown that bacterial-induced carbonate precipitation could be isolated not only from soils, but also from sands, waters, and rocks. We applied urea in isolation medium as a carbon source. When bacteria utilize urea, ammonia is formed during incubation that makes the reaction of these medium alkaline, producing a fuchsia color due to the presence of phenol red, a pH indicator. Aono *et al*. (1999) reported that certain structural component of the cell wall of some alkalophiles, such as teichuronopeptide, may contribute to pH homeostatis at alkaline pH and aid bacteria to survive in alkaline environment. Further, in B4 medium agar, isolates were able to form crystals that is produced by urease enzyme activity (Figure 1).

The urease activity can be measured by phenol hypochlorite. The blue color is a positive reaction of indophenol obtained at high pH of ammonia, phenol and hypocholorite. All 19 isolates produced a significant amount of urease. ID10-U016 isolate has lower urease activity (2.45 U/mL) than others. Bacteria hydrolyze urea by urease for (1) increasing the ambient pH (Burne & Marquis, 2000), (2) utilizing it as a nitrogen source (Burne & Chen, 2001), and (3) using it as a source of energy. Kantzas *et al.* (1992) reported that urease from *Bacillus pasteurii* (*S. pasteurii*) detected in the culture medium was extracelluler and offered an option of using the urease rather than the whole cell to consolidate sand with $CaCO_3$. The subsequent increase of pH in the surrounding medium due to the presence of ammonia ions and the additional release of CO_2 from the enzymatic urea hydrolysis further accelerate the rate of the urease-induced carbonate precipitation.

According to the results, ID10-U004 isolate have high urease activity that is 374.94 U/mL. We have not measured the activity every day, but we harvest the urease enzyme on maximum activity (120 hours) then it measured. Achal and Pan (2011) reported that the maximum urease enzyme activity was shown during the initial phase of bacterial growth and decreased when cells showed maximum growth, followed by slight increase in its activity when growth further declined. They used three isolates that are *Bacillus* sp. AP4, *B. megaterium* AP6 and *B. simplex* AP9, which showed maximum urease productivity at 120 hours for 534 U/mL, 553 U/mL, and 493 U/mL, respectively. The urease activity of ID10-U004 isolate was 294.77 U/mL at 120 h.

The present study showed a possibility that bacteria-induced carbonate precipitation isolated in this study can be exploited as a biologically induced mineralization. Bacteria-induced precipitation of calcium carbonate have been established tool for the *in situ* restoration of building and monuments (Castanier *et al.,* 1999; Stocks-Fisher *et al.,* 1999). Techniques to change soil properties by stimulating natural biochemical processes *in situ* are developed by many researchers (Whiffin *et al.*, 2005; van Meurs *et al.*, 2006; Ivanov & Chu, 2008). In this preliminary study, we used one isolate that is ID10-U004, which showed high urease enzyme activity as a model of biogrouting (biocementation) in the small scale level using a tray for the biogrouting experiment (Figure 4).

Visually, the results indicated that the surface of sand injected by bacteria and cementation fluid (1 M $CaCl_2$ and urea)

became more compact, strong, and stiff compared with the control negative (Figure 4). Calcite material from precipitate of $CaCO_3$ showed in white colour. However, the mechanical soil analysis of the study is needed. Putri *et al.* (2019) reported that by applying urease enzyme in sand, there is a rise in the cohesion value of the sand due to the addition of the clay mixture. A'la *et al*. (2020) also reported that the increase in shear strength of soil due to the addition of clay and the biocementation process of the urease enzyme.

Bacteria-induced carbonate precipitation are able to improve the mechanical properties of porous materials as reported by Nemati and Voordouw (2003) and DeJong *et al* (2006). In most studies, calcium carbonate precipitation was induced by the hydrolysis of urea in a solution with calcium chloride. Purified urease enzymes or whole bacterial cells containing the enzyme in high concentrations, were used to catalyze the hydrolysis of urea and produce ammonium and carbonate ions as stated in Reaction 1.

$$CO(NH_2)_2 + 2H_2O \rightarrow 2\ NH_4^+ + CO_3^{2-} \quad (1)$$

In the presence of dissolved calcium ions, the produced carbonate ions will precipitate and form calcium carbonate ions (Reaction 2).

$$Ca^{2+} + CO_3^{2-} \rightarrow CaCO_3\downarrow \quad (2)$$

When these crystals form bridges between the existing sand grains, they prevent movement of the grains and improve the materials's strength and stiffness of the properties (Ramakrishnan *et al.*, 2000; Lee, 2003; Whiffin, 2004; van Paassen, 2009).

