16. Molecular and Cellular Life Sciences: Infectious Diseases, Biochemistry and Structural Biology

by Sutini Sutini

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Molecular and Cellular Life Sciences:
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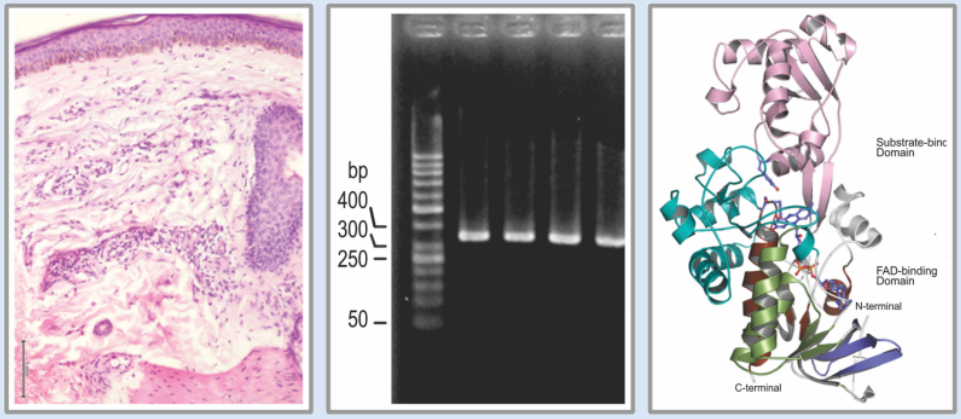

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Prof. Dr. Ni Nyoman Tri Puspaningsih
Prof. Dr. Maria Inge Lusida
Dr. Juniastuti
Dr. Mirny Lamid
Dr. Purkan
Ali Rohman

Molecular and Cellular Life Sciences (MCLS) Conference
Surabaya, 7 – 8 May 2015

Proceeding

Molecular and Cellular Life Sciences:
Infectious Diseases, Biochemistry and Structural Biology

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4
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Preface

50
The International Seminar on Molecular and Cellular Life Sciences: Infectious Diseases, Biochemistry & Structural Biology (MCLS 2015) was held in Hotel Pullman Surabaya City Centre, Surabaya, Indonesia, on 7 – 8 May 2015. This seminar was organized by the Institute of Tropical Disease, Universitas Airlangga, Indonesia in a very productive collaboration with the Institute for Protein Research, Osaka University, Japan. The seminar program included plenary lectures, invited lectures, oral presentations, poster exhibition, as well as a welcome visit to the house of the Mayor of Surabaya. In total of 208 scientific participants, 14 of whom are invited speakers, contributed to this conference. During conference, they had very effective shares and discussions in the fields of infectious diseases, biochemistry, and structural biology. MCLS 2015 was indeed in a truly international atmosphere. The participants came from 9 different countries, *i.e*. Australia, Indonesia, Japan, Malaysia, Netherlands, Singapore, Thailand, Taiwan, and Vietnam.

In order to spread the seminar outcomes, I would like to introduce you a conference proceeding (Molecular and Cellular Life Sciences: Infectious Diseases, Biochemistry and Structural Biology, ISBN 978-602-14292-4-2. A total of 62 papers were submitted to MCLS 2015 and each paper was reviewed by three peers. Of these reviewed papers, 32% were selected to be published in this proceeding. My grateful thanks to you all the peer-reviewers, who are Prof. Toshiharu Hase (Osaka University, Japan), Prof. Bauke W. Dijkstra (University of Groningen, Netherlands), Prof. Nicholas E. Dixon (University of Wollongong, Australia), Prof. Genji Kurisu (Osaka University, Japan), Prof. Ni Nyoman Tri Puspaningsih (Universitas Airlangga, Indonesia), Prof. Maria Inge Lusida (Universitas Airlangga, Indonesia), Dr. Juniastuti (Universitas Airlangga, Indonesia), Dr. Mirny Lamid (Universitas Airlangga, Indonesia), Dr. Purkan (Universitas Airlangga, Indonesia), and Ali Rohman (Universitas Airlangga, Indonesia).

On behalf of the organizing committee, in this joyful moment I would like to express my sincere gratitude to Prof. Fasich (Rector of Universitas Airlangga), Prof. Saburo Aimoto (Executive Vice President of Osaka University), Prof. Dr. Nasronudin (Director of the Institute of Tropical Disease, Universitas Airlangga), Prof. Haruki Nakamura (Director of the Institute for Protein Research, Osaka University), and Prof. Toshiharu Hase (the Institute for Protein Research, Osaka University) who facilitated this event to be smoothly taken place. I would also like to thank all the invited speakers for their discussions and sharing. They are Prof. Toshiharu Hase (Osaka University, Japan), Prof. Bauke W. Dijkstra (University of Groningen, Netherlands), Prof. Nicholas E. Dixon (University of Wollongong, Australia), Prof. Genji Kurisu (Osaka University, Japan), Prof. James R. Ketudat Cairns (Suranaree University of Technology, Thailand), Prof. Chun-Jung Chen (National Synchrotron Radiation Research Center, Taiwan), Prof. Bambang Sugiharto (Jember University, Indonesia), Prof. Kiyoshi Kita (University of Tokyo, Japan), Prof. Eiji Konishi (Mahidol University, Thailand and Osaka University, Japan), Prof. Atsushi Nakagawa (Osaka University, Japan), Prof. Robert C. Robinson (The Agency for Science, Technology and Research (A*STAR), Singapore), Prof. Hitoshi Sakakibara (Nagoya University, Japan), Prof. Ni Nyoman Tri Puspaningsih (Universitas Airlangga, Indonesia), and Prof. Maria Inge Lusida (Universitas Airlangga, Indonesia). Moreover, I wish to convey my sincere appreciation to the Mayor of Surabaya Madam Dr. (H.C.) Tri Rismaharini for inviting our distinguished guests to a warm welcome dinner in her official residence. An event such this conference requires a lot of work from many people. Therefore, I take this opportunity to thank to the Organizing Committee of MCLS 2015 and all people who supported this conference in various ways.

Last but not least, we hope that this proceeding provides a valuable contribution for the development of science and technology, especially in the area of infectious diseases, biochemistry, and structural biology.

Sincerely yours,

Maria Inge Lusida
Chairperson of the Organizing Committee

Scientific Committee of MCLS 2015

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HIV-1 Mutations Associated to RT Inhibitor Were Identified On Drug Naive and Arv Treated Subjects In Jayapura

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Abstract

Human Immunodeficiency Virus (HIV) type 1 cases in Papua province has categorized as high. The high incidence of HIV can be the result of ineffective antiretroviral (arv) therapy in patients. Ineffectiveness of ARV treatment might be the result of mutation that causes the virus to become resistant to a given arv. The objective of this study was to identify mutations associated with HIV against arv Reverse Transcriptase Inhibitors (RTI) group. The study was applied to 50 plasma samples of both arv naive and arv treated subjects. The 500 μl of each plasma sample was precipitated by centrifugating 14,000 rpm for 1 hour at 4°C. RNA was extracted from the precipitated pellet using membrane filtration technique. Each RNA was converted into cDNA by using One-Step RT-PCR kit and incubated at 50°C for 30 min, and 95°C for 15 minutes. Amplification of DNA fragment encoding Reverse Transcriptase (RT) was performed using PCR technique with the program 95°C, 4 minutes, 35x (95°C, 1 min; 53°C, 1 min; 72°C, 1 min); 72°C, 7 min. PCR products were purified and then characterized by migration analysis and sequencing. Sequencing results showed 5 samples were identified as mutants, 3 out of 5 were associated to RTI that occurred in subjects who were treated with ARV, 1 polymorphism in drug naive subject samples, and 1 RT inhibitor resistance-associated mutation in drug naive subject.

Keywords: Drug Resistance; HIV-1; Mutation

1. Introduction

The invention of antiretroviral (arv) has increased the expectation of Human Immunodeficiency Virus type 1 (HIV-1) infection eradication. The arv has been given to decrease the viral load in human CD4 cells. That makes arv as the primer choice to treat people living with HIV/AIDS. One of several arv group which is used in therapy program is Reverse Transcriptase Inhibitor (RTI). RTI basically works by inhibiting the activity of Reverse Transcriptase (RT) in viral multiplication. This therapy has been conducted as the first line therapy program in Jayapura. The decreation of HIV replication in blood has to be sustainable so that the patient can experience the immune system recovery. The recovery can be obtained by the patients with good adherence during the arv therapy. The patients' adherence is important to reduce the resistancy level of HIV-1 to the arv, and to prevent the transmission of HIV-1 mutant strain. The arv therapy is given as an arv combination to reduce the possibility of viral drug resistancy. Virus resistancy to arv occur because of the particular point mutation on viral DNA that change the sequence of amino acid of protein[1]. The arv therapy conducted to the patient in Clinic of Care, Support, and Therapy (CST) in Jayapura are the combination of nucleotide analog, Lamivudine (3TC)+Tenofovir Disoproxil Fumarate (TDF), Zidovudine (AZT)+3TC+ nonnucleoside analog Nevirapin (NVP) or Efavirenz (EFV).

Mutation is the alternation of DNA in the gene on chromosome and can be passed down to next generation. The mutation associated to drug resistancy can change the DNA sequence of the targeted pathogen so it will not be recognized by the drug. The mutations associated to NRTI resistancy are M41L, E44A/D, A62/V, K65R, D67N, T69D/G/N, T69 insertions, K70R, L74V, V75IA/M/S/T, Y115F, F116Y, V118I, Q151M, M184V/I,

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L210W, T215F/Y, and K219Q/N/E and mutation associated to NNRTI resistancy is A98G, L100I, K101E/Q, K103N, V106A/I, V108I, V179D/E, Y181C/I, Y188L/C/H, G190A/S/T, P225H, M230L, and P236L[2,3].

Mutations that often occur in people living with HIV/AIDS are selective mutations resulting from 3TC exposure. The mutation altered the methionine at codon position 184 changed into valine (M184V). Such changes lead to the decretion of viral susceptibility to 3TC. The therapy using 3TC also limit the use of arv drugs from the same group in the future. Miller *et al* in 1998 who conducted a study about resistancy of HIV against 3TC showed that mutations that cause HIV resistance to 3TC also trigger HIV resistance to abacavir, emtricitabine, and didanosine[1].

Another research study concerning to mutation associated to resistancy was conducted by Bansode in Karonga District, Malawi on 2011 showed the high level of mutation associated to RTI. There were 15 out of 75 individuals were identified carrying drug resistant HIV variants. The resistances was associated to mutation occur in codon number 118 where valin was changed in to isoleucine to form V118I motive. Another mutation is Y181C and G190A/E, K103K/N, Y181N/Y, V190I/V, dan H221H/Y[2]. Based on drug resistance database Stanford, G190A mutation causes a high level of resistance against EFV.

The database of the Stanford HIV drug resistance showed that resistance to TDF-associated mutations are K65R, Y115F, M41L, K70R, L210W, T215Y/F, T69 insertions, Q151M[4]. Based on research conducted by Wainberg in 1998, K65R motif occurs selectively when the HIV-1 cultured together with various concentration of TDF[5]. Another study done by the Rhee in 2006 showed that the presence of K65R lowering efficacy of TDF approximately 2 times when measured using the test PhenoSense[6].

This study focuses on arv resistance associated mutations identified in the DNA of HIV rt region by referring the nucleotide sequences to previous research data that has been published. HIV resistance testing against RTI was not conducted either in vitro or in vivo. The mutation data of HIV rt obtained from this study was able to describe whether the combination of arv used in CST in Jayapura still effective for this study subject.

2. Method

2.1. Study Subjects

The clinical isolate of this study included the arv naive patients (13 subjects) and the RTI group arv exposed patients (37 subjects).

2.2. Design of PCR primers

The rtHIV-1 gene sequences were collected from genbank. The sequences were: HIV-1 subtype A (AB052995.1), subtype B (AY180905.1), subtype C (AY162225.1), subtype D (AY322187.1), subtype E (AB052995.1), and subtype CRF01_AE (AY167123.1). The collected gene sequences were aligned using clustal W2 EMBL-ABI and primer design was focused on the most conserved region on nucleotide 30 to 700. A pair of primer was designed manually at 1839-2569 in full genome and was confirmed by using BLAST on http://ncbi.nlm.nih.gov.

2.3. HIV-1 RNA extraction

The five hundreds microliter blood plasma was prepared by precipitating the viral through centrifugation at 14,000 rpm, 4°C for 1 hour. Viral RNA was extracted from blood plasma according to QiaAmp Viral RNA Extraction Minikit (Qiagen) with modification in elution step. The elution of RNA was performed by applying preheated AVL buffer.

2.4. Amplification rtHIV-1 region by PCR

Prior to amplification step of rt of HIV-1 region, viral RNA was reverse transcribed using a pair of specific primer which was designed for this research to obtain specific cDNA. Synthesis of cDNA was performed in a total volume 25 μl containing 5xRT-PCR buffer, 10 uM dNTP, 0.5 uM pRTHIVF, 0.5 uM pRTHIVR, 200 ng RNA template, and 1 μl of SSIII-Platinum Taq Polymerase enzyme mix (Invitrogen). The reaction was performed by incubating the tube at 50°C for 30 minutes, and 95°C for 15 minutes to inactivate the SSIII enzyme. PCR amplification from cDNA as the template was performed in a total volume 50 μl containing 25 μl reverse transcription reaction, and 25 μl green PCR ready mix (Thermoscientific). Denaturation step was performed at 95°C followed by 35 cycles of (95°C; 57°C; 72°C). The final elongation was performed at 72°C. PCR product was characterized on agarose gel 1%.

2.5. Mutation analysis

PCR product was purified from agarose gel and sequenced. The sequencing was performed using ABI technology. Sequencing result was analyzed by assaying the homology of the product to the data from genbank. DNA fragment confirmed as rtHIV was proceeded into mutation analaysis using application on http://hivdb.stanford.edu. Every sample identified as mutant associated to resistance was referred back to the peak of chromatogram to assure the mutation.

3. Results

3.1. The rtHIV-1 from isolates were successfully amplified using the designed primer

Among the 50 samples sequenced, 5 samples were identified as mutant (table 1). Mutation M184V and Y115F were identified on 2 samples. Mutan K70R was identified on 1 sample who receive arv combination D4T and EFV.

Table 1 Mutation motive in sample subjects.

Sample code	Sex	Age (years old)	ARV/ Length of Therapy	Mutation Motive	Resistancies status
601229	P	26	TDF, 3TC / 6 months	Y115F, M184V	TDF: intermediate 3TC: high level
601243	P	23	Duvi (AZT+3TC), NPV / 3 months	K70R	AZT:intermediate 3TC: susceptible NPV: susceptible
601250	L	56	Duvi (AZT+3TC), NPV/ 2 years	Y115F, M184V	AZT: susceptible 3TC: high level NPV: susceptible
601253	P	30	-	M41L, D67E	Low level resistancies to ABC, AZT, D4T, DDI, TDF
601264	L	27	-	V106I	Polymorphisme

A pair of primer was manually designed to anneal specifically to DNA fragment encoded for *rt*HIV-1 at nucleotide (nt) 1839-2569 on HIV-1 complete genome. The expected PCR product was 730 base pair (bp). The sequence of primer is Prthivf 5'-TGTACAGAGATGGAAAAGGAAGGGAA-3' and Prthivr 5'-CCTCTAAGGAGTTTACATAATT GCCTTA-3'. The primer was confirmed by aligning the sequence on Basic Local Alignment Search Tool (BLAST) on website http://www.ncbi.nlm.nih.gov.

Mutation motive Y115F was identified on 2 subject samples together with M184V mutant. According to the study conducted by Sarafianos *et al*., on 1999, M184V mutation related to viral resistancy to 3TC. It is associated with the substitution of isoleucine for methionine at position of 184 of HIV-1 RT which results from single base change. The M184I is rapidly replaced by the variant M184I during 3TC therapy[9]. The combination of Y115F mutation with M184V increasing the HIV-1 resistance to 3TC, but still susceptible to AZT and D4T. K70R mutation was identified once in 1 subject sample receiving Duviral and Nevirapine. Duviral is antiretroviral that contains AZT and 3TC. The K70R mutation was associated to intermediate resistance to AZT.

Another mutation also identified in 2 RT inhibitors naive subjects. One of the subject carrying mutation associated to low resistance level to several types of NRTI, but still susceptible to 3TC, FTC, and also NNRTI antiretroviral group. Another naive subject carried nucleotide polymorphisme V106I which is not associated to any drug resistancies.

4. Discussion

HIV-1 replication is prone to error and it causes genetic variation. Viral genetik variation is important to help in escaping from host immune system, and also in distribution of the drug resistance variant. There are 3 stages in viral life cycel where mutation can occur : 1) in dividing infected host cell, the genomic proviral of the virus is copied by DNA replication system of the host cells. 2) at the time viruses are produced by infected cell, viral genomic RNA is made by RNA polymerase of the host cell. 3) when virus infects the host cell, RT converts viral RNA genome into DNA. Approximately, the error level in those process relatively high, about 10^{-4} error each base in 1 replication cycle. The highly variant of HIV-1 sequences occurs not only in individual virus but also in the infected subject. The variation of the viral in the patient showed that one individual can be infected only by 1 virus, this explained that the viral sequences variation is generally occured after the cell is infected. Though the main explaination of high viral diversity is because the amount of the infected cells, and the high rate of infected cell turn over, the mutation that occur during the viral life cycle is the main source of viral variation[3,8].

Reverse Transcriptase (RT) is viral essential component for replication which its activity is an etiological agent of Acquired Immunodeficiency Syndrome, AIDS. RT enzyme consists of 3 catalytic sites essential for converting viral genomic RNA into cDNA. However, RT is a polymerase with the lowest accuracy. Accuracy test conducted on 3 RT recombinant enzymes showed that lowest accuracy level identified on RT from HIV-1, 1 nucleotide per 1700 incorporated nucleotide, while the most accurate RT is from Moloney Leukemia Virus (MLV), 1 nucleotide per 30,000 incorporated[9].

This study described the presence of mutation identified in subject sample. The subject of this study is HIV patient receiving arv and RTI naive patient as well. The arv given to the patient is combination of TDF+3TC+EFV, TDF+3TC+NVP, D4T+TDF, and D4T+EFV. Therapy of infected HIV patients was conducted by giving 2 or 3 arv combination to decrease the mutation rate associated to arv resistancies. Arv contribution to decrease the viral load in patient blod is significantly increase the HIV-1 infected patient's health and quality of life. RT is an ideal target in designing arv. The incomplete reverse transcription step which is facilitated by RT, the preintegration complex will not be formed and the virus will fail to replicate. Good adherence in therapy will help arv to repress the viral load in years, but will not be totally eliminated.[4]

The identification result showed that 5 subjects carried M184I which is subtitution type of mutation. This mutation involve the enhanced differention mechanism in determining the normal dNTP from analogue nucleotide when NRTI-TP incorporated. The RT choose not to incorporate 3TC and FTC by steric hindrance[5,6]. M184V mutation selectively occur in HIV-1 exposed to 3TC. The study conducted by Petrella on 2004 showed that substitution type of mutation occured in cultured wild type HIV-1 as the concentration of 3TC was increased. M184V mutation was also related to several RT function alteration in vitro that altered the replication capacity[7]. M184V mutation decreases the viral susceptibility to ABC 2-3 fold, and more to 3TC in vitro[10,11]. M184V mutation is not related to viral response to ABC significantly[12,13]. However, M184V that arises with another analog thymidine mutant M41L, D67N, K70R, L210W, T215F/Y, and K219Q/E increasing the viral susceptibility to AZT[14].

Mutation in position amino acid 115 was identified in 2 subject samples. Position 115 in RT domain is part of dNTP binding. Mutation occurs in this domain associated to viral resistancies to ABC and TDF, but increasing the RT accuracy. In lentivirus group, amino acid number 115 in RT is tyrosine (Y), whileas in another retroviral is phenylalanine. In HIV-1, Y115F mutation associated to low resistant level to nucleotide analogue. The convertion from Y into F in amino acid 115 causes the deletion of hydroxil group. The RT error rate at Y115F is 1.0×10^{-4}, lower than wild type RT[15].

Motive K70R mutation was identified on 1 subject sample exposed to AZT and 3TC combination and EFV for 3 months. The mutation is thymidine analogue mutant and associated to resistancies to AZT, ABC, d4T, and DDI. AZT is one of the analogue thymidine arv. It works by inhibiting the DNA rt chain formation specifically, leading to the failure of RT synthesis[16]. In a very high dose, AZT is able to inhibit the human cell DNA polymerase in cell binary division stage, but unlike in viral replication system, human cell has the ability in DNA repairing[17]. In this study, it was unknown whether the mutation identified in the subject sample was selected by arv or transmitted from the other patient.

In the RT inhibitor naive subjects, there were 2 HIV-1 infected subject identified as drug resistance related mutant and polymorphism carrier respectively. The mutation associated to drug resistancies were identified was more frequent identified in subject receiving arv. Another mutation, M41L motive known as the result of thymidine analogue arv therapy. It reduces the viral susceptibility to all approved NRTI. On this study, M41L mutant was detected on naive subject following mutant D67E.

Nucleotide sequences on HIV-1 that causes the changes of amino acid may occur naturally on untreated patients. Polymorphic can be identified in whole HIV-1 genome, even in the arv targeted gene. In this study, a V106I polymorphic mutant was identified in one sample subject. This V106I polymorphic mutant is unable to alter the viral RT response to arv. A study conducted by Gatanaga on 2010 revealed that V106I polymorphic mutant combine with V179D might alter the viral susceptibility to EFV and NVP significantly[18].

Mutation associated to resistancies was also identified on an arv untreated non B subtype sample subject. It was assumed that virus carrying mutation associated to resistancies had a lower transmission level compared to the wild type virus. However, the current publication showed that 10.4% out of 2208 HIV-1 infected patients studied on a research conducted by Wensing on 2005 in 19 European countries was infected by more than 1 point mutation mutant. Most of the infection was subtype B originated from Europe and North America. Infected non subtype B HIV-1 infected arrying drug resistant associated mutant was 4.8%[19].

RT-HIV-1 must be able to complete the viral cDNA synthesis so it can be replicated and transmitted. Whileas the NRTI resistant RT has to maintain its ability to incorporate dNTP normally and efficiently by differentiating the normal nucleotide from NRTI. Mutation associated to arv resistancies has been a big challenge on HIV-1 eradication program. These mutants are transmitted and increasing the prevalance of resistant HIV on naive HIV-1 infected patients.

5. Conclusion

HIV-1 mutant associated to arv resistance has been identified in HIV-1 infected subject in Jayapura. However, the treatment by using the same arv combination can repress the HIV-1 multiplication.

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Total Lymphocytes and Basophiles Blood Count in Chicken after Being Infected with *Newcastle Disease* Antigen (Early Study on The Potency of *Curcuma domestica val* as Immunostimulator Candidate)

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Abstract

The aim of this research was to observe total lymphocytes and basophiles in chicken after has been administered with *Curcuma domestica val (Cdv)* and infected with *Newcastle disease Antigen (NDA)*. The research was performed to forty chicken which were divided into four groups. Group 1 received drinking water, group 2 received drinking water + 40% *Cdv* twice a day for 2 weeks, group 3 received drinking water + 50% *Cdv* and group 4 received drinking water + 60% *Cdv*. They were infected with *NDA* after 2 weeks and total lymphocytes and blood basophiles were counted. The research showed that 40% *Cdv* increased total lymphocytes and total basophiles.

Keywords: Lymphocytes; basophiles; Newcastle disease; immunostimulator; *Curcuma domestica*

Nomenclature

NDA	Newcastle Disease Antigen
Cdv	*Curcuma domestica val*

1. Introduction

Newcastle Disease (ND) is an acute and easily transmitted respiratory disease. The disease is caused by a virus that attacks poultry, especially chickens, and causes significant loss to poultry economy. This is because its morbidity and mortality rates are high. Up to 100% of ND infects poultry farms[2,3].

ND disease outbreaks often occur in groups of chickens that do not have immunity or a group that has low immunity due to late vaccination or because of the failure of the vaccination program. In other countries, such as in China, poultry farms have already applied immunomodulator prior to vaccination. It is intended to be able to raise or increase the immune response prior to vaccination because it is expected to provide immunity against diseases like ND.

Immunomodulator is a compound that can increase the body's defense mechanisms both specific and non-specific. Compounds that have a fairly good prospect to increase the activity of the immune system are usually of the flavonoid, curcumin, limonoids, vitamin C, vitamin E (tocopherol) and catechins. Results of in vitro test of the flavonoid class of flavones and flavonols have been shown to increase immune response[5]. One of the

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medicinal plants that can be used as an immunomodulator is turmeric. Several studies on the content of bioactive compounds in turmeric plant showed that turmeric contains curcumin compounds, demetoxykurkumin, bisdemetoxykurkumin and essential oils. This is supported by Napirah *et al*[8]. who found that the administration of turmeric powder in quail broilers feed showed immunomodulator activity ie as reflected in the increase of total leukocyte count, neutrophyl, lymphocytes, basophils and monocytes percentage.

Assessment of turmeric use as an immunomodulator in broilers in Indonesia is remains rare. Therefore, more studies regarding the application of turmeric as an immunomodulator are needed. This aim of this research was to prove the effect of turmeric on the number of basophils in broilers infected with Newcastle Disease (ND) antigen that causes Newcastle Disease (ND).

2. Methods

This research used experimental animals comprising 40 Day Old Chick (DOC) broiler obtained from PT. Charoen Pokphan. Materials used in this study were turmeric juice, and Newcastle Disease (ND) antigen (derived from PUSVETMA, Surabaya), 10% Giemsa solution, methanol, water, and commercial broiler chicken feed. Equipments used in the study included henhouse, food and drink container, 1 ml syringe, EDTA tube, glass objects, capillary pipette, and a light microscope for observation.

2.1. Sampling Techniques

Blood sampling was carried out once the DOC arrived. As many as four samples of blood were taken, and the volume taken from each sample was maximally 1 ml. DOC blood sampling was done through wing veins, and the blood was collected in EDTA tubes to prevent blood clotting. After rearing for seven days, chicken blood sample was taken again through the brachial vein and then the blood was collected in EDTA tubes to prevent blood clotting. Three days after being infected with Newcastle Disease (ND) antigen, chicken blood sample was taken again through the brachial vein and then the blood was collected in EDTA tubes to prevent blood clotting. Basophil counting was done in the Laboratory of Microbiology, Faculty of Veterinary Medicine, Wijaya Kusuma Surabaya University.

2.2. Research Procedure

Forty samples, two-week old chicken, were placed in experimental unit with ad libitum supply of food and water. The chicken were randomly divided into four groups, each of 10 chicken (n = 10). Group 1 (P0) served as control group, receiving drinking water without *Curcuma domestica val*. Group 2 (P1), group 3 (P2), and group 4 (P3) served as treatment groups I, II, and III. These groups received drinking water of respectively 40%, 50% and 60% *Curcuma domestica val* twice a day for 2 weeks and, thereafter, they were infected with Newcastle disease virus.

The drinking water was given with turmeric extract once daily in the morning and after that the chicken were given with regular drinking water. The research was carried out until the chicken were about 2 weeks old. Exposure to Newcastle Disease (ND) antigen was done intramuscularly or through drinking water to the three-day old chicken treatment group. Blood sampling was performed 3 times, first when the chicken (DOC) arrived, 7 days after the administration of turmeric juice in drinking water, and 3 days after the chicken were infected with Newcastle Disease (ND) antigen. Furthermore, the blood sample was taken to the laboratory to quantify basophil count.

Basophil counting was performed using blood smear preparation and stained with 10% Giemsa for 30 minutes. The blood sample was mixed homogeneously before it was taken with capillary pipette. Then, a small drop of blood was placed near the end of the object glass at the flat surface. The second object glass was placed with the tip touching the glass surface of the first objects glass forming an angle of 30-45°. The second object glass was pulled aside and allowed the blood to flow by capillary power to achieve the first 2/3 object area. Both object glasses were driven by the same angle so as to form a thin layer. Blood smear preparations were left to dry in open air.

Blood smear preparations were fixed with methyl alcohol for 3-5 minutes. Preparations were taken and allowed to dry in the air. Once dried, the preparations were soaked with Giemsa dye for 15-60 minutes. The mixture was washed with water several times and allowed to dry in a rack. Calculation of basophils percentage was conducted with an objective magnification of 100x, basophil classification was done in some fields of view and counted per 100 leukocytes. At the end of the experiment, the blood was collected intrabrachially, and the lymphocytes and basophiles were counted.

3. Result and discussion

After performing research on the effect of turmeric juice (*Curcuma domestica Val.)* on the number of basophils in broilers infected with Newcastle Disease (ND) antigen, the result can be seen in some of the following tables and will be further described in the section below.

3.1. Total Lymphocytes

Table 1 Average and Standard Deviation (X ± St. D) of Total Lymphocyte (/mm^3)

Treatment	Aged	X ± St. D
P0	0	$(42150.40 \pm 8545.57)^{a,a}$
	7	$(81548.80 \pm 25762.71)^{a,b}$
	14	$(41395.20 \pm 21766.75)^{a,a}$
P1	0	$(43699.20 \pm 9426.64)^{a,a}$
	7	$(45862.40 \pm 16360.71)^{a,b}$
	14	$(44005.60 \pm 16360.71)^{a,a}$
P2	0	$(42150.40 \pm 8545.57)^{a,a}$
	7	$(57212.00 \pm 20371.10)^{a,b}$
	14	$(27168.00 \pm 3857.03)^{a,a}$
P3	0	$(43699.20 \pm 9426.64)^{a,a}$
	7	$(67827.20 \pm 25163.09)^{a,b}$
	14	$(36729.60 \pm 4120.95)^{a,a}$

Description : The same superscript letters in the same column states non-significant difference at the level of $p \geq 0.05$

Table 1 shows that the average lymphocyte count in each treatment group did not differ significantly. The average lymphocyte count in age 0 day (DOC) ranges $(42150.40 \pm 8545.57)^{a,a}$ - $(43699.20 \pm 9426.64)^{a,a}$/mm^3. The average value of lymphocyte count in age 7 days $(45862.40 \pm 16360.71)^{a,b}$-$(81548.80 \pm 25762.71)^{a,b}$/mm^3 and the average value of highest lymphocyte count at this age is in treatment group P0 $((81548.80 \pm 25762.71)^{a,b}$/mm$^3)$. On age 14 days or 7 days after infection, the average lymphocyte count ranges $(27168.00 \pm 3857.03)^{a,a}$ - $(44005.60 \pm 16360.71)^{a,a}$/mm^3 and the average value of the lowest lymphocyte count at this age is in treatment group P2 $((27168.00 \pm 3857.03)^{a,a}$/mm^3).

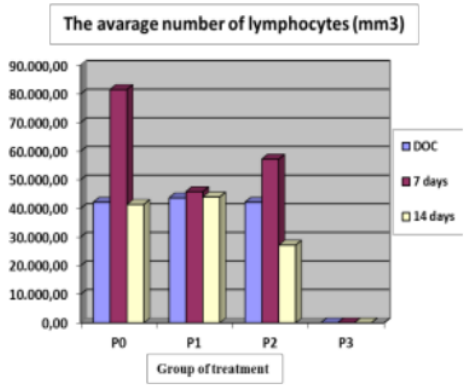

Fig. 1. The average of lymphocyte count (/mm3)

Fig. 1 shows that 7-day old chicken have a decrease in average lymphocytes count in the treatment group continued to increase at P1, P2 and P3 treatment groups. Chicken aged 14 days or 7 days after infection shows a decrease in the average lymphocytes count in treatment group P2.

3.2. Total Basophiles

Table 2. Average and Standard Deviation (X ± St. D) Total Basophiles (/mm^3)

Treatment	Aged	X ± St. D
P0	0	$(0.00 \pm 0.00)^{ab,a}$
	7	$(0.00 \pm 0.00)^{ab,a}$
	14	$(204.80 \pm 280.43)^{ab,b}$
P1	0	$(0.00 \pm 0.00)^{b,a}$
	7	$(0.00 \pm 0.00)^{b,a}$
	14	$(1616.80 \pm 2154.45)^{b,a}$
P2	0	$(0.00 \pm 0.00)^{ab,a}$
	7	$(0.00 \pm 0.00)^{ab,a}$
	14	$(204.80 \pm 311.57)^{ab,b}$
P3	0	$(0.00 \pm 0.00)^{a,a}$
	7	$(0.00 \pm 0.00)^{a,a}$
	14	$(0.00 \pm 0.00)^{a,b}$

Description: The same superscript letters in the same column states non-significant difference at the level of $p \geq 0.05$

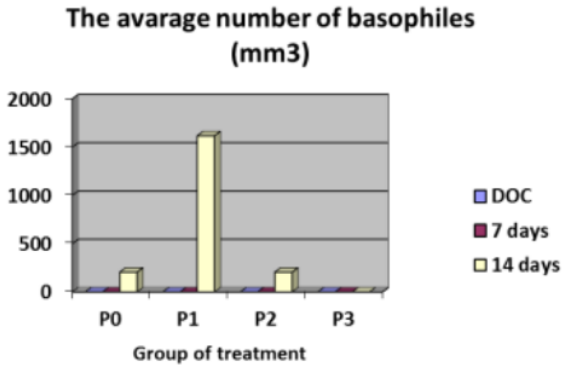

Fig. 2. The average basophile count (/mm^3)

Table 2 and Fig. 2 show that the basophils counts did not differ significantly, only in treatment group P1 aged 14 days or 7 days after infection shows significant increase in average basophil count. The average value of the number of basophils P1 at the age of 14 days or 7 days after infection ranges $(0.00 \pm 0.00)^{a,b}$ - $(1616.80 \pm 2154.45)^{b,a}$/mm^3 and the average value of the highest number of basophils at this age is in the treatment group P1 ($(1616.80 \pm 2154.45)^{b,a}$/mm^3).

At the age of 7 days (P0 : $(81548.80 \pm 25762.71)^{a,b}$/mm^3) the increase of average lymphocyte count was very significant compared to those in other treatment groups. Such improvement can be caused by a bacterial infection, where in treatment group there were a few chickens that died due to a bacterial infection, which was thought to have come from drinking water sources. According to Jackson[6], the increase in leukocytes and lymphocyte count in poultry or birds can be caused by inflammation (infectious or noninfectious), poisoning, bleeding in body cavity, fast-growing neoplasms, and leukemia.

At the age of 14 days or 7 days (P2: $(27168.00 \pm 3857.03)^{a,a}$/mm^3) after infection there was decrease in the average lymphocyte count as compared to those in other treatment groups. The decline may be caused by one of the curcumin compound in turmeric, the bisdesmetoxykurkumin. The nature of this compound is antagonistic to the work activity of curcumin and desmetokxykurkumin[1]. According to Kohli[7], one of the functions of turmeric (curcumin) is as an anti-inflammatory that can inhibit lymphocyte secretion into circulation. The inflammation comes from infected ND antigen and Newcastle disease incubation period is averagely 5-6 days[2,3], The provision of turmeric powder in certain dose can reduce lymphocyte count in blood since curcumin in turmeric can stimulate the secretion of glucocorticoid hormones that cause a decrease in lymphocytes count in the circulation[4].

The administration of turmeric juice in multilevel doses (40%, 50%, and 60% turmeric juice) until the age of 14 days had no significant result, but the average lymphocytes count increased compared to normal lymphocyte

count. Normal chicken lymphocyte count is 5.45 - 18.4 x 10^3/mm^3. This is consistent with the function of one of the compounds present in turmeric, in which curcumin, as an immunomodulator, can increase total leukocytes count, including lymphocytes[8].

The results of average basophils count showed that the administration of turmeric juice in graded doses (40%, 50%, and 60% turmeric juice) until the age of 14 days was not significant. The average basophils count in each treatment group ranged from $(0.00 \pm 0.00)^{a,b}$ - $(1616.80 \pm 2154.45)^{b,a}$/$mm^3$. According to Swenson[11], normal basophil count in chickens is 0.36 to 1.32 x 10^3/mm^3. Basophils are rarely found in chicken blood. Less presence of basophils is normal, considering basophils have a very low power of phagocytic and normally there is only very small number of these cells in blood circulation.

Increased average basophil count in the age of 14 or 7 days after infection in group P1 treatment ($(1616.80 \pm 2154.45)^{b,a}$/$mm^3$) is due to inflammation caused by infection with ND antigen. Basophils will usually appear with the release of mediators for bleeding and allergic activity. Basophils play a role in a wide range of allergic reaction and would release heparin into the blood to prevent blood coagulation[10]. Basophils also has a function to evoke acute bleeding at the site of antigen deposit.

4. Conclusion

Provision of a dose of turmeric juice results in 40% increase in average lymphocytes and basophiles counts in chickens aged 14 days or 7 days after ND antigen infection.

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In The Case of An Emphysematous Lung, Could MMP-9 Activity of Gingival Crevicular Fluid Substitute that of The Sputum?

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Abstract

The imbalance between the matrix metalloproteinase (MMP)-9 and the tissue inhibitor of metalloproteinase (TIMP)-1 may serve the proteolysis degradation of extracellular matrix (ECM). MMP-9 has been mainly produced by alveolar macrophages and granulated neutrophils leading to destruction of extracellular matrix (ECM). This study aimed to propose a less invasive relevant biomarker from gingival crevicular fluid (GCF). Fifteen smokers with emphysema underwent the physical assessment, the spirometry, and radiological examination. Both GCF and sputum were collected to measure MMP-9 activity. Results showed that the activity of MMP-9 between GCF and sputum differed insignificantly (p>0.05). The MMP-9 activity of GCF might substitute the one of the sputum among emphysematous respondents and be suggested as a biomarker for emphysema.

Keywords: ECM; GCF; Sputum; MMP-9; Biomarker

Nomenclature

BAL	Broncho-alveolar lavage
COPD	Chronic obstructive pulmonary disease
CT	Computerized tomography
EBC	Expiratory breath condensate
ECM	Extracellular matrix
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
GCF	Gingival crevicular fluid
GOLD	Global initiative for chronic obstructive lung disease
MMP	Matrix metalloproteinase
TIMP	Tissue inhibitor of metalloproteinase

1. Introduction

Smoking has been the major risk factor for the development of the chronic obstructive disease (COPD) among smokers. On the averagem COPD has developed into 20-25% among smokers. The more cigarette consumed is linear with the higher risk occuring among smokers[1]. Smoking also exposes oral cavity including the periodontal tissues[2,3].

Smoking induces inflammatory responses releasing neutrophils, macrophages, lymphocytes as well as pro inflammatory cytokines from epithelial tissues, fibroblast, and cultured macrophage. Prolonged exposure could

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make morphological and physiological changes in the lung parenchyma i.e. the disorder of alveolar septa, the enlargement of terminal bronchioles, the decrease of alveolar elastic recoil and more of other disorders of lung and respiratory tracts[4].

Biomarkers have been developed in order to assess the severity of the disease based on related molecules and agents from either cells or tissues. Cytokines, chemokines, oxidants and proteases are taken as biomarkers to evaluate pathophysiology, inflammation, destruction and lung remodeling in COPD. These biomarkers are commonly taken from bronchial biopsy, bronchoalveolar fluid (BAL), sputum, and expiratory breath condensate (EBC)[5,6].

Emphysema mostly occurs as the process of elastolytic destruction since MMP-9 activity increases and so does MMP-9/TIMP-1 ratio[7,8]. The evaluation for both MMP-9 activity and MMP-9/TIMP-1 ratio has been beneficial to assess the progressivity of emphysema among COPD patients[9]. Those parameters also occur in destructing periodontal tissues[10]. Some studies still engaged MMP-9 activity to assess the recovery of COPD and periodontitis among patients who quit smoking after treatment as MMP-9 activity has shown the elevated and less influenced recovery of diseases[11,12].

Due to patients' similar exposure and final state, this study aimed to associate the correlation between MMP-9 activity in periodontal and the one in lung tissues in order to evaluate the progressivity of emphysema.

2. Methods

The study methods and procedures have been officially approved by The Ethical Committee of Faculty of Medicine, Syiah Kuala University, Banda Aceh, Indonesia. Fifteen respondents were employed in this research. They were outpatients at Pulmonary Department of Zainoel Abidin Hospital, Banda Aceh-Indonesia. They were male, >50 year olds, smokers or former smokers, with 20 pack years and more, and without periodontal pocket. Those with asthmatic, infection of respiratory lower tract and pulmonary malignancies were excluded.

Furthermore, all eligible respondents had to undergo the physical examination, the spirometry test, and CT scan as well. This study showed FEV_1/FVC ratio <70%, FEV_1% predicted <50%. Next, respondents underwent sputum induction with nebulizer-assisted 3% NaCl solution collected into sterile pots. Then, GCF was collected by putting small piece of cut *Whatman®* filter papers (2 mm x 8 mm) into gingival sulcus stored into micro-centrifuge tubes[13]. All samples were preserved into 80°C freezer for further analysis. The activity of MMP-9 from both sputum and GCF was analysed with *Sensolyte® 520 Generic MMP assay kit Fluorimetric*.

3. Results and Discussion

3.1. Characteristics of respondents

Respondents' mean age (SD) was 59.53 (5.579) with 33.40 (6.468) pack years. Spirometry tests showed mean FEV_1/FVC ratio (SD) and FEV_1 predicted i.e. 44.648 (7.356) % and 34.33 (6.377) % respectively. This was in accord with the criteria of COPD based on GOLD[14]. The above data were shown in table 1.

Table 1. Characteristics of respondents based on age and pack year

Variables	N	Mean (SD)
Age (yr)	15	59.53 (5.579)
Pack year	15	33.40 (6.468)
FEV_1/FVC ratio (%)	15	44.648 (7.356)
FEV_1% predicted (%)	15	34.33 (6.377)

3.2. Activity of MMP-9 from GCF and sputum

Mean MMP-9 activity (SD) from GCF and sputum was 0.939 (0.722) µM and 1.653 (1.574) µM respectively. Activity of MMP-9 from sputum was higher than the one from GCF, as shown in table 2.

Table 2. Activity of MMP-9 from GCF and sputum

Variables	N	Mean (SD)
MMP-9 activity (µM) (GCF)	15	0.939 (0.722)
MMP-9 activity (µM) (Sputum)	15	34.33 (1.574)

3.3. Correlation of MMP-9 activity between GCF and sputum

After the data were calculated with paired sample t test, the correlation of MMP-9 activity between GCF and sputum showed insignificant differences (p>0.05) as seen in Table 3.

Table 3. Paired sample t test of MMP-9 activity between GCF and sputum

Variable	n	Mean (SD)	*p* value
MMP-9 activity (μM)	15	0.714 (1.948)	0.177

3.4. Discussion

Mostly, COPD occurs in midyears as conducted in the study including respondents whose mean age (SD) was 59.53 (5.579) years old. Respondents also had more than 20 pack years, i.e. mean (SD) 33.40 (6.468) pack years. They had been diagnosed with COPD as they had worse FEV1/FVC ratio and FEV 1% predicted according to GOLD[14] (Table 1). Smoking cessation among smokers was supposedly difficult to begin until they suffer from clinical signs and symptoms of airway obstruction. This condition was common among public to comply with the widespread smoking warning advertised and promoted. Supports from medical staffs helped smokers with COPD to quit smoking. However, healthy smokers still kept smoking although they had similar supports from medical staffs[15].

Emphysema is caused by destruction of lung parenchyma mainly known by the decrease of FEV_1. The compensatory of upper lobes of lungs might increase the perfusion and ventilation instead of the disorder of lower lobes. The decline of FEV_1 in emphysematous lung occurs since the closure of lower lobe airways is faster during the expiration due to collapsed airways and the extracellular matrix (ECM) degradation. Structures of lower lobes contribute more than those of upper lobes so that the decrease of FEV_1 reflects disorders of lower lobes as emphysema mostly occurs within these parts[16].

Regarding mean MMP-9 activity (SD) (Table 2), respondents had lower MMP-9 activity from GCF than the one from sputum. Lung parenchyma is more susceptible to suffer from degradation than periodontium since smoking might directly expose the lung and the respiratory tract. However, periodontitis would be mainly caused by prolonged exposure of smoking[3]. Age, pack year, and gender have influenced the spirometry and MMP-9 activity among smokers with COPD. MMP-9 activity is regulated tightly by genetic factor and its anti protease. The number of MMP-9 activities is not the key point to cause an emphysema among smokers as individual genetic susceptibility and amount of anti protease maintaining the balance might lead the fate of increasing MMP-9 activity among smokers[17]. The appropriateness of included respondents and matched inclusion criteria should be considered fairly since cigarette consumptions and ages may influence results. The insignificant difference of MMP-9 activity among healthy smokers was also found compared to smokers with COPD. It seems that healthy smokers still accumulate more exposures to keep increased induction of neutrophils within airway leading to the release of MMP-9[18].

Neutrophils, monocytes, and macrophages release MMP-9 from epithelial tissues were found in GCF in which increased amount of MMP-9 had effects in degradation of ECM within periodontium[10]. Smoking habit led to the increasing amount of neutrophils from airway, subsequently followed by increased activity of MMP-9. Neutrophil is well known as a source of MMP, particularly MMP-9[18]. Smoking injured tissues of periodontium and respiratory, and induced neutrophils, macrophages and lymphocytes to release and activate MMP-9. The severity of inflamed tissues was linear with release of MMP-9 into the circulation[17]. Although the study did not focus on periodontitis, ECM degradation has been mostly caused by the increased activity of MMP-9[10].

The imbalance of protease and anti protease, found in sputum, normally increased among COPD respondents. However, the correlation of MMP-9 activity was linear with the number of neutrophils from inflammed airways caused by prolonged exposure of smoking leading to the ECM degradation of lung parenchyma, commonly known as emphysema. Moreover, airway obstruction commonly occurs in small airways and lung parenchyma evaluated with radiological examination and spirometry[9,19,20].

The insignificant difference of MMP-9 activity (p>0.05) (Table 3) between GCF and sputum concluded that MMP-9 activity from GCF would substitute the one from sputum. Although periodontitis and COPD were independently associated with each other, smoking exposure would affect pathogenesis course of either periodontitis or COPD which then will activate various mediators of pro inflammatory mediators, macrophages and neutrophils releasing MMP-9. Active MMP-9 is able to cleave ECM of related tissues, i.e. periodontium and lung parenchyma. Additionally, periodontitis may serve as a risk factor for COPD as bacteria from periodontitis sites might spread rapidly to respiratory tract. Bacteria from those infected sites could lead the exacerbation among COPD patients. However, causality effect of both diseases remained obscure[3,21].

4. Conclusion

This study suggests that the MMP-9 activity from GCF be a useful biomarker to assess progressivity of emphysema. Then, further studies should be conducted in order to determine better proximity between periodontium and lung parenchyma.

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Cloning and Expression of cDNA Encoding Membrane Protein of Tachyzoite of *Toxoplasma gondii* Local Isolate in Bacteriophage

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Abstract

Toxoplasmosis is a disease caused by obligatory intracellular protozoa of *Toxoplasma gondii*. Generally, diagnosis relies on serological methods. However, immu diagnosis might be indistinct in immunocompromised patients. The aims of the research are to clone and to express cDNA encoding membrane protein of *Toxoplasma gondii* for developing molecular diagnosis. Total RNA and messenger RNA were isolated from tachyzoites growing up in Swiss strain mice by using PolyAtract®mRNA Isolation Systems (Promega) and cDNA was synthesized using Riboclone®cDNA Synthesis Systems (Promega). Complementary DNA was ligated with *Eco*RI adaptor using Riboclone®EcoRIAdaptor Systems (Promega). Bacteriophage of λgt11 ligated with cDNA was transfected in *E. coli* Y1090. Recombinant plaque was analyzed using polyclonal antibodies against membrane protein of tachyzoite of *T. gondii*. Over-expression of recombinant was analyzed by dot blot technique in order to identify cDNA recombinant expressing membrane protein tachyzoite of *T. gondii*. Transfection in *E. coli* Y1090 by λgt11 produced 7 clones with cDNA encoding membrane protein of tachyzoite of *T. gondii*.