In this study, the biocementation reaction showed that the pH was increased for 14-day incubation to near pH 9. The pH had a significant biochemical effect on urease activity, which exhibited an optimum activity on pH between 7 and 9. One of the roles of urease plays for *Sporosarcina pasteurii* (reference isolat) is to increase the external pH to 9.25 thus creating an environment conductive to growth (Wiley & Stokes, 1963).

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All the authors are the main contributor in this manuscript, who contributes for isolation, identification, characterization, and preservation the isolates, and writing the manuscript.

References

Achal, V., & Pan, X. (2011). Characterization of urease and carbonic anhydrase producing bacteria and their role in calcite precipitation. *Current Microbiology, 62*(3), 894-902. DOI: 10.1007/s00284-010-9801-4.

A'la, H., Rahayu, W., & Lisdiyanti, P. (2020, February). Effect of increasing urease enzim concentration on shear strength properties sand clay biocementation. *IOP Conference Series: Earth and Environmental Science, 426*(1), 012029. IOP Publishing.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research, 25*(17), 3389-3402.

Aono, R., Ito, M., & Machida, T. (1999). Contribution of the cell wall component teichuronopeptide to pH homeostasis and alkaliphily in the alkaliphile Bacillus lentus C-125. *Journal of Bacteriology, 181*(21), 6600-6606.

Boquet, E., Boronat, A., & Ramos-Cormenzana, A. (1973). Production of calcite (calcium carbonate) crystals by soil bacteria is a general phenomenon. *Nature, 246*(5434), 527-529.

Burne, R. A., & Chen, Y. Y. M. (2000). Bacterial ureases in infectious diseases. *Microbes and Infection, 2*(5), 533-542.

Burne, R. A., & Marquis, R. E. (2000). Alkali production by oral bacteria and protection against dental caries. *FEMS Microbiology Letters, 193*(1), 1-6.

Cappuccino, J. G. & Sherman, N. (2005). *Microbiology: a Laboratory Manual*, 2nd ed. The Benjamin Cummings Publishing Company, Inc. USA.

Castanier, S., Le Métayer-Levrel, G., & Perthuisot, J. P. (1999). Ca-carbonates precipitation and limestone genesis—the microbiogeologist point of view. *Sedimentary Geology, 126*(1-4), 9-23.

Castanier, S., Métayer-Levrel, G. L., Orial, G., Loubière, J. F., & Perthuisot, J. P. (2000). Bacterial carbonatogenesis and applications to preservation and restoration of historic property. In *Of Microbes and Art* (pp. 203-218). Springer, Boston, MA.

DeJong, J. T., Mortensen, B. M., Martinez, B. C., & Nelson, D. C. (2010). Bio-mediated soil improvement. *Ecological Engineering, 36*(2), 197-210.

DeJong, J. T., Fritzges, M. B., & Nüsslein, K. (2006). Microbially induced cementation to control sand response to undrained shear. *Journal of Geotechnical and Geoenvironmental Engineering, 132*(11), 1381-1392.

Friedrich, B. & Magasanik, B. (1977). Urease of *Klebsiella aerogenes*: control of its synthesis by glutamine synthetase. *Journal of Bacteriology, 131*(2), 446-452.

Fujita, Y., Ferris, F. G., Lawson, R. D., Colwell, F. S., & Smith, R. W. (2000). Subscribed content calcium carbonate precipitation by ureolytic subsurface bacteria. *Geomicrobiology Journal, 17*(4), 305-318.

Hammes, F., Boon, N., Clement, G., de Villiers, J., Siciliano, S. D., & Verstraete, W. (2003a). Molecular, biochemical and ecological characterisation of a bio-catalytic calcification reactor. *Applied Microbiology and Biotechnology, 62*(2), 191-201.

Hammes, F., Boon, N., de Villiers, J., Verstraete, W., & Siciliano, S. D. (2003). Strain-specific ureolytic microbial calcium carbonate precipitation. *Applied and Environmental Microbiology, 69*(8), 4901-4909.

Hammes, F. (2002). Key roles of pH and calcium metabolism in microbial carbonate precipitation. *Reviews in Environmental Science and Biotechnology, 1*(1), 3-7.

Harkes, M. P., Van Paassen, L. A., Booster, J. L., Whiffin, V. S., & van Loosdrecht, M. C. (2010). Fixation and distribution of bacterial activity in sand to induce carbonate precipitation for ground reinforcement. *Ecological Engineering, 36*(2), 112-117.

Ivanov, V., & Chu, J. (2008). Applications of microorganisms to geotechnical engineering for bioclogging and biocementation of soil in situ. *Reviews in Environmental Science and Bio/Technology, 7*(2), 139-153.

Ivanov, V., Stabnikov, V., & Kawasaki, S. (2019). Ecofriendly calcium phosphate and calcium bicarbonate biogrouts. *Journal of Cleaner Production, 218*, 328-334.

Joshi, S., Goyal, S., & Reddy, M. S. (2021). Bio-consolidation of cracks with fly ash amended biogrouting in concrete structures. *Construction and Building Materials, 300*, 124044.

Kaltwasser, H., Krämer, J., & Conger, W. R. (1972). Control of urease formation in certain aerobic bacteria. *Archiv für Mikrobiologie, 81*(2), 178-196.

Kantzas, A., Stehmeier, L., Marentette, D. F., Ferris, F. G., Jha, K. N., & Maurits, F. M. (1992, June). A novel method of sand consolidation through bacteriogenic mineral plugging. *Proceeding of the CIM Annual Technical Conference, 2*, 1-15. Petroleum Society of CIM, Calgary, Canada.

Lee, Y. N. (2003). Calcite production by Bacillus amyloliquefaciens CMB01. *Journal of Microbiology, 41*(4), 345-348.

Mobley, H. L., Island, M. D., & Hausinger, R. P. (1995). Molecular biology of microbial ureases. *Microbiological Reviews, 59*(3), 451-480.

Nemati, M., & Voordouw, G. (2003). Modification of porous media permeability, using calcium carbonate produced enzymatically *in situ*. *Enzyme and Microbial Technology, 33*(5), 635-642.

Putri, A. R., Rahayu, W., & Lisdiyanti, P. (2019). Effect of urease enzyme and clay mixture in shear strength properties of sand. *MATEC Web of Conferences, 280*, 04014). EDP Sciences.

Ramachandran, S. K., Ramakrishnan, V., & Bang, S. S. (2001). Remediation of concrete using micro-organisms. *ACI Materials Journal-American Concrete Institute, 98*(1), 3-9.

Santhosh, K. R., Ramakrishnan, V., Duke, E. F., & Bang, S. S. (2000). SEM investigation of microbial calcite precipitation in cement. *Proceedings of the International Conference on Cement Microscopy, 22*, 293-305.

Rodriguez-Navarro, C., Rodriguez-Gallego, M., Ben Chekroun, K., & Gonzalez-Munoz, M. T. (2003). Conservation of ornamental stone by Myxococcus xanthus-induced carbonate biomineralization. *Applied and Environmental Microbiology, 69*(4), 2182-2193.

Stocks-Fischer, S., Galinat, J. K., & Bang, S. S. (1999). Microbiological precipitation of $CaCO_3$. *Soil Biology and Biochemistry, 31*(11), 1563-1571.

Tiano, P., Biagiotti, L., & Mastromei, G. (1999). Bacterial bio-mediated calcite precipitation for monumental stones conservation: methods of evaluation. *Journal of Microbiological Methods, 36*(1-2), 139-145.

van Meurs, G., van der Zon, W., Lambert, J., van Ree, D., Whiffin, V., & Molendijk, W. (2006). The challenge to adapt soil properties. In 5th ICEG Environmental Geotechnics: Opportunities, Challenges and Responsibilities for Environmental Geotechnics: Proceedings of the ISSMGE's fifth international congress organized by the Geoenvironmental Research Centre, Cardiff University and held at Cardiff City Hall on 26-30th June 2006 (pp. 1192-1199). Thomas Telford Publishing.