Keywords: *Toxoplasma gondii*; tachyzoite; membrane protein; bacteriophage; cloning.

1. Introduction

Toxoplasmosis is a parasitic disease caused by *Toxoplasma gondii*. The disease is spread all over the world, and is more common in cold climate regions[1]. In acute infections, the disease is asymptomatic and only shows a slight pain symptom which is usually undiagnosed[2]. An infection in adults shows no clinical symptoms, but it sometimes shows lymphadenitis in cervical region and other regions accompanied by a mild fever[3]. In pregnant women and animals, severe infections can lead to miscarriage or congenital defects in infants born[1,4]. Primary *Toxoplasma gondii* infection in pregnant women in the first trimester results in 20% of infected fetuses and the third trimester of pregnancy, the fetus is infected 65%[5]. In the definitive host, toxoplasmosis with extraintestinal cycle, clinical symptoms are common, but the cycle intraepithelial clinical symptoms are frequently seen, such as anterior uveitis, posterior uveitis, fever, muscle suffered hyperaestesia, weight loss, not eating, breathing disorders, like attacking, and loss of balance[6,7].

Toxoplasmosis causes several clinical manifestations in individuals who suffer from immunosuppression and it can cause defects in infants born from infected mothers during pregnancy. In individuals with AIDS, *Toxoplasma* is an opportunistic parasite that causes a very serious problem[8]. Toxoplasmosis losses cause, either directly or indirectly, huge economic losses. In the United States, some researchers reported that toxoplasmosis in humans had caused economic losses covering medical costs and care for people with mental retardation[9]. The cost of the treatment of infants affected by congenital toxoplasmosis at the average was 67,246 dollars per patient[8]. Other economic losses due to toxoplasmosis are loss of income due to illness, death and reduction in work participation[1].

Toxoplasmosis is a disease that can be transmitted from animals to humans. It requires good disease control strategies which include knowledge of toxoplasmosis, treatment and vaccination of meat before eating. Prevention of toxoplasmosis can be done, for example, by cooking meat at the temperature of minimal 66°C for 20 minutes or by cooling it at the temperature of -20°C for several days. The use of gloves in the meat

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processing and washing hands after meat processing are recommended[6]. Prevention is better than treatment to avoid the risk of toxoplasmosis[10]. *Toxoplasma gondii* vaccines that have been used are in the forms of a live vaccine (tachyzoite), the surface protein[11], and nucleic acids[12]. Constraint on development of live vaccines is the development of the culture and the handling of tachyzoite which are relatively insecure and expensive; while, the surface protein is vaccine used as an antigen supply which is not easy. The use of inactivated vaccine and sub unit vaccine of *Toxoplasma* is one alternative to vaccination. However, the obstacles faced are contaminants, cost prohibitive in the provision, as well as toxic effects when administered in large quantities[13].

Diagnosis of toxoplasmosis is often based on the presence of antibodies against *Toxoplasma*. Humans and animals showing seropositive for *Toxoplasma* is not necessarily suffering from toxoplasmosis, although once infected with *Toxoplasma*. Diagnosis is confirmed by finding cysts on the brain or tissue and the presence of *Toxoplasma* circulating in the form of bradyzoite or tachyzoite[6]. The Presence of antigens in the body can be detected by techniques Enzyme Linked Immunoassay (ELISA)[6], Polymerase Chain Reaction (PCR)[14,15,16], Polymerase Chain Reaction - DNA Enzyme Immunoassay (PCR-DEIA)[17]. PCR diagnostics constraint-DEIA or hybridization tracker techniques provide *Toxoplasma* protein for the production of antibodies and DNA is used as a tracer.

Toxoplasma gondii is an obligate intracellular protozoan that infects several types of nucleated cells. Contacts among others, the receptors on host parasite with an initial entry of the parasite into the host[18]. P30 is a major membrane protein which is a ligand that is essential in *Toxoplasma*[19]. P30 is located on the membrane surface and rhoptri of tachyzoie[20]. Tachyzoite of *Toxoplasma gondii* has five major proteins. The fifth of these proteins is SAG1 (P30) which is the most dominant major protein while SAG3 is the least amount of protein and other proteins are minor third[21].

The development of recombinant DNA technology solves the problem of providing a surface protein that is immunogenic in a large amount. Therefore, the production of the vaccine may be cheaper. Recombinant DNA technology can also provide a solution in providing both nucleic acid used as a vaccine and material for hybridization tracers in the diagnosis of disease. Lambda gt11Vectors which are often used for engineering is a bacteriophage. Bacteriophages are viruses that infect bacteria. One of the bacteriophages that are often used in cloning technology is bacteriophage lambda. Bacteriophage lambda genome is double-stranded DNA with a length of about 50 kb. DNA bacteriophage particles form linear double-stranded molecule with edges which is a single chain complementary to the other end and at the end, 12 nucleotides are in length complementary. After infecting bacteria, complementary ends of both ends of cohesive joining partner base circular form molecules and these molecules are used as a template for the transcription process. The materials integrate with the cell genome and replicate together with the host cell. Bacteriophage lambda does not only replicate and infect bacteria causing lysis, but it can also integrate into the genome and the phenomenon is called lysogenization. Lysogenic *E. coli* bacteria replicate the foreign DNA as the genetic material itself during lytic function. Bacteriophage vector is often chosen because it has a cloning capacity large enough to insert foreign DNA. Genotype selection is easy and it has a lot of side polylinker with restriction[22]. Lambda gt11 is often used in cloning because it has the properties phage easily packaged in vitro and selection because it has a *lacZ* gene. Insertion of *lacZ* gene inactivates the gene encoding the β-galactosidase so it is not capable of changing the substrate X-Gal that plague pale colored transparent. Since there is no insertion of blue plaque. At λgt11, induction *lacZ* fusion protein will be expressed containing β-chain protein that is bound galactosidase on DEAE-cellulose[22].

2. Methods

2.1 Cultivation parasite in vivo

Three mice were injected with tachyzoite of *Toxoplasma gondii* local isolate by intraperitoneally with a dose of $1x10^7$. After 72-96 hours, the mice showed marked clinical symptoms with fur standing, weakness, no appetite and drinking, decreased respiratory rate and quick heart rate. Mice were then sacrified and the abdominal cavity was washed with physiological saline fluid to get tachyzoite. The washing was done three times, each was about 5 ml. Tachyzoite was then infected into 30 adult male mice with a dose of $1x10^7$ to get more tachyzoite in the same way as the previous way. Then, the results of washing abdominal cavity of mice were centrifuged at 3000 rpm (Beckman) at 4°C for 174 minutes. Obtained pellets were washed three times with PBS containing 0.1% diethyl pyrocarbonat (DEPC) by centrifugation at 3000 rpm at 4°C for 10 minutes and then pellets were ready to be used for total RNA preparation.

2.2 *Isolation of RNA*

2.2.1 Total RNA isolation

Some 10^{10} tachyzoites were coupled with 1.5 ml of guanidine thiocyanate and mixed by means of resuspended, then the mixture was transferred into a sterile homogenizer RNase-free tubes. Homogenization was done with the homogenizer 6 times, each with a speed of 1200 rpm for 30 seconds. Homogenization was done in a cold state in order to avoid damage to RNA. The results of homogenization were transferred into RNase-free sterile tube, then it was added with Na-acetate-tenth of the volume and it was mixed until blended. The solution was put in ice for 5 minutes and added with phenol chloroform as much. Mixing was done by flipping and turning the tube and incubated on ice for 15 minutes. Aquaeus phase was obtained by centrifugation with 4000 rpm on 4°C for 25 minutes. Aquaeus phase was transferred into new sterile RNase-free tubes and added with isopropanol (1:1) and mixed with way of flipping through the tube. The solution was then incubated at -20°C overnight. The solution was centrifuged at 4000 rpm at 4°C temperature for 25 minutes. Obtained pellet was washed with cold 75% ethanol and centrifuged at 4000 rpm at 4°C for 25 minutes. The supernatant was then discarded and the pellets were dried until completely dry at room temperature. The next pellet was resuspended with 250 µl RNase-free water.

2.2.2 mRNA isolation

2.2.2.1 Annealing probe

Messenger RNA was isolated using PolyAtract®mRNA Isolation System. Two hundred and fifty microliters of total RNA were coupled with RNase-free water until the volume became 500 µl. The solution was incubated at 65°C for 10 minutes. Immediately after the incubation, the solution was added with 3 µl biotinylated oligo (dT) probe and 13 µl 20XSSC. The solution was mixed and incubated at room temperature for 10 minutes.

2.2.2.2 Washing streptavidin-paramagnetic particles (SA-PMPs)

Streptavidin-paramagnetic particles (SA-PMPs) were resuspended to solution, then they were captured by placing a magnetic stand for 30 seconds until the SA-PMPs collected on the wall (the solution becomes clear). Streptavidin-paramagnetic particles were washed three times with 0.5x SSC (each wash as much as 0.3 ml) in a manner as was done previously and then were resuspended in 0.1 ml of 0.5x SSC.

2.2.2.3 Arresting and washing hybrid mRNA with oligo (dT)

The reaction solution annealing was inserted into the tube containing the SA-PMPs, then it was mixed and incubated at room temperature for 10 minutes. Complex SA-PMPs with hybrid mRNA and oligo (dT) were captured by placing a magnetic stand until the solution became clear. Supernatant was discarded carefully to avoid damage to SA-PMPs pellets. Streptavidin-paramagnetic particles were washed four times with 0.1x SSC (each wash as much as 0.3 ml) as shown above by placing a magnetic stand until the solution became clear.

2.2.2.4 Elution of mRNA

SA-PMPs pellet were resuspended in 0.1 ml RNAse free water, then they were captured by the magnetic stand. Aquaeus phase was taken and put in new sterile RNase-free tubes. Washing was repeated by adding 0.15 ml of RNase-free water. Then, SA-PMPs were captured by using a magnetic stand. Aquaeus phase was taken with caution and put in a new sterile tube which had been washed with distilled DEPC.

2.2.2.5 Precipitation and concentration of mRNA

Two hundred and fifty microliters of mRNA elution process results were coupled with 25 µl Na-acetate and 250 µl isopropanol, then they were blended until smooth and incubated on -20°C overnight. The reaction mixture was then centrifuged at 12000 rpm at 4°C for 25 minutes. Pellets were resuspended in 1 ml of ethanol; 75% cold and centrifuged at 12000 rpm at 4°C for 25 minutes. The supernatant was discarded and the pellets were dried at room temperature. Pellets were resuspended with 10 µl RNase free water and stored at a temperature -70°C. mRNA concentrations were seen by spectrophotometer at OD_{260}.

2.3 cDNA Synthesis

2.3.1 Single strand cDNA synthesis

Ten microliters of solution containing 2 µg mRNA, 1 µl oligo (dT) primer, and 4 RNase-free water were mixed. The reaction mixture was then incubated at 70°C for 5 minutes and centrifuged for 5 seconds at a speed of 12000 rpm for lowering all solution. The reaction mixture was then added with 5 µl single strand 5X buffer, 25 U rRNasin ribonuclease inhibitor, 2.5 µl 40 mM sodium pyrophosphate, 15 U AMV reverse transcriptase RNA, and RNA free water to a final volume of 25 µl. The reaction solution was mixed well and incubated at 37°C for 60 minutes. After the incubation, the solution was stored in ice to be used for double strand synthesis.

2.3.2 Synthesis of double-stranded cDNA

Twenty microliters of the reaction mixture on the synthesis of single-stranded plus an additional 10 10X µl second strand buffer, 23 U of DNA polymerase I, 0.8 U RNaseH, and nuclease free water were mixed to obtain a final volume of 100 µl. The reaction solution was mixed well and incubated at 14°C temperature for 3 hours. The polymerization reaction was stopped by heating at 70°C for 10 minutes. The tube containing the reaction mixture was centrifuged at 12000 rpm for 5 seconds to bring down all liquid and then it was placed on ice. The reaction mixture was further added with 2 U T4 DNA polymerase and incubated at 37°C for 10 minutes. Reaction stopped by adding 10 µl 200 mM EDTA and placed on ice. DNA extraction was done by added Phenol : isoamylalcohol chloroform, 1: 1. Solution was mixed well and centrifuged at 12000 rpm for 2 minutes at room temperature. Aquaeus phase was transferred into a new tube, then it was added half times the volume of 7.5 M ammonium acetate and two and a half times the volume of cold absolute ethanol. The solution was mixed with gentle and incubated at -20°C overnight. After incubation, the reaction mixture was centrifuged at 12000 rpm at 4°C temperature for 25 minutes. Pellets were resuspended with cold 75% alcohol and centrifuged at the same speed. The supernatant was discarded and the pellets were dried at room temperature until totally dry. Pellet was resuspended with 10-50 µl TE buffer. DNA concentration was measured with a spectrophotometer at OD_{260}.

2.4 Addition of EcoRI adapter in cDNA

The addition of the adapter *Eco*R I uses Riboclone®*Eco*R I Adaptor Ligation System. A total of 2.5 µl DNA (100 ng/µl) added 3 µl T4 DNA ligase buffer, 3 µl BSA (1 mg/ml), 1 µl *Eco*R I adapter, 2.5 U T4 DNA ligase and nuclease free water to a final volume of 30 µl. The reaction mixture was mixed well and incubated at 15°C temperature overnight. To stop the reaction, the reaction mixture was incubated at 70°C for 10 minutes.

2.5 Reaction of phosphorylation

Thirty microliters of DNA ligated with *Eco*RI adapter were added to 4 µl T4 polynucleotide kinase buffer 10X, 2 µl 0.1 mM ATP, 1 µl polynucleotide T4 kinase 10 U and nuclease free water to obtain a final volume of 40 µl. The reaction mixture was mixed well and incubated at 37°C for 30 minutes. The reaction mixture was extracted with phenol chloroform adding as much and blended well for 30 seconds at a speed of 12000 rpm centrifuged for 3 minutes. Aquaeus phase was transferred into a new tube and re extraction to obtain maximum results by adding 20 µl TE buffer. Phase aquaeus was then added with half times volume 7.5 M ammonium acetate and two volumes of cold absolute ethanol. The solution was mixed well and incubated at -20°C overnight. To obtain a pellet, a solution was then centrifuged at 3000 rpm at 4°C for 25 minutes. The supernatant was discarded and the pellet was washed with cold 75% ethanol. Pellets were dried at room temperature until completely dry and were resuspended with 10 µl TE buffer.

2.6. Elimination of excess adapters

Sephacryl S-400 which had been thoroughly mixed in a tube was inserted into the column and allowed a few minutes for all solvent buffers down to the bottom. Column tube was inserted into the tube, centrifuged at 2500 rpm, and washed with rotor SW for 5 minutes. Centrifugation was repeated when a column of Sephacryl still imperfectly dry. Column tube was taken and DNA samples were inserted into the column using a micropipette. The addition of DNA samples was done right at the top of the column and maximum volume of 60 µl. Column tube was inserted into the collector tube and centrifuged at 2500 rpm for 5 minutes. Supernatant were accommodated in the collector tubes ready for ligation reactions with the vector.

2.7 Transfection and analysis of results

2.7.1 Preparation of bacterial cultures

A single colony of *E. coli* Y1090 was cultured in 3 ml LB medium containing 0.2% maltose and 10 mM $MgSO_4$ and incubated at 37°C temperature with agitation speed of 200 rpm overnight. Five hundred microliters of an overnight bacterial culture results were added to 50 ml of LB media already containing 0.2 maltose and 10 mM $MgSO_4$ and incubated at 37°C until OD260 showing the growth ranging from 0.6 to 0.8. Bacterial cultures were then stored at 4°C overnight.

2.7.2 Ligation of cDNA into a vector λgt11

A total of 2 µl λgt11 *Eco*RI arms (0.5 µg/µl), were coupled with 3 µl nuclease-free distilled water and 1 µl T4 DNA ligase (10-15 U/µl). Positive control ligation reactions used 4 µl λgt11 *Eco*RI arms (1µg), 2 µl positive control insert (0.4 µg), 1 µl 10X ligase buffer, and 1 µl T4 DNA ligase. Negative control used the same arrangement with the arrangement of the cDNA sample, but was not coupled with cDNA samples. The composition of the reaction was incubated at 14°C for overnight and ready for packing.

2.7.3 Packing of ligated DNA

Extract packer thawed in ice. Immediately after melting, the result of ligation reaction was added to the extract packer. For a positive control reaction, only 0.5 µg DNA containing λc1857 sam7 was added to extract packer. The reaction mixture was incubated at room temperature (22°C) for 3 hours. The reaction mixture was then added with 445 µl page buffer and 2 µl chloroform and mixed by means of tossing and turning the tube. Phage already packed can be stored at -4°C for 7 days.

2.7.4 Titration phage and transfection

Phage was packed to the titration by doing some dilution ie: 1:10, 1:100, 1:1000 and 1:10000. A total of 100 µl phage dilution resulting solution was added to 100 µl Y1090 bacterial culture and mixed well. For the positive control, the bacteria used are LE393. Incubation was carried out at 3°C for 30 minutes. While waiting for the incubation, 3 ml prepared to order warm (45°C) which had been coupled with 21 µl 5 mg/ml X-gal, 21 µl 20 mg/ml IPTG and 3 µl 10 mg/ml ampicillin. After being incubated, the mixture of bacteria with phage was coupled with 3 ml of top agar, shaken and immediately poured onto LB agar plate which had previously been warmed 37°C. LB agar plates were then incubated at a temperature of 37°C for 8-10 hours, and the amount of plaque formed can be used to count packing efficiency.

2.7.5 Transfer of protein expression on nitrocellulose membrane

Nitrocellulose membranes are used and adapted to the plate used and labeled samples. Slowly, membrane placed on LB agar plate until blended. At the edge of the membrane, stitch was made as a marker of plaque on the LB agar plate orientation. Incubation was carried out at 37°C for 3 hours, and then cooled at a temperature 4°C for 20 minutes, so that the hardening occured. Nitrocellulose membranes were taken by carefully using tweezers and washed with TTBS to remove the remaining 0.5% top agar.

2.7.6 Plaque screening with antibodies

Nitrocellulose membrane was washed with TTBS 0.5%, next blocked with 1% BSA overnight at 4°C, continued washed with 0.05% Tween 100 in TBS for 10 minutes, and the washing was repeated 3 times. Then, membrane was introduced into a solution of polyclonal antibody in PBS (1:50) and incubated 1 hour at room temperature with sacker, and washed with 0.05% Tween 100 in TBS for 10 minutes. Washing was repeated four times in the same manner. Furthermore, the membrane was incubated in a solution of conjugate (1:3000) (Santa Cruz, USA) for 1 hour at room temperature with sacker, and followed by washing five times with 0.05% Tween in TBS and one time without Tween. Membranes were stained with a substrate Western Blue Ready. The reaction was stopped when protein bands were visible by adding distilled water. Membrane was dried on Whatman paper and ready to be documented.

2.8 Over recombinant expression in E.coli Y1090

Recombinant plaques of the plate in order to be taken by way of insertion and extraction with Pasteur pipette. Plaques fetched were put in 500 BC µl buffer and kept at that temperature overnight bacteriophage 4°C

regardless of the order. Preparation of bacterial culture Y1090 was done by growing a single colony of bacteria Y1090 in 5 ml LB medium containing 0.5% maltose and 10 mM $MgSO_4$. Incubation was carried out at 37°C with agitation of 200 rpm for overnight. A total of 500 μl culture overnight growth was added to 10-20 μl solution containing recombinant plaques. Incubation was carried out at 37°C for 20 minutes, and then the culture was put in 100 ml of warm LB medium containing 10 mM $MgSO_4$. Incubation was carried out at 37°C with agitation at a speed of 200 rpm for approximately 5 hours. If there were no cell lysis after 7 hours, the culture was added with 500 μl chloroform and agitated for 15 minutes at the same temperature. Lysate was taken and centrifuged at 2000 rpm 4°C temperature for 10 minutes. The supernatant was taken and stored at temperatures 4°C. Proteins with high concentrations were taken by precipitation by the addition of saturated ammonium sulfate (1:1). The reaction mixture was then incubated at 4°C overnight. Pellets were obtained by centrifuged at 4000 rpm at 4°C for 20 minutes. The supernatant was discarded and the pellet was resuspended with sterile aquabidest.

2.9 Immunobloting with dot blot technique

A total of 10 μl lysate supernatant of bacterial growth Y1090 infected with recombinant or 2 μl λgt11 protein precipitation results dropped on a nitrocellulose membrane and allowed to dry. Negative controls used recombinant protein lysate proteins and protein precipitation results from bacterial growth Y1090 were not infected with λgt11. Nitrocellulose membrane was allowed to dry and then blocked with 1% BSA overnight at 4°C. Then, it was continued washed with 0.05% Tween 100 in TBS for 10 minutes, and the washing was repeated 3 times. Membrane was then introduced into a solution of polyclonal antibody in PBS (1:50) and incubated 1 hour at room temperature with sacker and washed with 0.05% Tween 100 in TBS for 10 minutes. Washing was repeated four times in the same manner. Furthermore, the membrane was incubated in a solution of conjugate (1:3000) (Santa Cruz, USA) for 1 hour at room temperature with sacker and followed by washing five times with 0.05% Tween in TBS and 1 time without Tween. Membranes were stained with a substrate Western Blue Ready. The reaction was stopped when protein bands were visible by adding distilled water. Membrane was dried on Whatman paper and ready to be documented.

3. Results and discussion

Total RNA isolated from tachyzoite of *Toxoplasma gondii* after electrophoresis on 1% agarose gel can be seen in Fig. 1.

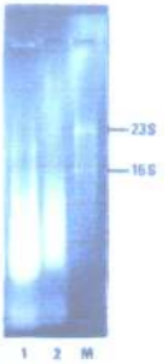

Fig. 1. Total RNA of *Toxoplasma gondii*
M: standard marker of *E. coli* rRNA; 1,2 RNA sample

In Fig. 1 visible sighting clears rRNA bands with bands visible mRNA smear bands between rRNA. In prokaryotes and eukaryotes including *Toxoplasma gondii*, the amount of mRNA contained in the cell is less than the other types of RNA. Messenger RNA isolated from total RNA obtains 1.5%. As a comparison, RNA in *E. coli*. *Escherichia coli* mRNA is only 5% of the total RNA, while the others are rRNA (80%) and tRNA (15%)[23]. Messenger RNA is the result of transcription of the DNAwhich will then be translated to form proteins. Messenger RNA has different length according to the genes that encode, resulting in the electrophoresis to form thin ribbons that look smear. Of 5% mRNA, only few percent that will be translated into proteins. In addition, there are several types of RNA: mRNA, rRNA, and tRNA. In length, Ribosomal RNA nucleotides are longer than the tRNA, so electrophoresis rRNA bands will form a clear tape24. In eukaryotes, it is obtained four kinds of rRNA : 5S; 5,8S; 18S; and 28S. rRNA which is in *E. coli* are 5S, 16S and 23S rRNA.

Messenger RNA is the result of transcription of DNA coding for a particular protein which is good for its

own purposes, secretion or as a surface protein that functions in the attachment to the host. Messenger RNA in eukaryotes has a poly A 3-prime chest. With the poly A at the 3-prime, mRNA can be easily separated by adding olido (dT) probe which has been added by biotin. With techniques developing Promega, complex poly AT has been added by biotin and mRNA molecules can be separated by adding puller made from paramagnetSpere streptavidin particles (SA-PMPs).

Transformation results in the form of blue plaques and white/transparent plaques. Plaque is a white plaque recombinant cDNA carrying inserts, and blue plaque is a plaque that does not carry cDNA inserts. The number of white plaques obtained from the transfection is about 40% of the total plaques formed. Plaque is formed on LB plate due to bacterial cell lysis results from infection λgt11. Lamda gt11 is a phage that has a growth cycle of lytic in *E. coli* Y109025. The arrival of λgt11 infecting host cells will lead to lysis of the bacteria. Lysis which occurs in bacteria is due to damage to the walls of bacteria because of enzymes released by phage[24]. At the end of the cycle of infection, Phage synthesizes lysozyme or endolysin which causes damage to the bacterial cell wall. The outbreak of bacterial cells, the particles, will be released around the new phage in bacterial growth medium. The results of λgt11 transfection into *E. coli* Y1090 can be seen in Fig. 2.

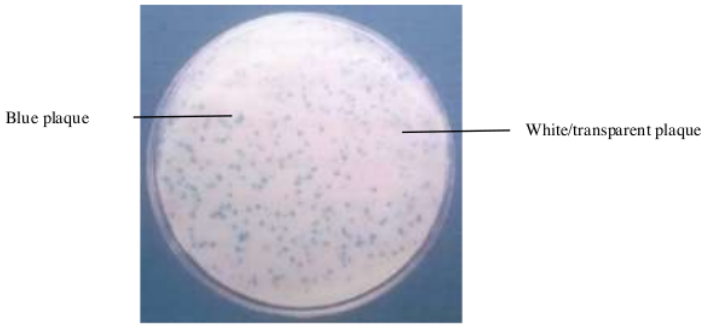

Fig. 2. The results of λgt11 transfection into *E. coli* Y1090

White plaques occur due to the side restriction of *Eco*RI in genes encoding β-galactosidase inserted cDNA. Their insertion of foreign DNA in gene encoding β-galactosidase fusion protein results in the formation of the protein expression of foreign DNA, and makes β-galactosidase inactive. A β-galactosidase which is inactive causes the X-gal substrate bias is not described, and chromogenic dye is not formed. Conversely, when the side of the *Eco*RI restriction on genes encoding β-galactosidase is not insersed with foreign DNA, it will form an active β-galactosidase. An active β-galactosidase enzyme causes the substrate X-gal can be revamped and releases chromogenic dye blue. White plaques possibility of carrying cDNA encoding membrane protein of *Toxoplasma gondii* tachyzoite or cDNA encodes other proteins. To determine plaque carrying cDNA encoding the membrane protein of tachyzoite, screening is required. The use of antibodies against membrane proteins of *Toxoplasma gondii* can be used to select gene encoding as expected. The immunoblotting result of recombinant plaque with polyclonal antibody can be seen in Fig. 3.

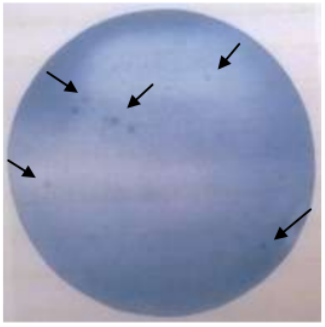

Fig. 3. Immunoblotting result of plaque recombinant with polyclonal antibody against *Toxoplasma gondii*. Arrows indicate the direction of plaque that expresses a membrane protein of tachyzoite *Toxoplasma gondii*

Selection of recombinant plaques carrying cDNA encoding membrane protein of *Toxoplasma gondii* is done by immunoscreening. Screening resulting in polyclonal antibody against the membrane protein obtains 7 clones carrying cDNA encoding membrane protein of *Toxoplasma gondii*, and which can be seen in Fig. 3.

Immunoblotting of recombinant plaque with polyclonal antibody against *Toxoplasma gondii* membrane protein is a blue black spot which is recombinant proteins that react with polyclonal antibodies against *Toxoplasma gondii* membrane protein. Polyclonal and monoclonal antibodies can be used to screen for the desired genes from libraries genes because the antibody has specific properties to foreign proteins known. Polyclonal antibodies used for the selection of clones are wider than the monoclonal antibody. Monoclonal antibody is specific for only recognizing one specific epitope on the protein to be expected[26]. The mutant P30 (SAG 1) of *Toxoplasma* is detected clones in the library. Parasite clones that do not carry genes P30 do not react with antibodies to P30. Relations between antibodies and membrane proteins have been reported by Harning *et al*. (1996) who stated the recombinant SAG 1 antigen expressing *E. coli* can be used to allow the detection of immunoglobulin G (IgG) and IgM specific to SAG 1.

Complementary DNA is inserted in λgt11 protein-coding genes of *Toxoplasma gondii* tachyzoite, and one of the tachyzoite protein-coding genes is a gene that encodes membrane proteins[21]. Tachyzoite of *Toxoplasma gondii* has five major surface proteins. These five major proteins include SAG1 (P30) which is the most dominant protein, SAG3 is the least amount protein, and the other three proteins are minor proteins. In addition, the third minor protein on the surface of tachyzoite is SAG1-related sequence (SRS). In such SAG1, SRS encodes proteins with glycosilphosphatidylinositol.

Seven spots of immunoblotting results obtain clones expressing membrane protein as a candidate for vaccine development and the diagnosis of toxoplasmosis. Further testing needs to be done to ensure these clones are carrying genes that encode P30 by using PCR and doing overexpression in the host Y1090. PCR results are known as long nucleotides and overexpression clones are known as molecular weight proteins encoded by these genes.

4. Conclusion

Transfection of *E. coli* Y1090 using recombinant λgt11 obtains 7 clones carrying cDNA encoding a membrane protein of *Toxoplasma gondii* tachyzoite local isolate. Overexpression and immunoblotting results show that recombinant clones are carrying genes that encode proteins of the membrane.

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Contribution Bioactive Flavan-3-Ol Callus *Camellia Sinensis* As A Candidate Healing Diarrhea Disease

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Abstract

Diarrheal diseases categorized infectious and non-infectious diseases. In Indonesia diarrhea caused by viruses, parasites and bacteria such as *E. coli* is transmitted through water, food contaminated with bacteria. The purpose of this paper was to determine the contribution of bioactive flavan-3-ol as diarrhea drug candidates. Flavan-3-ol can be obtained from plants, which are harvested when reaching age five. Then developed a method of in vitro callus flavan-3-ol include: initiation of callus with *Camellia sinensis* leaf explants, isolation and identification of qualitative-quantitative test the efficacy of *E. coli*. The results of the reviews flavan-3-ol can be as diarrhea drug candidate

Keywords: Camellia sinensis; in vitro culture; bioactive callus flavan-3-ol.

Nomenclature

Tea	*Camellia sinensis*
MS	murashige skoog
E. coli	*Escherichia coli*
2,4-D	2,4-diklorofenoksiasetat acid
NAA	naftalenasetat acid

1. Introduction

In some developing countries diarrhea is still a problem[1]. The disease is caused by several conditions: environment inadequate, people do not maintain hygiene habits, low education levels, low socioeconomic. The above conditions will be the cause of bakteri[2], viruses, parasites, infectious and non-infectious transmission media through the mouth, contaminated food and water which is a conductor for the incidence of diarrheal diseases. Associated with diarrheal disease authors conducted a study to produce callus flavan-3-ol of *Camellia sinensis* leaf is expected to be a candidate diarrheal disease prevention.

The purpose of writing paper was to determine the contribution of the bioactivity of flavan-3-ol to prevent diarrheal disease. Bioactivity flavan-3-ol produced in vitro, in the laboratory large scale. While the benefits of such bioactive paper writing flavan-3-ol has the potential to enrich the medicinal product, in an effort to prevent/treat diarrhea.

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2. Methods

This research was conducted for one year by using ingredients: *Camellia sinensis* leaf, NaOCl, 5.25%, MS medium containing plant growth regulators NAA and 2,4-D, materials for sterilization (sterile distilled water, tween 20, agreept, alcohol 90%. Materials for extraction (hot water, chloroform, ethyl acetate). The activities consisted of several stages as follows:

2.1. Initiation of callus with camellia sinensis leaf explants

Shoots in the first position with a symmetric shape, and position of the two is used for explant cleaned using running water for thirty minutes. Shoots that have cleaned, sterilized by soaking in a solution while gently shaken 5.25% NaClO, tween-20 for 30 minutes. Rinsing with sterile distilled water, while rocked gently for 5 minutes, flushing was repeated 3 times. The leaves are sterile petri moved on, bones and edges of leaves removed and then cut into pieces the size of 0.5-1 cm. Pieces grown as much as 4-5 explants in culture bottles containing MS medium enriched with plant growth regulators (PGR) NAA and 2,4-D. Incubation of the culture bottles at room temperature 25°C, then sub cultured to multiply times callus that formed[3].

2.2. Isolation and identification of qualitative-quantitative

Isolation and identification of qualitative-quantitative microscopy and high performance liquid chromatography (HPLC). Identification qualitatively by observation using a binocular microscope and triocular by comparing the shape of callus to form flavan-3-ol standard. Isolation performed by extraction using hot water, chloroform, ethyl acetate. Assay of callus tea extract with HPLC conditions: temperature 300°C setup, wavelength 274.8 nm, the mobile phase consisted of methanol : water : acetic acid = 20 : 75 : 5, mobile phase flow rate of 1 mL/minute[4].

Manufacture of standard solution of flavan-3-ol by means of carefully weighed 0.5 mg flavan-3-ol standard, then dissolved in methanol and 5 mL, in order to obtain a solution with a concentration of 100 ppm as the mother liquor. From the mother liquor of 100 ppm, made a wide range of concentrations to levels 5, 10, 15, 20, and 25 ppm[5], then each injected concentration of 100 uL on HPLC instruments with operational conditions that have been the setting, it will be obtained chromatogram. From the chromatogram created curve concentration (x) and area (y) then determined the regression line equation[5]. Samples callus tea extracts and standards flavan-3-ol dissolved in methanol sonicated for 5 minutes to remove gases contained in solution so as not to clog the column, then the solution is filtered with filter paper Whatmann 0.2 mm. To clarify the chromatogram peaks, the callus tea extract solution was added 15 ppm standard. Levels of flavan-3-ol obtained by injecting samples of 100 mL of tea callus on HPLC.

2.3. Test the efficacy of flavan-3-ol in E. coli

At the efficacy test (review flavan-3-ol in *E. coli*), prepared nutrient broth medium (NA) by means of a ose needle scraping containing bakteri[6], aseptically on media tube and then covered with a cotton swab every week rejuvenated and kept at a low temperature[7]. The method is performed is agar[8] diffusion, wherein flavan-3-ol impregnated on paper discs with a diameter of 6 mm. This paper disc placed on the surface of the media who have been inoculated bacteria *E. coli* that will tested[9]. Furthermore incubated for 24 hours at temperature 35-37°C, observed the area around the paper disc barrier[10]. Regional barriers formed a clear zone around the paper disc, which indicates bacterial pathogens or microorganisms that have been inhibited by the compounds tested flavan-3-ol which diffuses into the order of a paper disc. On Extraction taken with a concentration of 10% w/v, for soaking paper disc with a diameter of 6 mm[11]. A positive response to the activity shown by the clear zone (clear zone) at about medium which has been inoculated with the bacteria *E. coli*, which is a clear zone inhibition zone conducted by flavan extracts and compounds.

3. Results and discussion

Callus results of this study will be tested efikasikan in *E. coli* but in this article efficacy test are still reviewing the results of other studies researchers as written in the following chapters.

3.1. Initiation of callus with camellia sinensis leaf explants

Results tea callus initiation in vitro with plant growth regulators NAA and 2,4-D and using media MS at the age of 4 weeks to 12 weeks of age[12], the response callus growth can be seen in Fig. 1.

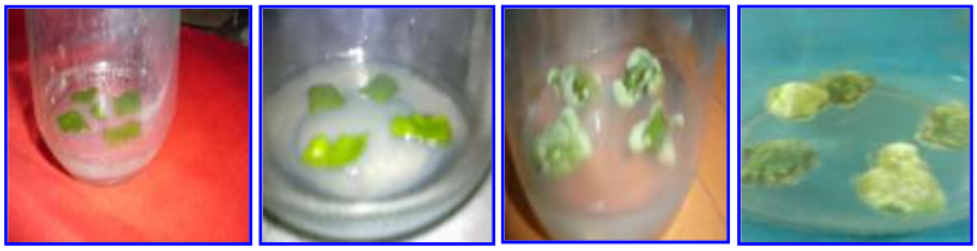

Fig. 1. Callus growth response with growth regulator 2-4 D and NAA

In Fig. 1, appears morphological changes from left to the right to successive leaf explants with the width of approximately one centimeter which turned into a frown, bulging, writhing then appears callus that is started from the edge of next explants formed callus that meet all explants. This research is relevant to Iriawati[13] that the use of in vitro culture methods that can successfully reproduce callus that induce alkaloid secondary metabolites as well as induce somatic embryo of the plant earth peg *(Eurycoma longifolia* Jack). Research conducted by Parthraj[14] is also mentioned that callus can be induced through this in vitro culture with MS media and on the plant growth regulator substances Swertia lawii Burkill.

3.2. Isolation and Qualitative–Quantitative Identification

Isolation and qualitative identification is by using HPLC chromatogram can be seen in the area and retention time of flavan-3-ol[15] as described in Fig. 2 and 3.

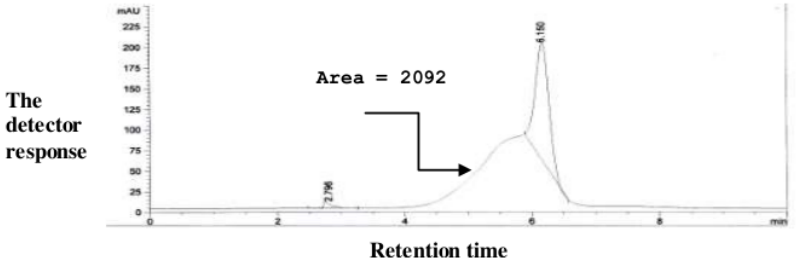

Fig. 2. Chromatograms area standard flavan-3-ol-catechol

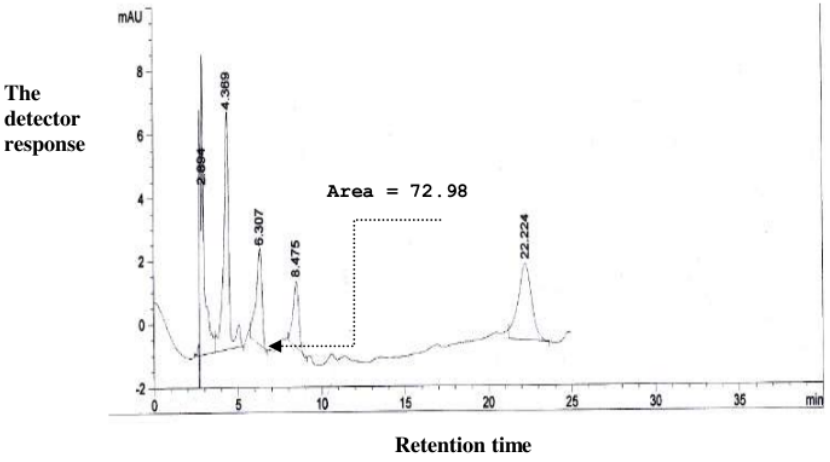

Fig. 3. Retention time of flavan-3-ol

Quantitative identification by calculating the levels of flavan-3-ol calculated as ratio of the chromatogram area samples and standards that follow the method of Mustafa[16] levels obtained the level of flavan-3-ol at 3:48 ppm. This research is relevant to the research of Dakah[17] who produce polyphenols successfully through in vitro culture *Ziziphora tenuior* L. that can be used for anti-oxidants.

3.3. Callus form observation using a binocular microscope

Callus form observation using a binocular microscope as described in Fig. 4 in which the form and the part of callus color is similar to a standard form of flavan-3-ol-cathecol.

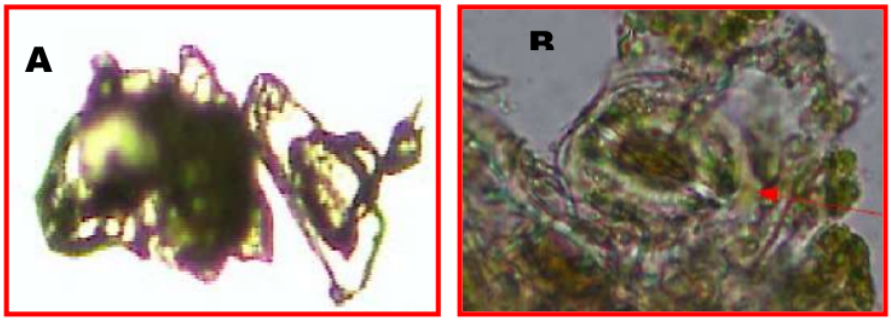

Fig. 4. Observation using a binocular microscope, magnification 10 X: standard form of flavan-3-ol-cathecol (A), and Callus form (B)

Fig. 4 shows the similarity of the shape and color of callus with standard. This case indicates that bioactive flavan-3-ol-cathecol can be produced through callus culture.

3.4. Callus cell observation using a triokuler microscope

Callus cell observation using a triokuler microscope like those in Fig. 5 which is similar to the standard of callus flavan-3-ol-polyphenol.

Fig. 5. Callus cell observation using a triokuler microscope magnification 400x :
the form of standard flavan-3-ol-polyphenols (A) and Callus cell flavan-3-ol-polyphenol arrow (B)

Fig. 4 and 5 show the form of cell that is similar to standard cell shape which indicates callus that contains bioactive metabolites flavan-3-ol but it also appears the form of callus which is considered to form an embryo. The embryonic form is same with the research of Lü[18] that the in vitro culture of *Camellia nitidissima Chi* (Theaceae) forms somatic embryos.

3.5. Efficacy test flavan-3-ol on E coli

Test the efficacy of flavan-3-ol / polyphenols from tea leaves concentration of 500 mg can inhibit the growth of *E. coli* bacteria (strain ATCC 25 922) 5000 mg/mL. Bacteria inhibition of growth can be seen in Fig. 6.

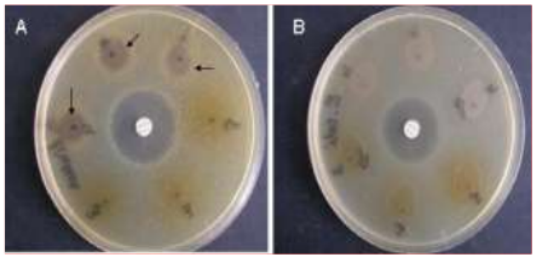

Fig. 6. Response activities *E.coli* bacteria (A) indicated positive arrows/clear zone. (B) negative no clear zone[19].

Research conducted by Hoshino that test the efficacy of flavan-3-ol / catecin and catecin-Cu complex can penetrate and damage the cell surface lipopolysaccharide *E. coli* seen in the illustration Fig. 7.

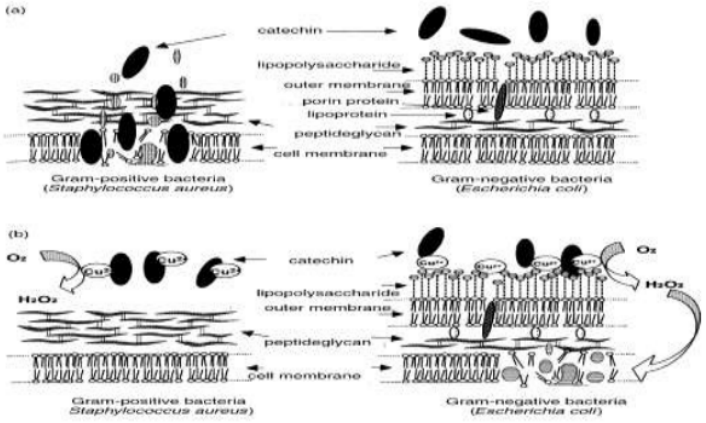

Fig. 7. Mechanism of flavan-3-ol / catechin and complex Cu catechin-penetrate and damage the cell surface lipopolysaccharide *E. coli* (Hoshino[20]).

4. Conclusion

Flavan-3-ol can be produced by in vitro culture methods using PGR 2-4D and acquired NAA levels of flavan-3-ol 348 ppm[21]. Flavan-3-ol/polyphenols from tea leaves concentration of 500 mg can inhibit the growth of *E. coli* bacteria (strain ATCC 25 922) 5000 mg/mL[19].

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In Vitro Antimalarial Activity of Ethanol and Methanol Extract *Carthamus tinctorius* L

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Abstract

The extracts ethanol and methanol from *Carthamus tinctorius* L. flowers showed the inhibition against *Plasmodium falciparum* 3D7 clone. Ethanol extract had the highest inhibition of 95.97% with IC_{50} 1.06 µg/mL compared to an untreated control, while methanol extract had inhibition of 62.39% with IC_{50} 15.89 µg/mL. Extracts of ethanol and methanol were obtained through maceration for 3x24 hours. The antimalarial activities of the extract were determined by the procedure described by Budimulya *et al*. Stock solution of the samples were prepared in DMSO and were diluted to the required concentration with complete medium until the final concentration of samples at well culture plate were : 100; 10; 1; 0.1; 0.01 µg/mL. The malarial parasite *P. falciparum* 3D7 clone was propagated in a 24-well culture plate in the presence of a wide range of concentrations of each compound. The antimalarial activity of each compound was expressed as an IC_{50} value, defined as the concentration of the compound causing 50% inhibition of parasite growth relative to an untreated control. The result showed that ethanol extract deserved further investigation toward development of a promising antimalarial drug.

Keywords: Antimalarial activity, In vitro, *Plasmodium falciparum* 3D7, *Carthamus tinctorius* L.

1. Introduction

Malaria is an infectious disease caused by protozoa of the genus *Plasmodium*. This disease contributes to the high rate of mortality among infants, under-five children, and pregnant women. Annually, more than 500 people worldwide are infected by malaria and 1 million died because of this disease. The majority of malarial cases occur in Africa, as well as in several countries in Asia, South America, Middle East, and Europe. To deal with the disease, the 60th meeting of World Health Assembly (WHA) has come up with a global commitment on the elimination of malaria in all countries. Guidance on how to go about doing this has been provided by the World Health Organization (WHO) through its *Global Malaria Program*[1].

Indonesia is one of the countries at the risk of malaria. Most of Indonesian regions are malaria-endemic, in particular the eastern part of the country including Papua, Maluku, Nusa Tenggara, Sulawesi, and Kalimantan, as well as some areas in Lampung, Bengkulu, and Riau. In some areas of Java and Bali, despite low endemism, cases of malaria outbreaks still occur frequently. In 2007, 396 out of 495 regencies in Indonesia are malaria-endemic, and it was estimated that 45% of the population live in areas that are at high risk of malaria infection. In addition, malaria morbidity in the country is reported to rise from year to year. To overcome malaria disease, various efforts have been made both in global and national scale. Malaria is one of indicators of Millennium Development Goals (MDGs), with an aim at putting a stop to the spread of malaria and reducing malaria-related incidents in 2015, to be indicated by a decrease in morbidity and mortality caused by malaria[2].

One factor leading to some problems faced in the eradication of malaria is a decrease in the efficacy of antimalarial drugs that have commonly been taken. *Plasmodium falciparum* has been reported to be resistant to antimalarial *chloroquine*. Reports also indicate that resistance to *chloroquine, sulfadoxine-primetamin, mefloquine, halofantrin,* and *quinine* occur in some areas in Southeast Asia. In certain areas within the region, a combination of *artemesinin* and its derivatives is the only effective medication[3]. Other factors leading to the still high rate of morbidity and mortality include large-scale human migration, environmental and climate change, poor healthcare system, and the emergence of strains of insecticide-resistant Anopheles mosquitos[4].

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An alternative way of preventing malaria is by developing a concept of immunotherapy medication, that is, an administration of drugs in the forms of either synthetic or natural compounds (plants) that are capable of optimizing the functions of all components in human body immune system to combat malarial infection. Immunity to malarial infection involves cellular and humoral responses. A cellular immune response is mediated by T. lymphocytes, which plays an important role in the fight against the infection from intracellular sporozoites (skizogoni extra-erythrocytic).