Van Paassen, L. A. (2009). *Biogrout, ground improvement by microbial induced carbonate precipitation*. Delft (NED): Delft University.

Warren, L. A., Maurice, P. A., Parmar, N., & Ferris, F. G. (2001). Microbially mediated calcium carbonate precipitation: implications for interpreting calcite precipitation and for solid-phase capture of inorganic contaminants. *Geomicrobiology Journal, 18*(1), 93-115.

Weatherburn, M. W. (1967). Phenol-hypochlorite reaction for determination of ammonia. *Analytical Chemistry, 39*(8), 971-974.

Whiffin, V. S. (2005). Biogrout and Biosealing, Pore-space Engineering with Bacteria. *Geo-Strata-Bio-Geo: In-situ Processes*, 13-16.

Whiffin, V. S. (2004). *Microbial $CaCO_3$ precipitation for the production of biocement* (Doctoral dissertation, Murdoch University).

Wiley, W. R., & Stokes, J. L. (1963). Effect of pH and ammonium ions on the permeability of Bacillus pasteurii. *Journal of Bacteriology, 86*(6), 1152-1156.

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Abstract. Abstract should be a brief summary of the paper and should present the most important results and conclusions in no more than 200 words. Abstract should be written in English and in Indonesian.

Keywords. Three to five, should be supplied after the abstract.

Abbreviation and Symbol. Abbreviation and symbol should be defined at first mention in the abstract and again in the main body of the text and used consistently thereafter.

Introduction. The Introduction is a short section in which the authors should state the reasons for the performing the work with brief reference to relevant previous work.

Material and Methods. The Materials and methods should be detailed enough for readers to reproduce the experiment.

Results. The Results section should be presented as concisely as possible, where appropriate in the form of tables or figures. Full details of replication of results and statistical significance of differences claimed should be given.

Discussion. The discussion section should deal with the significance of the results and their relationship to the object of the work. Comparison with relevant published work should be made and conclusions drawn.

Citations in the text (refer to APA(American Psychological Association) Style)

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Journal article:

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Brownlie, D. (2007). Toward effective poster presentations: An annotated bibliography. *European Journal of Marketing, 41*, 1245-1283. doi:10.1108/03090560710821161.

Giraffa, G., Chanishvili, N., & Widyastuti, Y. (2010). Importance of lactobacilli in food and feed biotechnology. *Research in microbiology*, *161*(6), 480-487.

Book

De Rond, M., & de Rond, M. (2003). *Strategic alliances as social facts: Business, biotechnology, and intellectual history*. Cambridge, UK: Cambridge University Press.

Chapter in a book

Kersters, K., Lisdiyanti, P., Komagata, K., & Swings, J. (2006). The family *Acetobacteraceae*: the genera *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter*, *Gluconobacter*, and *Kozakia*. *The Prokaryotes: Volume 5: Proteobacteria: Alpha and Beta Subclasses*, 163-200. Berlin: Springer.

Proceedings

Margawati, E. T., Indriawati, Subandriyo, Fullard, K., & Raadsma, H. W. (2009). Detection of Quantitative Trait Loci. QTL) Affecting Carcass Traits in Backcross Family of Indonesian Thin Tail Sheep. In *Proceedings of the 4th Indonesian Biotechnology Conference* (pp. 565-575). ISBN: 978-979-799-401-3.

Thesis/Dissertation:

Young, P. R. (2004). *Molecular analyses of candidate carotenoid biosynthetic genes in Vitis vinifera L* (Doctoral dissertation, Stellenbosch: Stellenbosch University).

Website:

Jürgens, R. (2005). *HIV/AIDS and HCV in Prisons: A Select Annotated Bibliography*. Retrieved from http://www.hc-sc.gc.ca/ahc-asc/alt_formats/hpb-dgps/pdf/intactiv/hiv-vih-aids-sida-prison-carceral_e.pdf.

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