Kasumba turate (*Carthamus tinctorius* Linn) flower from the Astreraceae family has empirically been used as traditional medication by people in South Sulawesi to heal measles by brewing the plant in hot water before taking its juice, the consumption of which is believed to help increase body's immunity system. A study on the ethanol extract of kasumba turate discovered that the extract highly significantly increases both immunoglobulin G (IgG) and A (IgA) activities. Dried flowers of this plant can also be used as emmenagogue, laxant, or stimulant. In addition to consumption, its seed oil is used in mixture of cosmetic materials[5]. When tested on mice to determine its cellular immune response, the immunomodulatory effect of juice extracted from kasumba turate flower (*Carthamus tinctorius* Linn) was remarkably significant. A test on nonspecific immune response showed that a dosage of 1.95 and 3.90 mg/kg bodyweight could increase phagocytosis speed, as was indicated by the phagocytosis index: 1.72 and 1.88 (K>1.5), and this was considered as a strong immunostimulation. This increases very significantly when the flower is brewed with hot water[6].

Based on the immunomodulatory activitiy exhibited by kasumba turate flower (*Carthamus tinctorius* Linn), it is of interest to find out whether ethanolic and methanolic extract from kasumba turate flower (*Carthamus tinctorius* Linn), which have been known to function as an immunomodulator, could also increase the activity of immunocompetent cells that play important roles in developing body's defense against malarial infection. This interest is worth pursuing, since it is important to build a scientific ground upon which kasumba turate (*Carthamus tinctorius Linn*) can be justifiably developed as malarialimmunotherapy.

2. Methods

2.1 Plant Material

Flowers of kasumba turate (*Carthamus tinctorius* Linn) were collected from Bone, a regency in South Sulawesi, and have previously been determined by Purwodadi's LIPI in Pasuruan.

2.2 Extraction

The flowers of kasumba turate (*Carthamus tinctorius* Linn) were ground into powder before they were divided into two parts, each weighed 300 grams. Simplicia powder was macerated in ethanolic and methanolic liquid for 3 x 24 hours. The filtrates were collected and evaporated by using evaporator, reducing pressure until thick extract was obtained.

The powder extracted from the flowers of kasumba turate (*Carthamus tinctorius* Linn) was macerated in ethanolic and methanolic liquid. Two hundred and fifty grams of the powder of kasumba turate flower were extracted by using methanol, resulting in 23.32 grams of thick yellow-brownish methanolic extract with 9.33% of yield, whereas one hundred and twenty-five grams of kasumba turate flowers powder was dissolved in ethanolic liquid, resulting in 20.48 grams of thick yellow-brownish ethanolic extract with 16.38% of yield.

2.3 In Vitro Antimalarial Activity Assay

The antimalarial activities of the extract were determined by the procedure described by Budimulya *et al*[7]. Breeding is done in a petri-dish and done aseptically. Plasmodium falciparum 3D7 culture obtained from frozen deposits in-thawing and bred. After culturing Plasmodium falciparum synchronized then be tested as follows: Material testing ethanol and methanol extracts *C. tinctorius* L flowers dissolved in DMSO and sterile filtered with a membrane filter of 0.22 lm. This test solution is then inserted into the microplate that contains the parasite suspension to the level of 1% parasitaemia then diluted in series with a medium until obtained final concentration of test substance in the microplate as follows: 100; 10; 1.0; 0.1 and 0.01 mg/mL. Negative controls used DMSO diluted in the same manner as diluting materials above test, in order to obtain final DMSO concentration is not more than 0.5%. Mixture and suspension test parasites (test preparation) is then inserted into the candle-jar and incubated in a CO_2 incubator at a temperature of 37°C for 48 hours. After incubation for 48 hours, made thin blood smear on glass object. Smear dried at room temperature, fixed with methanol, then once dry stained with Giemsa and counted under a microscope parasitemianya with 1000 times magnification. Calculations performed on 5000's erythrocytes.

3. Data Analysis

The data obtained from in vitro antimalarial activities assay above is in the form of number of parasite-infected erythrocytes (counted on around 5000 erythrocytes) were subsequently converted into parasitemia levels (percent parasitaemia) and the percent inhibition of the test substance on the growth of the parasite.

Percent parasitaemia calculated by the formula:

$$\% \text{ Parasitemia} = \frac{\Sigma \text{ infected erythrocytes}}{\text{The number of erythrocytes}}$$

The percentage inhibition of parasite growth is calculated using the formula:

$$\% \text{ Inhibition} = 100\% - \left(\frac{Xp}{Xk} \right) \times 100\%$$

Based on the data about the inhibition and concentration of the tested materials, IC_{50}, or the concentration of test materials that inhibit parasite growth by 50%, were then calculated by using the probity analysis.

4. Results and discussion

Methanolic extracts of kasumbaturate flower. Results of this test can be seen on the table and figure below. As can be seen, ethanolic and methanolic extracts had an inhibitory power over the growth of *Plasmodium falciparum* 3D7. The test on 100 μg/mL concentration show that, compared to the negative control, the inhibitory power of ethanolic and methanolic extracts were 95.97% and 62.39% respectively. This inhibitory power reduced as tested concentration was also reduced, indicating that the inhibitory power of ethanolic and methanolic extracts depend on concentration.

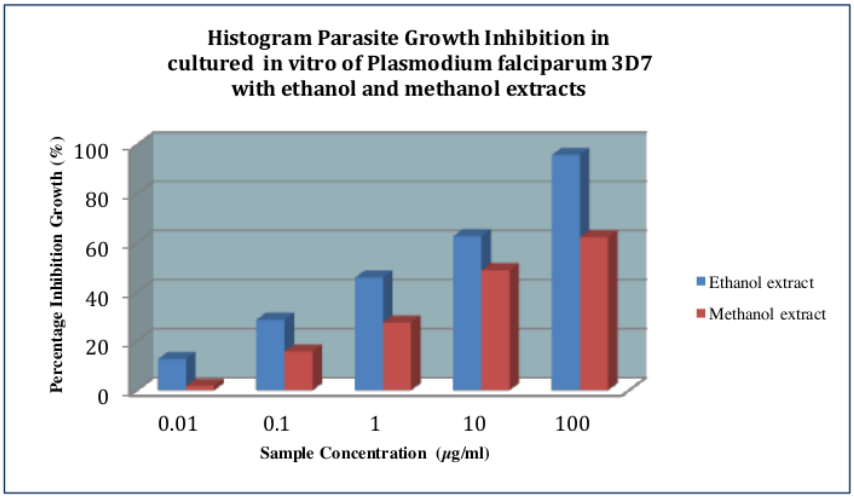

Fig. 1. Histogram parasite growth inhibition in culturedin vitro *Plasmodium falciparum* 3D7 clone of ethanol and methanol extracts with variation concentrations.

To determine the potential growth inhibition of ethanolic extract, methanolic extract, and water extract, IC_{50} values were calculated by using probity analysis. The results of this analysis were presented on the following table.

Table 1. Average Percent Inhibition Growth and IC_{50} ethanol and methanol extracts against *Plasmodium falciparum* 3D7 clone

Extract	Growth inhibition at variation of doses (μg/mL)					IC_{50} (μg/mL)
	100	10	1	0.1	0.01	
Ethanol extract	95.97	62.85	45.95	28.78	12.68	1.06
Methanol extract	62.39	48.94	27.61	15.79	1,77	15.89

According to Kohler (2002), an extract whose IC_{50} value is lower than 50 (μg/mL) and a fraction whose IC_{50} value is lower than 25 (μg/mL) are considered effective antimalarials. Data on the table above show that the IC_{50} values of both ethanolic and methanolic extract are lower than Kohler's criteria. It can therefore be stated that the extracts of ethanol and methanol can serve as effective antimalarials. However, since the IC_{50} value of ethanolic extract is lower than that of methanolic extract, it can be concluded that ethanol has a more potential antimalarial activity than does methanol. Therefore, it is necessary to isolate the flower of kasumba turate (*Carthamus tinctorius* Linn) according to the *bioassay guided isolation* principle in order to obtain active compounds that can be used as a marker in the standardization of antimalarial materials, so that flowers of kasumba turate can be developed into phytopharmaca products.

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Vanillin Production from Lignocellulosic Extract of Oil Palm Empty Fruit Bunch Fiber (OPEFBF) by *Pycnoporus cinnabarinus*

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Abstract

This study was conducted to produce vanillin via fermentation by *Pycnoporus cinnabarinus* MUCL 39533 on lignocellulosic extract of oil palm empty fruit bunch fiber (OPEFBF). Lignocellulosic extract of OPEFBF was obtained by treating 10% wt. (*d.b*) of OPEFBF with 50 g L^{-1} sodium hydroxide at 120°C for 2 hours. Soluble lignin in the extract was recovered through acidification, centrifugation and drying. About 21.87±3.44 gram of lignin had recovered from each liter of extract. The obtained dried lignin fragment and phenolic-rich black liquor was then fed to *P. cinnabarinus* separately for vanillin production through fermentation. Based on the results obtained, vanillin production was only achieved with the fed of lignin. With the presence of cellobiose and resin Amberlite XAD4, about 0.65±0.08 mg of vanillin was successfully yielded from 1 gram of lignin. The overall bioconversion process that developed in this study successfully produced 0.15 g of vanillin from each kg of OPEFBF.

Keywords: Black liquor; fermentation; lignin; oil palm empty fruit bunch fiber (OPEFBF), vanillin.

Nomenclature

OPEFBF	Oil Palm Empty Fruit Bunch Fiber	ppm	part per million
HPLC	High Performance Liquid Chromatography	g L^{-1}	gram per liter
d.b	dry basis	rpm	revolutions per minute
%wt.	percentage weight/weight		

1. Introduction

Concept of waste to wealth has been promoted in Malaysia since late 1990, to convert unwanted agricultural waste into value added product/chemicals, while reducing waste generation and improve environmental sustainability[1]. Under 10th Malaysia Plan (2011-2015), oil palm industry and oil palm-related products was identified as one of the National Key Economy Area (NKEA) to chart the country development towards a high income nation. To achieve this mission, initiative has taken to develop palm oil industrial clusters (POIC) into integrated sites for promoting downstream activities such as production of biofuel, oleochemicals, specialty food products, pharmaceuticals and other products[2].

Among various oil palm wastes, empty fruit bunch fiber (EFBF) is the most abundant biomass[3]. Therefore, EFBF has been selected as the targeted substrate in this study. Moreover, in order to maximize the efficiency of biorefinery, the recovery of lignin and aromatic compounds from the generated black liquor was focused. Untreated black liquor form oil extraction is a major agricultural pollutant of oil palm industry. Black liquor is a complex aqueous solution comprised of organic components such as lignin, carbohydrate fragments, low molecular weight resin and other phenolics as a valuable resource from the treated biomass and inorganic

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component such as ion salt[4]. Several studies had reported the production of vanillin from lignin and its derived aromatic compounds such as ferulic acid via either chemical or biotechnological means[5,6,7,8].

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is an aromatic flavoring compound widely used in food, pharmaceutical and cosmetic industry. Nonetheless, due to the low yield and high cost of natural vanillin extraction from *Vanilla sp.* orchid, the current vanillin demands have to fulfill by synthetic vanillin. However, according to EU and US legislation, vanillin synthesized from natural resource through biotechnological means can be labeled as natural vanillin[9]. With this in mind, the successful biotransformation of lignocellulosic extract of EFBF into vanillin via fermentation not only will broaden the EFBF utilization, but also increases the revenues of biorefinery process of oil palm industry.

In this study, efficiency of vanillin production via fermentation by *Pycnoporus cinnabarinus* was compared between the different feeding rate of recovered lignin and black liquor obtained after alkaline treatment of EFBF. In addition, the effects of resin Amberlite XAD4, cellobiose and its combination on the yield of vanillin from fermentation were also studied.

2. Methods

2.1 Materials

Oil palm empty fruit bunch fiber (OPEFBF) obtained from Seri Ulu Langat Sdn. Bhd., Dengkil, Selangor, Malaysia was used as the substrate of this study. The fresh OPEFBF was washed, and then sun-dried until moisture content reached at approximately 10-11% wt. (*d.b*). The dried OPEFBF was then cut into size 5-8 cm, and kept refrigerated at 4°C until further usage. Strain of *Pycnoporus cinnabarinus* MCUL 39533 was purchased from BCCM/MUCL (Agro) Industrial Fungi & Yeast Collection, Belgium. The strain was maintained on malt extract slant agar containing 20 g L^{-1} malt extract and 15 g L^{-1} agar at 4°C. Other chemicals used included sodium hydroxide,

HPLC grade methanol, magnesium sulfate, dipotassium hydrogen phosphate, calcium chloride (Merck, Germany), vanillin, D-(+)-maltose monohydrate, diammonium tartrate, yeast extract (Sigma Aldrich, USA) etc. All chemicals were analytical grade and obtained from commercial source.

2.2 Methods

The OPEFBF treatment was carried out by soaking 10% wt. of OPEFBF into 50 g L^{-1} sodium hydroxide solution. The reaction was conducted at 120°C for 120 minutes in an autoclave. The black liquor generated after treatment was separated from the treated OPEFBF through vacuum filtration by using muslin cloth. pH of the recovered black liquor was adjusted to pH 5 by using concentrated sulphuric acid, and then autoclaved at 121°C for 15 minutes. This sterile black liquor was then served as the feeding substrate of the fermentation. For lignin preparation, the collected black liquor was adjusted to pH 2, and then centrifuged at 10000 rpm for 10 minutes using centrifuge Eppendorf 5804 (Hamburg, Germany). The lignin pellet was oven-dried at 105°C overnight. The dried lignin fragment was crushed and ground into fine powder using mortar and pestle. The ready-to-use lignin powder was kept in a tight capped bottle at room temperature until further usage[11].

P. cinnabarinus culture was grown in 100 mL basal medium containing 50 g L^{-1} glucose, 1.842 g L^{-1} diammonium tartrate, 5 g L^{-1} yeast extract, 0.2 g L^{-1} potassium dihydrogen phosphate, 0.0132 g L^{-1} calcium chloride and 0.5 g L^{-1} magnesium sulphate at 30°C and pH 5 with 150 rpm agitation for 10 days[12]. Then, the mycelium was ground with Waring blender and the cell concentration was determined. Mycelium suspension with dried cell weight at 6-7 mg cell mL^{-1} suspension was prepared. About mL of mycelium suspension was inoculated into 100 mL basal medium containing 20 g L^{-1} maltose, 1.842 g L^{-1} diammonium tartrate, 0.5 g L^{-1} yeast extract, 0.2 g L^{-1} potassium dihydrogen phosphate, 0.0132 g L^{-1} calcium chloride and 0.5 g L^{-1} magnesium sulphate at pH 5, and incubated at 30°C with 150 rpm agitation. After 3 days incubation, about 10 mL of black liquor and 1 g of sterile lignin powder was aseptically inoculated into the medium respectively. Sampling started at day 4 and the fermentation ended on day 5. Effect of the presence of resin Amberlite XDA4 and cellobiose on vanillin yield during fermentation were also studied. Either 2 g of resin Amberlite XAD4 or 2 mL of 0.05 mg mL^{-1} cellobiose or both was added into the fermentation medium an hour before feeding respectively[13,14]. Vanillin adsorbed onto the resin Amberlite XAD4 was recovered by addition of methanol during regeneration of the resin. Vanillin yield is the sum of free vanillin presence in the medium and the recovered vanillin from resin Amberlite XAD4 after fermentation. The analysis of vanillin was conducted using HPLC[10].

3. Results and discussion

Initially, the yield of total recovered lignin, vanillin and vanillin precursors, included vanillic acid and ferulic acid presence in the black liquor were quantified. Based on the results obtained, about 21.87±3.44 gram of lignin was recovered from each liter of produced black liquor and 11.46 ppm vanillic acid, 16.61 ppm vanillin and

35.66 ppm ferulic acid was detected. During alkaline treatment, hydroxide anions cleaved the α- and β-aryl ether bonds in the lignin network. As consequences, lignin polymer became fragmented into smaller water/alkaline-soluble fragments. The lignin fragmentation process continued with the cleavage of linkages that holds the phenylpropane units, and thus additional free phenolic hydroxyl group generated[15]. Under alkaline condition, alkaline labile ester bond which linked hydroxycinnamic acid such as ferulic acid within the lignin-hemicellulose network cleaved, and free ferulic acid released into the black liquor[16]. Besides, free ferulic acid also tends to further oxidized to vanillin and vanillic acid in the alkaline medium[17]. Therefore, quantification of vanillin, vanillic acid and ferulic acid content in black liquor prior feeding is necessary to avoid over-estimation of fermentation efficiency of *P. cinnabarinus* in black liquor. Results of the *P. cinnabarinus* fermentation on lignin and black liquor were showed in Table 1.

Table 1. Vanillin yield from the fermentation of lignin and black liquor from OPEFBF by *P. cinnabarinus* ($p<0.05$; n=3).

Compounds	Lignin			Black liquor		
	Before feeding (ppm)	After feeding (ppm)		Before feeding (ppm)	After feeding(ppm)	
		Day 4	Day 5		Day 4	Day 5
Vanillin	n.d	2.64±0.07^{a}	2.53±0.34^{a}	16.61	7.06±0.55^{a}	10.46±0.29^{b}
Vanillic acid	n.d	2.56±0.22	<0.00	11.46	10.99±0.55^{a}	13.63±1.34^{b}
Ferulic acid	n.d	<0.00	<0.00	35.66	15.20±0.44^{a}	22.08±1.23^{b}

Note: a-b : Different alphabets in the same row indicated that there are significant differences ($p<0.05$) between the yield of the compounds from different day of fermentation.
n.d : represents not detected.

The presented results clearly indicated that the recovered lignin was a better substrate than black liquor in *P. cinnabarinus* fermentation for vanillin production. Fermentation of black liquor by *P. cinnabarinus* did not improve the vanillin content. On the contrary, it reduced the yield of initially present vanillin and ferulic acid significantly ($p<0.05$). Consumption of ferulic acid did not productively transform into vanillin by *P. cinnabarinus*. Therefore, we strongly suspected that the presence of series of aromatic compounds in the black liquor exerted some degree of inhibition in the metabolic pathway of *P. cinnabarinus* during fermentation. Content of both ferulic acid (substrate) and vanillin (product) in the fermentation medium affected the productivity and yield of products. Accumulation of end product of its biochemical pathway stops the synthesis of vanillin, while other compounds, such as vanillyl alcohol and vanillic acid, will be generated[18]. Thus, this explained the reason why the yield of vanillic acid had increase on day 5 fermentation. In addition, slight increase of ferulic acid on day 5 fermentation might be due to the release of ferulic acid from lignin present in the black liquor. Absence of the vanillin/vanillic acid/ferulic acid in the medium at the early stage fermentation prevents the end product inhibition to occur with the feeding of lignin.

Due to the superior potential of lignin in vanillin production, further study on the improvement of vanillin yield from OPEFBF was conducted by using lignin as the fermentation substrate. Table 2 showed the vanillin yield from the lignin fermentation by *P. cinnabarinus* in the presence of resin Amberlite XAD4, cellobiose and both resin and cellobiose respectively.

Table 2. Comparison of vanillin yield from lignin fermentation by *Pycnoporus cinnabarinus* in the presence of resin Amberlite XAD-4, cellobiose and both resin and cellobiose respectively ($p<0.05$; n=3).

Fermentation setting	Vanillin yield			
	Day 4		Day 5	
	ppm	mg g^{-1} lignin	ppm	mg g^{-1} lignin
Resin only	5.30±0.27^{Ac}	0.53±0.03^{Ac}	5.08±0.08^{Ab}	0.51±0.01^{Ab}
Cellobiose only	2.67±0.23^{Aa}	0.27±0.02^{Aa}	2.87±0.10^{Aa}	0.29±0.01^{Aa}
Resin + Cellobiose	3.25±0.28^{Aa}	0.33±0.03^{Aa}	6.50±0.75^{Bc}	0.65±0.08^{Bc}

Note: a-c : Different alphabets in the same column indicated that there are significant differences ($p<0.05$) between the yield of vanillin from different fermentation setting.
A-B: Different alphabets in the same row indicated that there are significant differences ($p<0.05$) between the yield of vanillin from different day of fermentation.

Based on the results obtained, it was demonstrated that the highest yield of vanillin was achieved in the fermentation with the presence of both resin Amberlite XAD4 and cellobiose. The vanillin yield was increased by 2.6-fold, compared to the lignin-fed fermentation. During *P. cinnabarinus* fermentation, vanillic acid was produced from ferulic acid via ferulic acid propenoic chain degradation. With the presence of cellobiose, vanillic acid metabolism of *P. cinnabarinus* has been channeled to the production vanillin and vanillyl alcohol via reductive pathway. Cellobiose act as an energy source for the expression of reductive pathway and as an inducer of cellobiose:quinone oxidoreductase which inhibits vanillic acid decarboxylation in methoxyhydroquinone generation[19,20,21]. Addition of cellobiose without Amberlite XAD4 failed to significantly ($p>0.05$) improved vanillin yield. Thus, resin Amberlite XAD4 has been proven to play a crucial role in improving vanillin yield during fermentation. As the accumulated vanillin was toxic to fungi[22], its removal by the adsorbent resin Amberlite XAD4 was necessary to accelerate the production of vanillin in fermentation.

Overall, in this study, about 0.15 g of vanillin was successfully produced from 1 kg of OPEFB. Even though the productivity of vanillin from OPEFBF was still low, the potential of this biotransformation technology in biovanillin production couldn't denied. High accessibility of OPEFBF from the local oil palm industry has created great opportunity for the development of oil palm biorefinery in Malaysia[23,24]. Further study on the optimization of biovanillin production from OPEFBF via fermentation is necessary.

4. Conclusion

Through this study, recovered lignin was found to be more effective than black liquor, as a substrate for biovanillin production via *Pycnoporus cinnabarinus* fermentation. Only 2.53±0.34 ppm of vanillin was produced by *P. cinnabarinus* in 5 days fermentation. However, the vanillin yield had multiplied by 2.6-fold to 6.50±0.75 ppm, when cellobiose and resin Amberlite XAD4 were added during the fermentation. Resin Amberlite XAD4 has been proven to play a significant role in improving vanillin production from OPEFBF lignin. Nevertheless, the yield of vanillin obtained in this study still not achieve at a satisfactory level. Therefore, further study on the optimization of this fermentation was suggested.

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A High Prevalence of Oral Manifestations and Its Profile among HIV/AIDS Patients at UPIPI Dr. Soetomo General Hospital Surabaya 2014

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Abstract

AIDS is one of the greatest public health threats for the human race. Regarding HIV/AIDS infection, oral manifestation can be used as a clinical biomarker to determine stage and progression, as well as to predict the onset of opportunistic diseases. Aiming to discern the profile of oral manifestations among HIV/AIDS patients at UPIPI (Infectious Disease Intermediate Care Unit) of Dr. Soetomo General Hospital, this study applies descriptive observational research with cross-sectional and total sampling method. Samples for this study were taken from medical records of 88 HIV/AIDS patients from July to August 2014. Subjects have the following characteristics: male - female ratio of 1.8:1, aged 26-35 (42.05%), married (75%), highschool educated (52.27), entrepreneur (35.22%), heterosexual oriented (95.45%), and HIV transmission through sexual intercourse (95.45%). Examination of the subjects' oral cavity results in 59.1% of the patients receiving antiretroviral therapy with 69.32% having <200 $CD4^+$ count, 120 cases of oral manifestation, 65 cases (54.17%) of Candidiasis Oral, 31 cases (25.83%) of Angular Cheilitis, 15 cases (12.5%) of Oral Hairy Leukoplakia, 7 cases (5.83%) of Linear Gingival Erythema, and 2 cases (1.67%) of Necrotizing Ulcerative Periodontitis. In conclusion, oral Candidiasis is the most common oral manifestation strongly associated with HIV/AIDS that is mostly transmitted through sexual intercourse by heterosexuals.

Keywords: Epidemology; Oral Manifestation; HIV/AIDS

1. Introduction

Acquired Immune Deficiency Syndrome (AIDS) is a group of symptoms characterizing diseases caused by the decrease or damage of the immune system, whose etiology is the Human Immunodeficiency Virus (HIV)[1]. The number of patients with HIV infection increases annually, infecting the entire world population and becoming a global health problem. HIV has been endangering human health for more than 20 years. By the end of 2012, there were about 35.3 millions of people living with HIV/AIDS worldwide[2]. From 1987 to September 2014, as many as 150,296 people living with HIV and AIDS with a mortality rate equal to 55,799 for HIV and 9,796 for AIDS. In Indonesia, East Java Province is the second province with the highest HIV-infected amounting to 19,249 patients and 8,976 AIDS patients[3]. Dr. Soetomo General Hospital in Surabaya, East Java, is one of the type-A Hospitals which is in charge of handling the largest referral patients throughout eastern Indonesia, including referral handling and treatment of HIV/AIDS[4]. The hospital has a division for treating HIV/AIDS patients called the Infectious Disease Intermediate Care Unit (UPIPI). In 2014, the number of people suffering from HIV/AIDS admitted to UPIPI reached a total of nearly 1300 patients, including 50 new patients every month[5].

Oral health plays an important role in assessing the systemic health of a person's overall health status, with no exception in HIV/AIDS case[6]. Oral manifestations often show early clinical signs of systemic diseases

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including HIV/AIDS. Oral manifestations of HIV infection can be used to classify the stage of the disease, predict morbidity, and show overall health status of a patient[7]. In addition, oral manifestations may become clinically important biomarkers in HIV infection, such as Oral Candidiasis (OC), Angular Cheilitis (AC), Oral Hairy Leukoplakia (OHL), Linear Gingival Erythema (LGE), Necrotizing Ulcerative Gingivitis (NUG), Necrotizing Ulcerative Periodontitis (NUP), Kaposi's Sarcoma (SK), and Non-Hodgkin's Lymphoma (NHL)[6,7]. Accordingly, research on the profile of oral manifestations associated with HIV/AIDS in some regions of the world is important to explain of the epidemic of HIV/ AIDS and can be used as reference for further researches.

This study was conducted to determine and reveal the profile of oral manifestations in HIV/AIDS patients at UPIPI Dr. Soetomo General Hospital Surabaya in 2014.

2. Methods

A descriptive observational study with cross-sectional and total sampling method is applied in this study. The samples consisted of 120 cases in 88 HIV/AIDS patients treated at UPIPI Dr. Soetomo General Hospital from July to August 2014 based on the set criteria. Patients agreed to participate were required to fill in informed consent. Diagnosis of the oral manifestation was based on clinical appearances, and the oral cavity was examined by dentists specializing in Oral Medicine. $CD4^+$ counts and other data were obtained from the patients' medical records and personal interviews.

3. Results and discussion

Table 1. Total Sampling of HIV dan non-HIV Patients at UPIPI from July to Agust 2014

Sample	Total
HIV+	88 (100%)
HIV-	0

Table 2. Gender Distribution of HIV/AIDS Patients

Gender	HIV
Men	57 (64.77%)
Women	31 (35.23%)
Total	88 (100%)

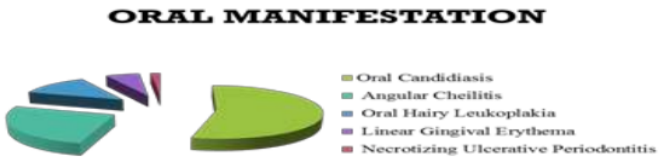

Fig. 1. Prevalence of Oral Manifestation in HIV/AIDS Patients

Table 3. Prevalence of Oral Manifestations among HIV/AIDS Patients at Inpatient and Outpatient Wards of UPIPI

Oral Lesion	Inpatient	Outpatient	Total Cases
Oral Candidiasis	53 (44.17%)	12 (10%)	65 (54.17%)
Angular Cheilitis	31 (25.83%)	-	31 (25.83%)
Oral Hairy Leukoplakia	13 (10.83%)	2 (1.67%)	15 (12.5%)
Linear Gingival Erythema	6 (5%)	1 (0.83%)	7 (5.83%)
Necrotizing Ulcerative Periodontitis	2 (1.67%)	-	2 (1.67%)
Total	105 (87.5%)	15 (12.5%)	120 (100%)

Table 4. Distribution of HIV/AIDS Patients Based on Educational Background

Education	Total
Elementary	17 (19.32%)
Junior High school	16 (18.18%)
Senior High school	43 (48.86%)
Bachelor's degree	10 (11.36%)
Uneducated	2 (2.27%)
Total	88 (100%)

Table 5. Distribution of HIV/AIDS Patients Based on Occupation

Job	Total
Private Employee	24 (27.27%)
Entrepreneur	29 (32.95%)
Civil servants	4 (4.55%)
Labor	22 (25%)
Unemployee	9 (10.23%)
Total	88 (100%)

Table 6. Distribution of HIV/AIDS Transmission at UPIPI

HIV Transmission	Total
Sexual Intercourse	84 (95.45%)
Injected Drug User	4 (4.55%)
Total	88 (100%)

Table 7. Distribution of HIV/AIDS Patients at UPIPI Based on Sexual Orientation

Sexual Orientation	Total
Heterosexual	84 (95.45%)
Homosexual	4 (4.45%)
Total	31 (100%)

Table 8. Distribution of HIV/AIDS Patients at UPIPI Based on Marital Status

Marital Status	Total
Married	66 (75%)
single	22 (25%)
Total	88 (100%)

Table 9. Distribution of ARV user in HIV/AIDS Patients at UPIPI

ARV	Total
Yes	52 (59.1%)
No	36 (40.91%)
Total	88 (100%)

Table 10. The distribution of age, gender and CD4 + count value in patients with HIV

Age (year)	$CD4^+ < 200$ sel/mm^3		$CD4^+$ 200-500 sel/mm^3		$CD4^+ > 500$ sel/mm^3		total
	Male	female	male	Female	male	female	
16-25	7	3	1	1	2	0	14 (15.91%)
26-35	15	8	5	4	3	2	37 (42.05%)
36-45	9	4	1	2	0	1	17 (19.32%)
46-55	6	3	1	1	1	0	12 (13.64%)
>55	4	2	1	0	0	1	8 (9.1%)
Total	41	20	10	7	6	4	88 (100%)
	61 (69.32%)		17 (19.32%)		10 (11.36%)		

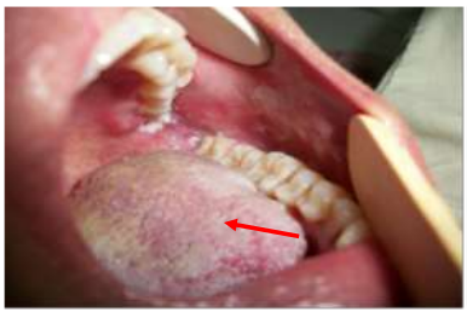

Fig. 1. Oral Candidiasis

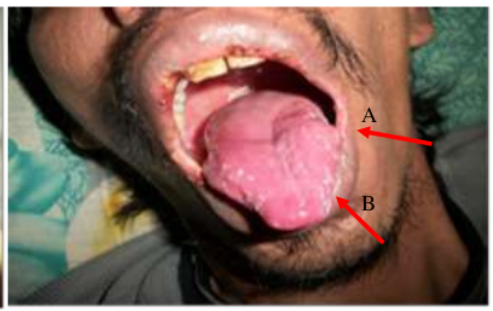

Fig. 2. Angular Cheilitis (A) and Oral Candidiasis (B)

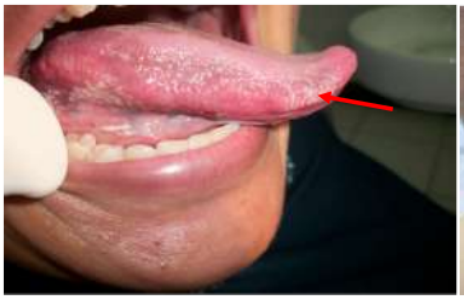

Fig. 3. Oral Hairy Leukoplakia

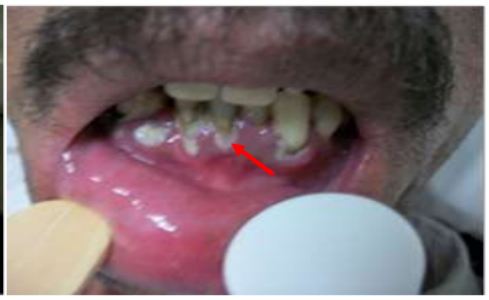

Fig. 4. Necrotizing Ulcerative Periodontitis

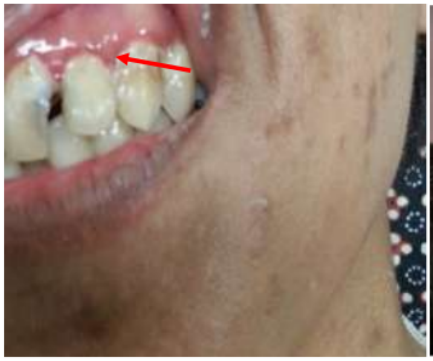

Fig. 5. Linear Gingival Erythema

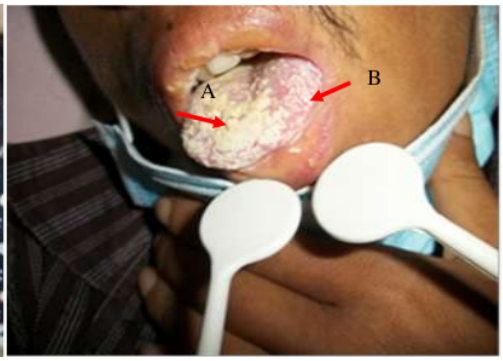

Fig. 6. Oral Candidiasis (A) and Oral Hairy Leukoplakia

Human Immunodeficiency Virus (HIV) is a retrovirus. Retroviruses are viruses that have the ability to use the host RNA and DNA to form DNA virus with an incubation period of about 5 to 10 years. HIV infects the body with a long incubation period (latent period), and is able to cause symptoms of a disease called Acquired Immunodeficiency Syndrome (AIDS)[1]. HIV infection is a viral infection that lowers the immunity of the body significantly and progressively. HIV-specific has an affinity with $CD4^+$ (helper T Cells). HIV attacks and disrupts homeostasis efforts made by the body so that the host is susceptible to opportunistic infections. People with HIV/AIDS sustain immunodeficiency syndrome that cause a variety of clinical manifestations including clinical manifestations that appear in the oral cavity[8]. Disorder of immunity system can lead to an unbalanced condition of ecosystem in the oral cavity. Normal flora in the oral cavity from commensal becomes pathogen that it can cause opportunistic infections in the oral cavity[12]. Oral manifestations may indicate systemic conditions of person and an initial clinical signs that can predict the progression of HIV infection into AIDS[9].

This study was conducted at the Infectious Disease Intermediate Care Unit (UPIPI) Dr Soetomo General Hospital with regards to the fact that the hospital is the second largest referral center in Indonesia, serving Eastern Indonesia. The hospital is owned by the Government of East Java province and has served in the health sector since 1938. It also has become one of the seven hospitals designated by the Ministry of Health as a Pilot Project of HIV/AIDS service since 2010[5].

This study reveals (see Table 1) that there were 120 cases of oral manifestations in 88 HIV/AIDS positive patients. Table 2 shows that the number of people living with HIV/AIDS consisted of 57 male patients (64.77%) and 31 female patients (35.23%). Meanwhile, the subjects on a previous study by Hartanto[10] at UPIPI Dr. Soetomo General Hospital in 2011 consisted of 69% male and 31% female patients. These differences occur due to differences in lifestyle, habits, and behavior between the sexes. As men are more likely to have multiple partners, they have higher risk for contracting HIV/AIDS, whereas female patients are usually victims who contracted HIV/AIDS from husbands who frequently change partners.

In Fig. 1, OC was commonly found in HIV/AIDS, with as many as 65 cases (54.17%) and followed by the AC with 31 cases (25.83%). Table 3 shows 65 cases of OC (54.17%), consisting of 53 OC cases in Inpatient (44.17%) and 12 cases of OC in Outpatient (10%). The number of OC cases encountered was more than that in Hartanto's study[10] on Inpatient of Dr. Soetomo Hospital in 2011, which was 31 cases (66%). According to Suyoso[11], OC often arises in people with HIV/AIDS due to immunodeficiency which enables fungal colonies in the oral cavity turn from commensal into pathogenic. Thus far, OC in HIV/AIDS is believed as opportunistic infections as a result of decreased immune system and closely related with HIV/AIDS infection.

Table 1 shows that there were 31 cases found in the overall AC Inpatient (25.83%). The number of such cases was more than that of Hedge[5] in India in 2012 with 16 cases (12.8%) and Davoodi[12] in Iran in 2014 with 17 cases (17%). The difference in results was attributed to differences in customs and behaviors in each community groups, including habits related to healthcare. According to Kartono *et al*[13], AC occurs due to HIV/AIDS infection resulting in immunodeficiency so that bacteria such as *Staphylococcus aureus*, streptococci and commensal fungal Candida in the oral cavity become pathogenic. Candida has corresponding specific receptors on the cell wall of bacteria *Staphylococcus aureus* adhesin and agglutinin receptor-like Protein Sequence 3 (AlS3P) - one of the hyphaespecific genes.

In Table 3, there were 15 cases of OHL (17.04%) consisting of 13 cases of OHL in Inpatient (10.83%) and 2 cases of OHL in Outpatient (1.67%). The number of such cases was less than that that found in Davoodi[12] in Iran in 2014 with 17 cases (17%) and Hedge[14] in India with 16 cases (12.8%). These differences depend on the levels of $CD4^+$ in HIV/AIDS patients at the time of inspection. OHL reflects the condition of decreased level of $CD4^+$ so that these lesions can be used as a clinical biomarker of $CD4^+$ condition with HIV/AIDS[10]. OHL often arises in people with HIV/AIDS who have $CD4^+$ count of 200-500 cells/mm^3, especially in $CD4^+$ cell <300/mm^3. OHL appears in HIV/AIDS patients with a low immune system so that the EBV virus that was initially dormant

becomes pathogen[12].

The most common periodontal manifestation patients with HIV/AIDS was Linear Gingival Erythema as found in 7 cases (7.95%), consisting of 6 cases of LGE in Inpatient (5%) and 1 case of LGE in Outpatient (0.83%). Meanwhile, there were only 2 cases (1.67%) of Necrotizing Ulcerative Periodontitis (NUP) found in the Inpatient. The number of such cases was more than that found in Hartanto's[10] study in 2011, in which there were only 5 cases (11%) of Inpatient UPIPI LGE and no case of NUP. Such difference may occur due to the use of a larger sample in this study. It also indicates a tendency of oral manifestations of differences arising from each time. According to Reznik & O'Daniels[15], oral manifestations of periodontal lesions tend to rise not because of the condition of plaque or calculus but due to immunodeficiency that occurs in people with HIV/AIDS. NUG, NUP and LGE may arise when the number of $CD4^+$ count is less than 200 cells/mm^3. The decrease of immunity and inflammatory responses has resulted in disruptions that develop commensal microorganisms into pathogens that cause NUG and NUP. NUG occurs when there is necrosis of gingival tissue due to marginal gingivitis as *Bacteroides melaninogenicus* and commensal bacteria symbiosis, such as *Bacillus fusiform* and *Borellia vincentii*. NUG will continue to develop into NUP which eventually results in periodontal destruction. Clinically, it can be seen from the surface layer of the tooth root surface or cementum that can cause teeth mobility. According to Hedge[5], LGE arises due to Candida infection in subgingival which triggers an inflammatory response in the free gingival margin so it will appear like a red line and sometimes involve attached gingiva. According to Aas[16], microbiological examination of subgingival plaque on LGE in HIV/AIDS patients with a high viral load and low $CD4^+$ levels detected *Saccharomyces cerevisiae* as the only species of fungi in LGE, whereas *C. albicans* was not detected. *C. albicans* were detected on LGE in patients with HIV/AIDS with a low viral load and high levels of $CD4^+$. Nonetheless, Pathobiogenesis of LGE has not been fully explained yet.

Table 4 reveals that people with HIV/AIDS educated in senior high school comprised as many as 43 patients (48.86%). Accordingly, Spiritia[17] also found 50.8% people with HIV/AIDS with senior high school education. Education is an important tool to gain knowledge, including knowledge regarding HIV/AIDS. It is generally assumed that the higher the level of education a person has, the more concern he or she has over his or her health. However, in fact there are many people living with HIV. Therefore, counseling and prevention of HIV/AIDS are required to raise awareness of HIV/AIDS.

Table 5 shows that most HIV/AIDS patients work as Entrepreneur with a total of 29 patients (32.95%), while the rest works as civil servant, employee, student, or labor. Occupation surely has an effect on lifestyle and behaviors as they relate to income. The higher the income will likely affect a person to lead a more modern lifestyle that includes free sex and drug use, which are risk factors for HIV/AIDS[17].

Table 6 illustrates the prevalence of HIV/AIDS transmission among the patients, in which the majority or as many as 84 patients (95.45%) were infected through sexual intercourse, while the other 4 patients (4.55%) were infected through drug-way syringe (IDUs). These data are consistent with the data from KPAI[18] in 2012 in which as many as 204 patients (53.40%) contracted through sexual intercourse, 161 patients (42.15%) due to injecting drug use, and 6 patients (1.57%) through perinatal, while for the remaining 11 patients the cause was unknown (2.88%). In terms of the mode of transmission, the proportion of HIV/AIDS through sexual intercourse (both heterosexual and homosexual) dominated, reaching up to 60%, and through the syringe by 30%, while the rest varied from perinatally infection, blood transfusions and through exposure at work. This finding reflects an iceberg phenomenon where the actual incidence may still be higher than the actual number reported.

In Table 7, based on the sexual orientation, the majority of people with HIV/AIDS were heterosexuals. Based on Spiritia[17], in 2014 the highest percentage of AIDS risk factors are at risk of heterosexual sex (67%), MSM (Men Sex Man) (6%), the use of non-sterile needles to IDUs (6%), and from mother to child HIV positive (4%). Meanwhile, Table 8 shows that people with HIV/AIDS were mostly married; therefore, the transmission of HIV/AIDS occured between married couple from husband to his wife or from wife to her husband through sexual intercourse with heterosexual orientation.

In Table 9, there were as many as 52 HIV/AIDS patients (59.1%) using ARV in order to suppress viral load and increase $CD4^+$ counts. In this study, oral manifestation was most prevalent in the 26-35 year age group with as many as 37 patients (42.05%). Finally, Table 10 summarizes Bodhade's[19] research in India on HIV/AIDS patients over the age of 30. Symptoms of HIV/AIDS infections usually appear within 7-10 years after HIV infection, hence it is estimated that the age of the patient at the time of infection is still fairly young at around 18-27 years old, a fairly productive age group as workers as well as reproduction. The large number of people living with HIV/AIDS in young age indicates that during this period of mental development to the maturation stage, people tend to become easily affected by environmental conditions and negative lifestyles, including sexual promiscuity and the use of narcotic drugs and syringes, which are high risk factors for contracting HIV/AIDS[17]. This further proves that in the productive age group, oral manifestation is mostly found in people with HIV/AIDS whose immune system is low. In people with HIV/AIDS, low immune system affects the function and the number of macrophages and neutrophils. Accordingly, phagocytosis process that can eliminate fungal infections, bacteria, viruses become obstructed.

4. Conclusion

Based on discussion above, the prevalence of oral manifestations in patients with HIV/AIDS at UPIPI Dr. Soetomo General Hospital in 2014 revealed Oral Candidiasis as the most common oral manifestation strongly associated with HIV/AIDS. In this study, HIV/AIDS was mostly transmitted through sexual intercourse infecting persons with heterosexual orientation. In conclusion, oral manifestation that is closely related to HIV/AIDS infection can be used as a promising clinical biomarker to determine stage and progression, as well as to predict the onset of opportunistic diseases.

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The Isolation of Duck Ceca Bacteria as Probiotics to prevent Salmonellosis

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Abstract

This study aims to find out bacteria that can prevent Salmonella infection. Several healthy ducks were slaughtered and then approximately 1 g of ceca juice was taken from them. Each juice was diluted several times with sterile distilled water and grown in MRS media. It was then incubated 37°C for 48 hours. The morphology and inhibitory activity of the grown isolates against Salmonella bacteria were examined by superimposing isolates on the media that have been overgrown with *Salmonella* bacteria (1×10^7 CFU/ml). Four colonies of bacteria were obtained, all of which were Gram positive. One isolate was coccus form and three isolates were rod form. Three isolates showed inhibition against *Salmonella pullorum* with 15-17 mm diameter of clear zone, while one isolate against *Salmonella enteritidis* has 7-9 mm clear zone diameter. This suggests that the isolates obtained from duck ceca are more dominant in inhibiting the proliferation of *Salmonella pullorum* than of *Salmonella enteritidis*.

Keywords: bacteria isolation; duck ceca; probiotic; *Salmonella pullorum; S. enteritidis*.

1. Introduction

Livestock product is a source of nutrition for human life, but it can also endanger health if it is not safe. Therefore, food security is an absolute requirement to prevent zoonotic and food-borne disease[1]. Food-borne disease is a human disease caused by food contaminated with pathogenic bacteria. Bacterial diseases that could potentially cause food-borne diseases are Salmonellosis, Shigellosis, Campilobacteriosis, Botulismus and *Eschericia coli* infection[2]. Chicken is one of important sources of transmission of *Salmonella*. *Salmonella* can be transmitted to human, especially when people consume foods that are not well cooked or from infected animals[3].

Probiotic in poultry serves as a growth promoter[4]. It also maintains the balance of beneficial microbes in the digestive tract, eliminates microbial pathogens including *Salmonella*[5], increases the surface area of the intestine, as well as reduces the macroscopic and microscopic lesions caused by bacterial infection[6]. Alkhalf *et al*[7] points out that the probiotic in broiler chickens can lead to the increased levels of antibodies to the ND virus significantly.

Microbes in the ceca can protect the poultry from bacterial infections and affect the function of the small intestine to improve the absorption of nutrients affecting the growth rate[8]. The inoculation of bacterial cultures from adult chicken ceca is proven to increase the growth rate of broiler chickens in the first 3 weeks and prevent *Salmonella* infection[9]. Therefore, the bacteria in the poultry ceca can be used as a source of probiotics. Ducks are known to be more resistant to infection of Avian Influenza virus than chickens[10]. This is likely due to bacteria in the duck ceca are able to stimulate immunity so that ducks infected with AI virus show no symptoms of illness.

This study aims to isolate bacteria from the duck ceca, which has the potential to inhibit the growth of *Salmonella* bacteria that can be used later as probiotics to protect broilers from *Salmonella* infection.

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2. Methods

The research materials are adult ducks purchased at the animal market in Surabaya, MRS media (Becton, Dickinson) and Bactoagar (Becton, Dickinson).

2.1 Isolation of probiotic bacteria

Ten healthy adult ducks were slaughtered. The ceca were taken aseptically afterward. The juice of each cecum was weighed 1 g and diluted with 10 ml of sterile distilled water. The dilution was repeated 10 times. Each of isolates was further grown in MRS medium (Man Rogosa Sharpe) and incubated at 37°C for 48 hours. The isolates were refined several times in the MRS medium to make them pure[11].

2.2 Inhibition test against pathogenic bacteria

The isolates were then grown in MRS broth and incubated at 37°C for 24 hours. Colonies that grew later were laid on sterile paper disks and were incubated for 5 minutes. Isolates were then superimposed on the media that had been overgrown with *Salmonella* bacteria (1×10^7 CFU ml^{-1}) and incubated at 37°C for 24 hours. Isolates which showed wide diameter of clear zone would be chosen as seed isolates.

3. Results and discussion

The culture of duck cecum juice on MRS medium (Man Rogosa Sharpe) shows that some of bacteria colonies grow. Pure bacteria culture is subsequently colored with Gram stain. In Gram stain, it is commonly known that these bacteria exist in the form of cocci, short rods and long rods. All isolates are Gram-positive as shown in Table 1.

Tabel 1. Macroscopic and Microscopic picture of Four Kinds of Cellulolytic Bacteria Colonies Isolates

Character	Isolat A-2	Isolat A-3	Isolat C-2	Isolat I-2
Macroscopic			173	
Color	White	White	White	White
Form	Round	Round	Round	Round
Edge	Smooth	Smooth	Smooth	Smooth
Elevation	Convex	Convex	Convex	Convex
Texture	Slimy	Slimy	Slimy	Slimy
Microscopic				
Gram	Positive	Positive	Positive	Positive
Form	Coccus	Short-Rod	Rod	Rod

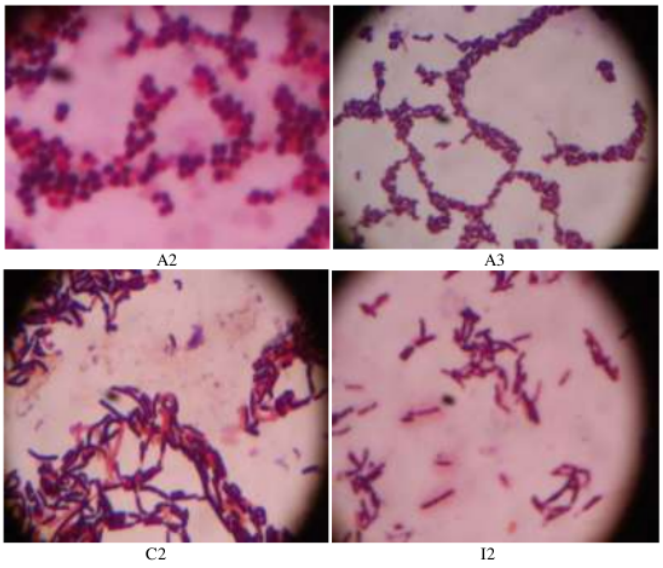


Fig. 1. Bacterial isolates in duck ceca on Gram staining.

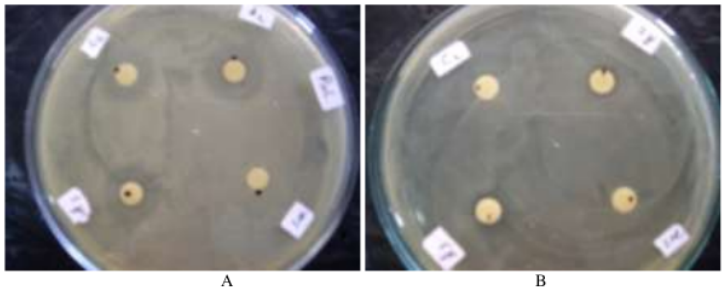
Each paper disk that already contained isolates was taped over with *Salmonella* culture. After an incubation period was completed, isolates isolated in the paper disk successfully inhibited the proliferation of *Salmonella* bacteria by forming a clear zone as shown in Table 2.

Table 2. Inhibitory Activity of Duck Ceca Bacteria Against *Salmonella pullorum* and *Salmonella enteritidis*

Isolate	Clear Zone Diameter (mm) in *Salmonella pullorum* Culture	Clear Zone Diameter (mm) in *Salmonella enteritidis* Culture
A2	17	9
A3	17	9
C2	15	7
I2	5	5

Jozefiak *et al*[12] stated that cecum is a fermentative organ and the highest microbial populations in the gastrointestinal tract. However, it is also a place that allows the infection of pathogenic bacteria such as *Salmonella* sp. to take place. There are five isolates of bacteria found in the poultry ceca. They are *Lactobacillus, Bifidobacterium, Salmonella, Campilobacter* and *Eschericia coli*.

Fig. 2 shows that some isolates have inhibitory effect on *Salmonella pullorum* by forming a clear zone with the diameter of 15-17 mm. On the other hand, isolates which are against *Salmonella enteritidis* only form a clear zone with the diameter of 7-9 mm. This suggests that the isolates obtained from duck ceca are more dominant in inhibiting the proliferation of *S. pullorum* than of *S. enteritidis*.



A B

Fig. 2. Inhibitory test of duck ceca bacteria to *Salmonella pullorum* (A) and *Salmonella enteritidis* (B).

Surachon *et al*[11] found 56 isolates that have inhibitory effect on *S. enteritidis*, but only 13 isolates have a clear zone of 19 mm diameter. Three isolates can survive at pH 2.5 for 18 hours, and one of the isolates was identified as *Lactobacillus salivarius* TP 4.2-2.

Pullorum disease spreads widely and almost all countries in the world are already infected[13]. In some developing countries, the infection of *S. pullorum* is familiar and Pullorum disease is a threat to the poultry farm[14,15]. Economic losses caused by Pullorum disease are decreasing, particularly for egg production, hatchability reduction, as well as morbidity and mortality which can reach 80-100% [16].

S. pullorum persists in the reproductive tract of chicken, as the consequences could be spread vertically to the chicks via trans-ovarian transmission of bacteria into the hatching egg[17]. The horizontal spread can be transmitted through the water, feed, cage equipment, or through intermediaries such as insects or rodents[18], as well as wild birds[19].

S. enteritidis is the causative agent of gastroenteritis in humans. Patients usually become infected by eating unwell-cooked poultry products[20]. In chickens, *S. enteritidis* does not show significant symptoms disease, but it can infect multiple organs such as ovaries that can infect eggs. *S. enteritidis* in chickens is a series of important *Salmonella* infection. Some strategies are developed by the researchers to eradicate *Salmonella* from chicken farms, for example by biosecurity, vaccination, acidification of feed or drinking water, food modification, and the use of feed additives including probiotic, prebiotic and synbiotic[21].

Lactic Acid Bacteria (LAB), particularly *Lactobacillus* sp., which play a role in the health and the digestive system in animals and humans, is an important probiotic among all probiotic bacteria. LAB have the ability to ferment, produce lactic acid and antimicrobial substances and colonization in the digestive tract of the hosts[22,23,24]. In the poultry industry, LAB have commonly been used as probiotics to stimulate the growth of and control the pathogenic agent in the gut (eg. *Salmonella* sp.) for several decades[25].

It can be concluded that several bacterial isolates can be isolated in the duck ceca to potentially inhibit the proliferation of *S. pullorum* and *S. enteritidis*. It is shown that the isolates are more dominant in inhibiting the proliferation of *S. pullorum* than of *S. enteritidis*.

4. Conclusion

In this study we found that all *V. alginolyticus* strains isolated from the environment and diseased fish produced several extracellular enzymes and the results of AP-PCR patterns also demonstrated that these isolates are genetically heterogeneous. However, further research on protein expression of this species may clarify their role in bacterial pathogenesis.

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Predominant Genotype of Hepatitis B Virus in HBsAg Positives Patients on Maintenance Hemodialysis, in Surabaya, Indonesia

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Abstract

Genotype analysis of Hepatitis B Virus (HBV) in HBsAg positives patients on maintenance hemodialysis in Surabaya was performed. Twenty two HBV DNA were detected from 22 serum samples from hemodialysis patients with positive HbsAg. All 22 positive HBV DNAs were sequenced and phylogenetic tree was constructed using Genetyx10 version computer program. The predominant genotype of HBV from HBsAg positive patients on maintenance Hemodialysis in Surabaya Indonesia in this study belonged to genotype B.

Keywords: Hemodialysis; HBsAg positives; Hepatitis B Virus; Predominant Genotype.

1. Introduction

Hepatitis B virus (HBV) infection is a major health problem and a major cause of liver diseases in the world. Infection of HBV can be transmitted parenterally[1]. Patients on maintenance hemodialysis are considered a high risk group to acquire hepatitis B virus (HBV) infection[2].

Based on complete or partial genomes, HBVs were classified into 10 genotypes, designated as A–J[3,4,5]. Classification of the HBV genotype was based on >8% intergenotype and <4% intragenotype divergences nucleotides. In the previous studies, HBV genotype B, C and D were found in Indonesia[6].

The aim of this study was to determine the predominant genotype of HBV in HBsAg positive patients on maintenance hemodialysis, in Surabaya, Indonesia.

2. Methods

Serum samples were obtained from 22 hemodialysis patients on maintenance hemodyalisis in Dr. Soetomo Hospital Surabaya, Indonesia. All of the serum samples were Hepatitis B Surface Antigen (HBsAg) positive using AUSAB enzyme immunoassay (Diagnostic Division, Abbot Laboratories), which were then subjected to Polymerase Chain Reaction (PCR) using pairs of primers based on surface region of HBV genes. Alanine aminotransferase (ALT) serum levels were determined by using ALT (ALAT/ GPT) FS* (IFCC mod.) test kit (Diagnostic Systems International (Diasys), according to the manufacturer's instruction.

First-round PCR was conducted for over 35 cycles, each of which consisted of 1 minute at 94°C, 1 minute at 55°C, and 2 minutes at 72°C. The primers used to amplify a portion of the surface region of HBV were selected on the basis of the sequences reported to be conserved among many HBV isolates, P7 (sense) and P8

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(antisense)[7]. The PCR products were electrophoresed in an agarose gel containing ethidium bromide and visualized by UV illumination. If there was negative result, second-round PCR was performed under the same condition, using the primers HBS1 (sense) and HBS2 (antisense)[8]. The PCR products were then electrophoresed as mentioned previously.

Direct sequencing of the DNA positive samples were performed using Sequencing kit (Perkin-Elmer) and ABI 310 sequencer DNA (Applied Biosystems, Inc.). Phylogenetic analysis of the HBV nucleotides obtained in this study and those available from international DNA data banks were compared by multiple sequence alignment. Phylogenetic tree was later constructed using Genetix 10 version computer program.

3. Results and discussion

Characteristics of the sampels are presented in Table 1. The genotype of HBV in this study belong to genotype B, presented in phylogenetic tree Fig 1.

Table 1. Characteristics of patients on maintenance hemodialysis

Patients	Male	Female	Total
No. of patients	16/22 (72.7%)	6/22 (17.3%)	22 (100%)
Mean Age ± SD (years) (range)	48.53 ± 11.59 (29 – 71)	47.5 ± 6.09 (38-55)	46.05 ± 10.18) (29 -71)
Mean SGPT ± SD (IU/L) (range)	19.51 ± 11.59 (16 - 53)	18.83 ± 5.91 (11 – 22)	19.33 ± 10.22 (11 – 53)
Mean HBsAg ±SD (range)	1.68 ± 1.10 (0.115 – 2.588)	1.68 ± 1.17 (0.156 -2.425)	1.68 ± 1.09 (0.115 – 2.588)

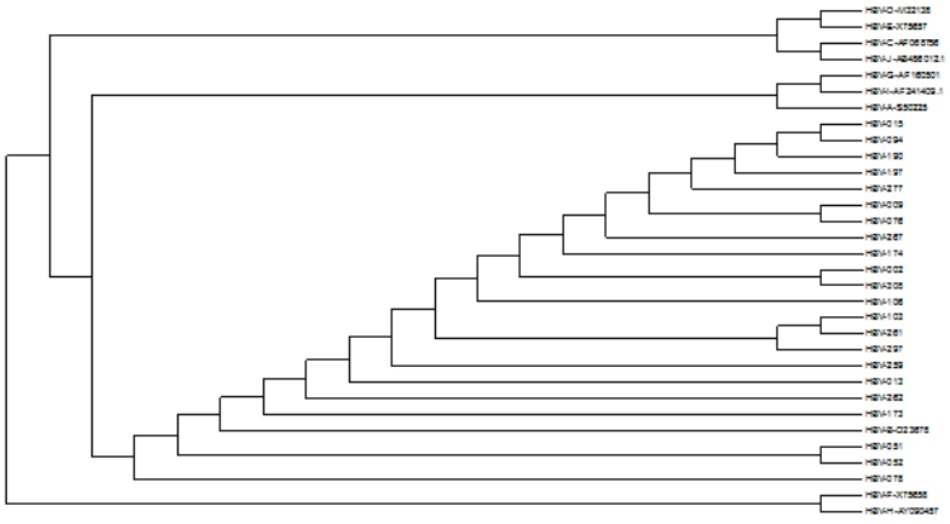

Fig 1. Phylogenetic tree nucleotides sequences of HBV isolates from patients on maintenance Hemodialysis populations in Surabaya, Indonesia, and reported sequences of 10 HBV genotypes A to J.

Serum samples with HBsAg positive were obtained from 22 hemodialysis patients, ranging from 29 to 71 years old with mean age of 46.05 years old, on maintenance hemodialisys in Dr. Soetomo Hospital Surabaya, Indonesia. The number of male patients in this study was greater than that of female. The mean age, ALT, and HBsAg levels were almost the same between male and female.

Twenty two HBV DNAs were detected from 22 sera obtained from hemodialysis patients with positive HBsAg. All 22 HBV DNA positive that have been sequenced and analysed were HBV B genotype. Even though there were HBV genotype B, C, and D found in Indonesia[6], the predominant subtype of HBV in hemodialysis patients in this study was HBV genotype B. The multiple alignment figure of these nucleotides result showed

some consistent differences of nucleotide sequences from Indonesian samples compared to nucleotide of HBV B genotype Acc. No. D23878 from Japan (figure not shown). It is possible that nucleotides caused the different amino acids and serotype. Further analysis of the prediction of amino acid were required to know the serotype of those HBV.

HBV genotypes are widely distributed in different geographic area. The predominat HBV genotype in hemodialysis patients in Brazil[9] and Khuzestan Iran[2] was HBV D genotype. The predominat HBV genotype in a group of patients may reflect the general predominat HBV genotype in that various geographical area.

4. Conclusion

HBV DNA were detected in all HbsAg positive serum samples and the predominant genotype of HBV was B genotype in hemodialysis patients' sera, in Surabaya, Indonesia.

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The Prevalence of Anti-Hepatitis C Virus (anti-HCV) Antibody among Blood Donors in Tuban, East Java, Indonesia

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Abstract

A study was conducted to determine the prevalence of Anti-Hepatitis C Virus (anti-HCV) Antibody among blood donors in Tuban, East Java, Indonesia. By using an EIA test Kit, anti-HCV antibodies were from 500 blood donors in Tuban, consisting of 375 males (75%) and 125 females (25%). Blood donors younger than 20 years old were formed the largest age group. Anti-HCV Antibodies were detected in 7 (1.4%) blood donors, all men. The result in this study showed a lower prevalence than the previous study in Indonesia.

Keywords: Hepatitis C Virus; Anti-HVC; Blood Donor; Tuban Indonesia.

1. Introduction

Hepatitis C Virus (HCV) infection is still a global public health problem. According to the WHO, about 3% or 170 million people, worldwide are infected with hepatitis C. The cases of new Hepatitis C virus infections every year[1]. Infection of HCV transmitted parenterally, so it can be spread through blood transfusion and it also has been known that Hepatitis C virus (HCV) is a major etiologic agent of post transfusion as well as sporadic non-A, non-B hepatitis worldwide[2]. It has been known that HCV is a major causative agent of chronic liver disease such as chronic hepatitis and liver cirrhosis, which often leads to hepatocellular carcinoma. The majority of individuals who acquire HCV infection are a symptomatic. Hepatitis C is a silent killer that may produce no symptoms for a long time and often the first sign of illness occurs when a person's liver stops working or they develop liver cancer[3]. Anti-HCV antibody is a common serological marker for detection of HCV infection. The prevalence of anti-HCV antibodies been reported to vary in different blood donor centers in different areas of the same or different country[4,5,6]. The aim of this study was to determine the prevalence of anti-HCV antibody in Tuban Red Cross Blood Donor Center, East Java, Indonesia.

2. Method

This research laboratory experiment is a cross-sectional study. In total, 500 serum samples were collected from blood donors in Tuban Red Cross Blood Donor Center, East Java, Indonesia, from January 2015 until March 2015. Blood samples were collected in a tube without anti coagulant and allowed to clot, then all samples were transported to the Institute of Tropical Disease, Universitas Airlangga, where the serum sample separation was carried out. Anti-HCV antibodies were detected using a EIA test kit.

3. Result and discussion

The age range of blood donors in this study from the Tuban district were between under 20 years and 70

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years, with an average age of 30.66 years. The youngest was 17 years and the oldest was 69 years old. The majority, 375 (75%) were men and the largest male age group was 21-30 years (20.6%). The largest group of women blood donors was under 20 years (10.4 %). The ages and sex distribution of Tuban Blood Donors are presented in Table 1.

Table 1. Ages and Sex Distribution of Tuban Blood Donors

Sex	Age (Years)						
	≤20 (%)	21-30 (%)	31-40 (%)	41-50 (%)	51-60 (%)	61-70 (%)	Total (%)
Male	100/500 (20.0%)	103/500 (20.6%)	78/500 (15.6%)	72/500 (14.4%)	14/500 (2.8%)	8/500 (1.6%)	375/500 (75%)
Mean	17.77	26.95	34.32	45.13	53.5	64.5	31.32
SD	0.89	2.84	3.00	2.90	2.50	2.62	11.88
Female	52/500 (10.4%)	25/500 (5.0%)	20/500 (4.0%)	20/500 (4.0%)	8/500 (1.6%)	-	125/500 (25%)
Mean	17.71	25.44	34.65	45.25	53.88	-	28.77
SD	1.08	3.38	3.17	2.75	3.09	-	12.24
Total	152/500 (30.4%)	128/500 (25.6%)	98/500 (19.6%)	92/500 (18.4%)	22/500 (4.4%)	8/500 (1.6%)	500 (100%)
Mean	17.75	26.66	34.39	45.15	53.64	64.5	30.66
SD	0.95	3.00	3.02	2.86	2.67	2.62	12.01

In total, 7 (1.4%) of the 500 blood donors were detected as positive for anti-HVC sera and all were men. The results show that the prevalence of HCV is higher in males than females.

Table 2. The Result of Anti-HVC Antibody Detection in Blood Donors

Sex	Anti HVC	
	Positive (%)	Negative (%)
Male	7/500(1.4 %)	368/500 (73.6%)
Female	0/500 (0 %)	125/500 (25.0%)
Total	7/500(1.4 %)	493/500 (98.6%)

Individual blood donors need to be in a healthy condition, because transfused blood from donors must be free of infectious diseases. All 500 serum samples were obtained from 375 male (75%) and 125 female (25%) blood donors in Tuban, East Java, Indonesia, with the mean ± standard deviation of age 30.66 ± 12.01 years. The age range of blood donors in Tuban Red Cross Blood Donor Center varied from under 20 years to 69 years. As people over 60 do not fulfill the criteria for blood donors, 8 (1.6%) blood donors were not allowed to continue as blood donors. The number of younger people (under 20 years old) donating blood was quite high in Tuban (30.4%) with an average of age 17.75 ± 0.95. In this study, the age of blood donors tended to be younger (30.4% under 20 years old), especially women blood donors. The largest age group (10.4%) for women blood donors was the youngest group age (younger to 20 years old) with the mean ± standard deviation of age 17.71 ± 1.08 years. The largest age group (20.6%) for male blood donors was 21-30 years with the mean ± standard deviation of age 26.95 ± 2.84 years (Table 1). This result is in line with Makroo[7] results in North India, in which younger blood donors (18-30 years) were more numerous than older groups.

Hepatitis C Virus (HCV) is a blood-borne virus that causes chronic hepatitis, cirrhosis, and hepatocellular carcinoma. The most common routes of transmission of HCV in developed countries include intravenous drug use, blood transfusions, haemodialysis, needle-stick injuries, tattooing, sexual intercourse and peri-natal infections[3]. Infection of HCV is transmitted parenterally, therefore it can be spread through blood transfusion. In this study, anti-HCV antibodies were positive in 7 sera (1.4%) and all were from male blood donors. This prevalence of a positive anti-HCV antibody in blood donors in Tuban is lower than the prevalence of an anti-HCV antibody (2.3%) in 2234 blood donors in a previous study in Surabaya, Indonesia[3]. Inoue (2000), in a study conducted in Indonesia, showed that 161 out of the 7572 (2.1%) blood specimens were positive for antibodies of HCV[5]. Blood specimens in the Inoue study were collected from 21 of the 27 provinces in Indonesia during a survey of a total of 24 blood banks from November 1992 to February 1993. The prevalence rates of anti-HCV antibodies differ from place to place in Indonesia. The prevalence rates reported from some African countries also differ from place to place[8]. In previous studies, prevalence of 1.44% of 4650 positive anti-HCV blood donors in Cameroon was reported[6]. 6% of 200 blood donors in Nigeria[8] and 1,57% of 15898 blood donors in New Delhi, India[9]. A study from West Pakistan reported that of 1.131 volunteer blood donors studied, 46 (4.1%) were positive for anti-HCV antibodies[10] and in North India, 795 (0.39%) out of 206.022 blood donors were positive for the anti-HCV antibody[7].

Prevalence rates of positive anti-HCV in developed countries are different to those in developing countries. In developed countries, prevalence rates of positive anti-HCV are low and in developing countries, the prevalence rates of positive anti-HCV are higher. This condition involves different socio-cultural practices

involving the use of sharp instruments contaminated by blood and body fluids for procedures such as scarifications, tattooing, circumcision and so on, which are common practices in many developing countries[3]. On the other hand, most developed countries now have low prevalence rates of positive anti-HCV because blood and blood products for transfusion are routinely tested for various blood-borne pathogens including HCV and measures such as the use of sterilized instruments and needle exchange programs for intravenous drug users have also served to reduce the prevalence of HCV infection[3].

In the current study, the age range of blood donors with positive anti-HCV in Tuban Red Cross Blood Donor Center was 29-47 years, and all were men. Makroo (2013) showed that the highest positive anti-HCV prevalence was in the age group 18 to 30 years (0.41%) with the lowest in the age group 51-60 years (0.26%), and showed a decreasing trend with age[7]. Egah (2004) in Nigeria showed that 95% of blood donors were males with the age between 21 and 50 years and 6% positive-anti HCV were also all male[8]. Results regarding the gender of donors are in line with Sajed (2014), reporting from Lahore, Pakistan, who showed that the positive anti-HCV prevalence in men was higher than in women[3]. Jain (2003) reported the age distribution of anti-HCV reactivity showed a maximum prevalence rate of 1.8% in the age group of 20-29 years[9]. In addition, there is a clear declining trend for positive anti-HCV with an increase in age. Khattak (2008) reported that the age of blood donors in Pakistan infected with HCV tended to be higher in ages more than 27 years if they had a history of injections or intra venous drip in the past[10]. Mean while according to Jain[9] and Gao[11], there was no significant difference between men and women in HCV infection.

Based on research in Semarang, Indonesia, from 5800 samples with Transfusion Transmitted Disease obtained from blood donors at the Semarang Red Cross from January 2008 to December 2012, HCV reactive was the third highest cause of infection (821 out of 5800), or 14.1%. The other causes were HBsAg reactive: 3198 (54.94%); Syphilis reactive: 1138 (19.5%) and HIV reactive: 673 (11.5%), as the 1st, 2nd, and 4th causes of infection respectively[1].

It is surmised that the lower Anti-HCV antibody detected in this study was caused by better health management and prevention of infection.

4. Conclusion

The prevalence of positive anti-HCV antibody in blood donors in Tuban, East Java, Indonesia is 1.4%.

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Analyze Activation Marker of Azurophilic Granule (CD63) and CFSE CD11C Expressions of Salivary Neutrophils Insevere Early Chilhood Caries

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Abstract

Early childhood cariesis is a very serious health problem because it is a chronic infectious disease that is infectious. In recent years the view has changed dramatically neutrophils, where neutrophils which are a key component of the first line of defense against pathogen through the process of phagocytosis. Neutrophils also release their granule contents such as defensins, Defensins (α defensins) are stored in the azurophilic granules have microbicidal function shown in enable macrophages to release tumor necrosis factor (TNF) and interferon γ (IFNγ) which promote pro inflammatory. α Defensin also increases the permeability of the epithelial monolayer in vitro and chemotactic effects on T cells, mast cells and dendritic cells. Purpose this research is to analyze activation marker of azhurophilic granules and CFSE CD11c expression of salivary neutrophils in severe early childhood caries (S-ECC). Two groups ie, results mouthwash NaCl 1.5% samples of 20 early childhood caries-free and 20 severe early childhood caries. Salivary neutrophils that collected from severe early childhood caries analyzed using flow cytometry to detect the CD63 and CFSE CD11C expressions. Based on the average value is known that salivary neutrophils expressing $CD63^+$ in early childhood caries-free higher (2.67% ± 0.46) in comparison to the severe early childhood caries (2.32% ± 0.57), likewise salivary neutrophils expressing $CFSECD11c^+$ in early childhood caries-free higher (2.44% ± 0.52) in comparison to the severe early childhood caries (1.57 ± 0.39). Decreased activation marker azhurophilic granules (CD63) and phagocytosis markers (CFSE CD11c) expressions may be one cause in the S-ECC.

Keywords: severe early chilhood caries; neutrophils; azhurophilic granules; phagocytosis.

1. Introduction

Early childhood caries is a very serious health problem. In 2003 the American Academy of Pediatric Dentistry (AAPD) has declared severity of the disease because it is a chronic infectious disease that menular[1]. Dental caries begins after deciduous teeth grow and develop on the surface of the tooth with a very fast and progressive with manifestations of pain, acute and chronic abscesses, fever, swelling of the lips so that the appetite decreased[2].

Severe early childhood caries with decay exfoliation filling of teeth (def-t) > 6 is a form of very destructive because it involves several teeth, including maxillary anterior teeth[3], with the signs is the smooth surface decay on children under three years of age and usually begins as soon as the first tooth eruption and growing rapidly to cavitation stage performance only 6 to12 months[4].

Early childhood caries prevalence is still very high, the results showed that the prevalence reached 80% in developing countries with a percentage five times more than asthma, seven times more than allergies, and fourteen times more than chronic bronchitis[2,5].

In Indonesia, Fitriani research results in 2007 in Semarang, show that at 90.5% in urban and 95.9% rural early childhood suffered from dental caries, while in Surabaya in 2006, the results of research conducted Indawati showed that 74% early childhood suffered from dental caries[6].

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Dental caries is a chronic disease, is multi factorial which began with the shift of microbiology in the complex biofilm (dental plaque) were affected by the consumption of sugar, salivary flow, and behavior[7,8]. The shift toward bacterial plaque flora acidogenic and aciduric in a long time so that the pH of the plaque to be low which is accompanied with many foods containing sucrose and the factors involving oral hygiene, the aging, genetic factors, and changes in the immune system, making conditions the plaque that support the increasing number of species acidogenic and acid uric like *Streptococcus mutans* (*S.mutans*) or laktobasilus[9].

Untreated dental caries can cause more severe effects on the body, including mortality[10]. The poor nutritional intake associated with pain in early childhood can reduce proper diet so that the immune system weakens and becomes more susceptible to infection. Various prevention of dental caries has been done, for example by brush their teeth, fluoridation by topical application and manufacture of vaccines to date have not shown the expected results[11].

In recent years the view has changed dramatically neutrophils, where neutrophils which are a key component of first line of defense against mikroba[12]. Neutrophil not only act as the killing of microbes by phagocytosis, release of reactive oxygen species (ROS) and its antimicrobial peptide but also regulate activation of neutrophils immune responses[13], besides neutrophils has resulted in a variety of cytokines, chemokines and growth factors that become a major contributor in the production pro inflammatory cytokines in the area of infection[14].

Important function of neutrophils in killing pathogenic microbes is phagocytosis, which was significantly more effective due process of opsonization by antibody and complement contained in the surface of microbes. Phagocytosis against microbes can causes oxidative burst process of producing reactive oxygen species are accompanied by degranulation of cytoplasmic granules in the phagosome containing microbes that contains antimicrobial peptides and protease[15].

During phagocytosis, there was internalization of microbes into the phagosome in neutrophils. Receptors involved in the recognition of microbes also activate neutrophils to kill microbes are ingested. A combination of the phagosome with neutrophil granules (lysosomes) leads to the formation of phagolysosome and at this point most of the microbicidal activity[16].

Neutrophil contains three types of primary granules, granules azurophilic (primary granules) contain antimicrobial proteins such asdefensins (HNP 1-3), elastase, cathepsinand proteinase-3 and contains CD63 highly potent in killing microbes both Gram positive and Gram negative, including *S.mutans* which is one of the predominant microbial cause of dental caries.

Based on the above, this study aims to identify risk factors for caries of the aspects of the immune system to identify neutrophil function as innate immunity effect or cells in preventing dental caries in early childhood.

2. Methods

Samples were obtained from kindergarten children in Surabaya region. Examination of dental caries was conducted by measuring its index of def-t, and after it has been examined subjects were divided into two groups: caries-free group and the S-ECC group with def-t more than 6. All subjects at the time of sampling old between 4 to 6 years. Prior to sampling in a sample, the distribution of questionnaire and signed a written informed consent by parents respectively.

Samples of saliva were taken to the isolation of *S.mutans* and from the mouth wash NCL 1.5% for the determination of phagocytic function analyzed by CFSE expression of CD11c and CD63 expression is a marker granulation azurophilic granules on the surface of neutrophils saliva. Sampling was done by researchers and trained person elusing a standard protocol. Subjects should not eat, drink, chew gum, or brushing teeth for 60 minutes before sampling. Having collected the samples was frozen at -80°C for analysis[17].

2.1. Streptococcus mutans isolation from saliva

Isolation of *S.mutans* conducted from saliva samples taken from both preschool children identified as severe caries and caries-free conducted in the following way: isolation and biochemical characterization of *S.mutans* saliva samples were diluted in Brain Heart Broth (BHI), after incubation 24 hours.

Samples were grown on Triptone Yeast Cysteine (TYC) agar medium. Suspected colonies of *S. mutans* maked subculture to conducted biochemical tests using fermented mannitol, raffinose, sorbitol, salicin, esculin and arginine. Isolates were identified as *S. mutans* if positive for sugar fermentation and negative for arginine and subsequently confirmed by Gram staining and catalase test. Isolates of *S. mutans* is stored on -80°C[17].

2.2. Isolation and Counting Total Salivary Neutrophils

Neutrophils in saliva obtained by subjects instructed to rinse with 10 mL of sterile 1.5% NaCl solution as he gargled his mouth but not swallowed for 30 seconds, then expectorationed in sterile glass. This procedure was repeated 4 times. The next sample is centrifuged at a speed of 450 g for 15 minutes, at a temperature of 4°C. The results of further centrifugation pellets are mixed with 2 ml of RPMI medium then the sample in vortex then

filtered sequentially with 20 and 11µm nylon filter19. The results of the filter in the form of a suspension of cells is then counted using a hemocytometer.

2.3. Preparation of the Streptococcus mutans (S. mutans)

S. mutans isolated subsequent cultured in media Brain Heart Infusion (BHI) so that the tube for 48 hours in an aerobic jar. *S. mutans* culture results subsequent taken 1 ose and cultured back in BHI liquid medium for 48 hours in an incubator. S. *mutans* bacteria cultures are then put into the microtube and centrifuged at 12,000 rpm for 10 minutes at a 4°C. Pellets were then washed with PBS and centrifuged again with the speed, time, and temperature are the same. Pellets were then stained with 50 µl solution CFSE: PBS in the ratio 1:20. Bacteria that have dyed subsequent incubated in a 37°C temperature for 30-60 minutes in the dark (microtube coated aluminum foil). After incubation, the bacteria then centrifuged at a speed of 12,000 rpm, for 10 minutes, at a temperature of 4°C. Pellets were then washed with PBS 2 times by means of centrifugation back at the same speed. *S. mutans* bacteria were then killed by heating 60°C for 30-60 minutes. The bacteria were then washed with PBS and measured at 620 nm (0.35). Bacteria opsonisationed subsequent use as much 500 µl serum FCS and incubated at 37°C for 30 minutes. The next steps are conducted centrifugation with the same speed as before. Pellets containing bacteria, calculated using the haemocytometer to be taken and planted in the method of phagocytosis.

2.4. Phagocytosis activity Salivary Neutrophils against *S. mutans*

The principle underlying the process of phagocytosisis caused by bacteria can be labeled with fluorescein isothiocyanate (FITC) and then bacteria difagosit by neutrophils, after it was given a second dye in the form of ethidium bromide (EB) which serves to bind DNA. EB is what will give color to the *S. mutans* is not in phagocytosis because the microbes that have internalized protected by a membrane of phagocytesthat is not exposed to the dye. At Flowcytometer light scattering can be used to isolate a population of neutrophils. The combination of fluorescence and dissemination will identify *S. mutans* that has been labeled, neutrophils, and neutrophil containing *S. mutans* is ingested.

Neutrophils that have been isolated and regulated concentration calculated to be 2 X IO^6/ml. Make sure that the cells are predominantly neutrophils (>95%) and the life (>95%) after the test with trypan blue propidium iodide by flow cytometer, thus cells are now ready to test phagocytosis.

In the phagocytosis test, the addition of 5 ml suspension as much as 2 X IO^6 neutrophils/ml of bacteria to the tube. Then 1 ml was transferred into a tube containing 1 ml of ice cold, 0.9% and 0.02% EDTA saline as a control. The same procedure was repeated every 15 minutes with new aliquot. This will give the results of phagocytosis over a period of 1 hour.

The engulfed of bacteria is determined by measuring green fluorescence at 525 nm in flow cytometry, using 488 nm excitation. To estimate the extra cellular fluorescence, immediately after running each tube add 1 ml of 3 mg/ml trypan blue, and then mixed and measured fluorescence signal again.

2.5. Measurement of CD63 Expression On Salivary Neutrophils

Profile measurement of neutrophil cells using CD63 antibody which is a marker of neutrophil activation. CD63 is the primary granule membranes were expressed on the membrane surface of neutrophils and increased due to the stimulation of the neutrophil cells (Faurschou and Borregaard, 2003) with the signs that can be measured by flow cytometry with the method that has been modified Bjornsson11. The sample used is a sample result of 1.5% NaCl mouth wash that has been isolated neutrophils its. Neutrophil cell suspension that was isolated and then inserted into the microtube that has been filled as much 500 µl PBS. The cell suspension was then centrifuged at a speed of 2500 rpm, for 5 minutes at a temperature of 4°C. Pellets obtained were then stained with antibody extracellular much 50 µl (Biolegend antihumanα-CD63PE), and α-PI Biolegend PE conjugated antibody ratio : PBS is 1: 200.

Cells that have been added antibody is then stored at 4°C for 30 minutes. Then the cell suspension added 1 ml PBS and centrifuged at 2500 rpm, for 5 minutes at 4°C. Pellets then added Biolegend Cytofix Cytoperm as 100 µl and homogenized until well blended. Do incubation in the dark and 4°C for 20 minutes. After incubation, cells were then added 1 mL Biolegend Washperm 1X and then centrifuged at a speed of 2500 rpm, for 5 min at 4°C. Pellets were then added intracellular antibodies, including BD antihuman α-CD64 PerCP conjugated, subsequentin corporated the cell suspension into flow cytometer cuvette, added as much as 300 µl PBS, and mounted on then ozzle to conducted BDFACS Calibur flow cytometer using the machine running. Samples were analyzed by flowcytometry (FACS Calibur flow cytometer, BD BioSciences, SanJose, CA).

Neutrophil gate identified by using density and size with aside angle light scatter and then forwarded to the forward angle light scatter. This compensation is achieved by using FITC and PE antibodies dilebel with the individual. The results are expressed as mean fluorescence intensity (MFI). FACS Calibur from Becton

Dickinson Cell Quests of ware program used for the analysis.

3. Results and discussion

3.1. CFSECD11C expression In Salivary Neutrophils at S-ECC and Free Caries

The test results of two independent samples T test showed that there was a significant decrease in the expression of CFSECD11c in salivary neutrophils S-ECC shown in Table1.

Table 1. The mean and standard deviation salivary neutrophils activated conduct phagocytosis of *S. mutans* were labelled by CFSE dye express $CD11c^+$ in S-ECC and caries-free (%)

Group	n	Mean± standard deviation standard deviation	SD	P Value
Free Caries	20	2.44% ± 0.5	0.52732	0.000 (p<α)
S-ECC	20	1.57 ± 0.39	0.39038	

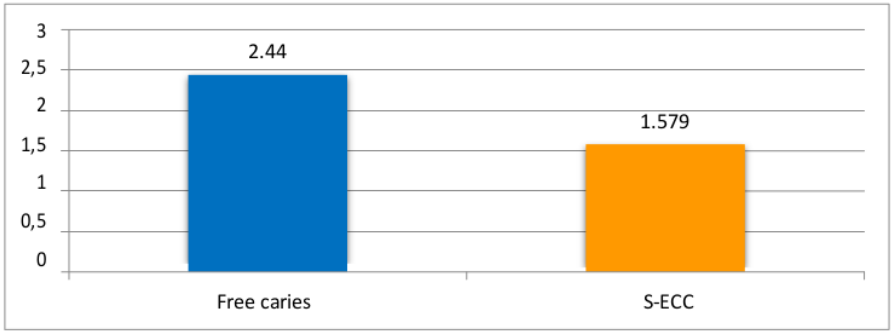

Fig.1. Phagocytosis of bacteria *S. mutans* were labeled by CFSE dye express $CD11c^+$ in S-ECC and free caries (%).

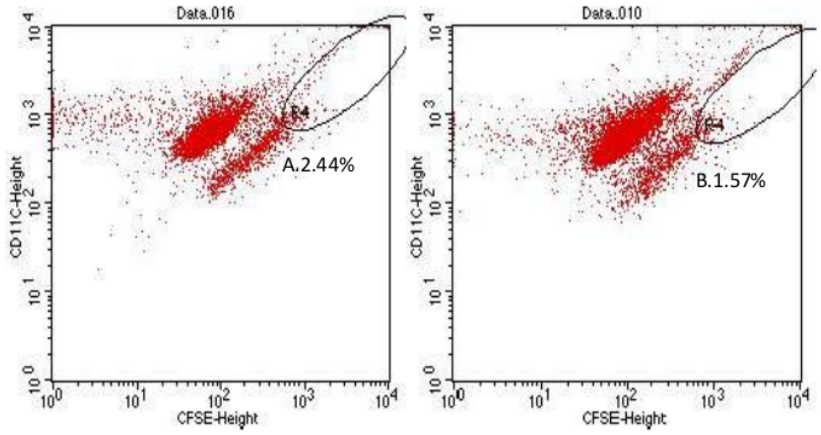

Fig.2. Activated salivary neutrophils conduct phagocytosis against *S. mutans* which in CFSE label edexpressing $CD11c^+$ were detected using flow cytometry on early chihood caries-free (A) and S-ECC (B)

3.2. $CD64^+CD63^+$ Expression On Salivary Neutrophilsin S-ECC and Free Caries

Results of the analysis of activated salivary neutrophils ($CD64^+$) that expresses $CD63^+$ in early Childhood free caries and S-ECC are shown in Table 2.

Table 2. The mean and standard deviation of activated salivary neutrophils ($CD64^+$) that expresses $CD63^+$ in early Childhood free caries and S-ECC (%)

Group	N	Mean± Standard deviation	95%CI	p Value
Free Karies	20	2.67 ± 0.46	2.37–2.96	0.040 (p<α)
S-ECC	20	2.32 ± 0.57	1.96–2.68	

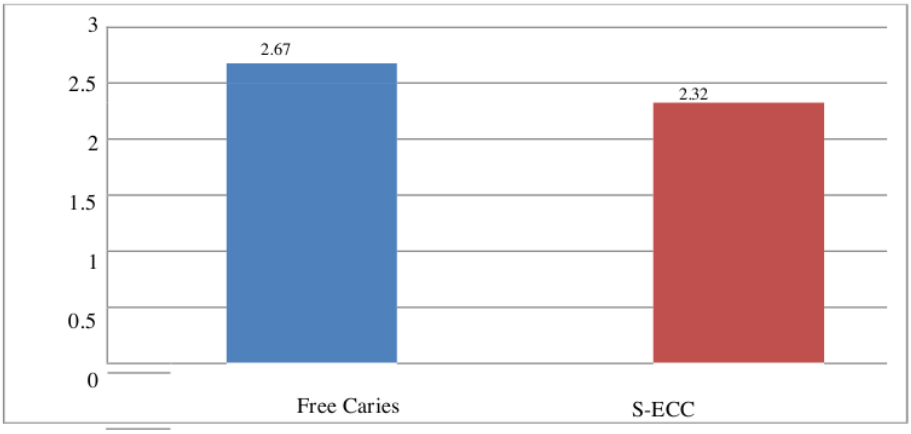

Fig.3. The mean and standard deviation saliva activated neutrophils ($CD64^+$) that expresses $CD63^+$ in early childhood caries-free and S-ECC (%)

The results of flow cytometry analysis using saliva activated neutrophils ($CD64^+$) that expresses $CD63^+$ which has been done using a different test independent 2 samplest-test showed significant value which is smaller than α, this means that there are significant differences in the exp ression of $CD63^+$ between the two groups. Based on the average value of salivary neutrophilsis was known that expressing $CD63^+$ in early childhood caries-free higher (2.67% ± 0.46) compared in S-ECC (2.32% ± 0.57).

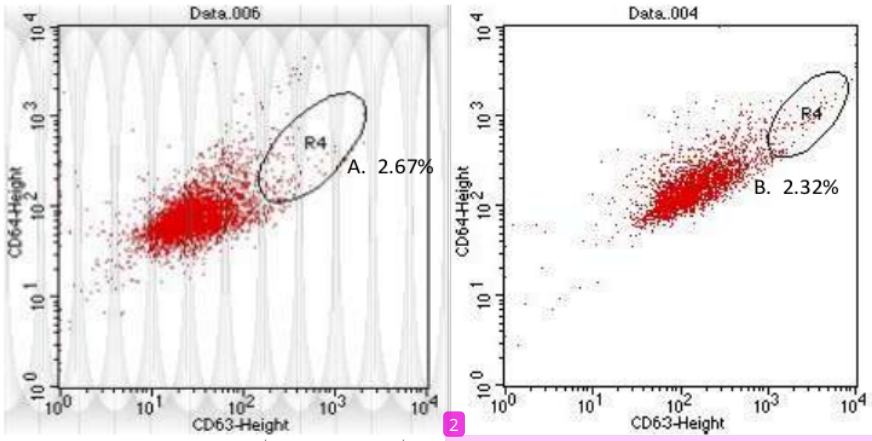

Fig.4. Salivary Neutrophil activated ($CD64^+$) expressing $CD63^+$were detected using flow cytometry in early childhood free caries (A) and in S-ECC (B)

Streptococcus mutans plays an integral role etiology of dental caries in preschool children is an infectious disease transmitted[18], so that *S. mutans* is considered an important predictor as cariogenic bacteria because it is acidogenic (able to produce acid) and acid uric (able to survive in an acidic environment)[3].

In salivary neutrophil first line of defense is the most prominent of immune cells for defense against pathogens microbial. The importance of neutrophils in the host immune system in patients with neutropenia or deffects in neutrophil function which leads to a tendency for the occurrence of serious infections[19].

Neutrophil recruitment process, transmigration, phagocytosis, and activation of highly coordinated to prevent or eliminate infection in humans. In the area of infection, neutrophils bind and engulf microbes through a process known as phagocytosis. Neutrophils recognize the surface-bound or free molecules secreted bacteria, including peptidoglycan, lipoprotein, lipoteichoic acid (LTA), lipopolysaccharide (LPS), CpG-containing DNA, and flagellin. This pathogen molecules known as PAMPs, interact directly with a number of Pathogen Recognition Receptors (PRRS) expressed on the surface of cells, including Toll Like Receptors (TLRs)[20].

Dental caries severity varies greatly between individuals, and this variability may be due to differences in the microflora as well as differences in the immune system in response to oral microflora in general, the immune system in the oral cavity to prevent the invasion of the microflora[21].

Neutrophils are important effect or cells in the first line of defense against pathogenic bacteria through the process fagositosis[22], the disruption phagocytic function against cariogenic bacteria may play an important role in the initiation and progress ion of dental caries. This, our study shows that the percentage of neutrophil phagocytosis against *S. mutans* which is the bacteria that cause dental caries showed significant declines in the S-ECC than in early childhood caries-free.

The ability of the pathogen to avoid internalizing and killings played a central role in the virulence strategies. Pathogens that have been internalized through a process of phagocytosis by neutrophils to developing three strategic defense system to avoid killing intracellularly is the first escape from the phagosome, the second blocking phagosome fusion-lisosome, and the third is the use of a mechanism to allow for the survival of the phagolysosomes[22]. Some species of bacteria including Streptococcus and Staphylococcus have evolved mechanisms to avoid opsonophagocytosis or action of the system complemen[23]. The failure of the existing defense systems in saliva in early childhood in killing the *S. mutans* bacteria due to the possibility that there are strains of *S. mutans* in the oral cavity S-ECC has successfully developed a mechanism to fight the hydrolytic activity of lysozyme. This is supported by studies that say that some Streptococcus have an effective strategy to reduce the bactericidal effect of lysozyme.

Phagocytosis is active receptor-mediated process, in which cells internalize microbial later there cytoskeletal rearrangements, neutrophil plasma membrane extends around the target, initiate a process that ultimately creates a membrane-bound vacuoles called the phagosome. Neutrophil release granule mediators that are released upon degranulation orexocytosis of membrane-bound secretory granules. Neutrophils also have the capacity to release a variety of antimicrobial proteins and intracellular enzymes into membrane-bound organelle called the phagosome containing microbes.

Primary granules (azurophilic) contains many antimicrobial compounds such as myeloperoxidase (MPO), defensin in the form of human neutrophil peptides1-3 (HNP1-3), lysozyme, azurocidin, and serine proteinase elastase, cathepsin G, proteinase 3, esterase N, and so on. These granules associated with phagocytic vesicles release their contents in the phagosome causing microbes that have in phagocytosis[24].

The low expression of CD63 neutrophil saliva in severe early childhood caries may be due to *S. mutans* that has been internalized by phagocytosis of neutrophils through a process that is mediated through FcαR (CD89) or CR1 (CD35) might be able to develop three strategic defense system to avoid killing intracellular namely that the first escape phagosome, the second blocking phagosome fusion-lisosome, and the third is the use of a mechanism to allow for the survival of the phagolysosomes[22].

Phagocytosis by neutrophils causes translocation of granulesto the phagosome which is a marker for the release of the granules contents. Caused by the release of granule contents as the mediator of granulocytes whicha re strictly controlled by a mechanism leading to exocytosis which took place in several phase[25], namely the recruitment of granule exocytosis from the cytoplasm to the membrane targets depend on rearrangement of actin and microtubulecy to skeleton followed by vesiclete the ring and docking, which leads to contact the outer surface of the lipid bilayer membrane around the granules with the inner surface of the target membrane[26].

The role of neutrophils as innate immunity lies in the lysosomal compartment shown in congenital abnormalities which cause the secretion of lysosomal events and lysosomal proteolysis disrupted as happens in Chediak-Higashi[29] Syndrome. And Lefevre Papillon-syndrome[28]. The function of lysosome-associated membrane protein-2(-2LAMP) contained in neutrophil function is critical to the self-cleaning of the oral cavity that regulates natural defense against biofilm formation in the oral cavity. Phagosome fusion-lisosome is very important for the degradation of the internalized pathogens efficiently with lysosomes thus play an important role in the killing of bacteria that do not depend on oxygen[29]. Neutrophil-deficient LAMP-2 caused deficiency of distribution and azurophilic granule fusion, cellular localization lactoferrin contained in the secondary granules which showed a reduction inco-localization of causing disruption of biogenesis, traffic, both types of granulesis a function that contributes to killings *S. mutans*[30] and it is a possibility that lysosome-associated membrane protein-2(LAMP-2) to contribute to fusion with neutrophil granules in phagosomes. Based on the low expression of CD63S-ECC possibility of deficiency LAMP-2 as found in mice that were suffering from periodontitis therefore LAMP-2 deficiency.

4. Conclusion

Decreased activation marker azhurophilic granules (CD63) and phagocytosis markers (CFSE CD11c) expressions may be one cause in S-ECC.

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Insilico Docking Analysis Of *Imperata cylindrica* Pure Compound and Human Thymidylate Synthetase Mutant as Choriocarcinoma Anti Cancer Candidate

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Abstract

Choriocarcinoma is a malignant, belongs to gestational trophoblastic disease. Floxuridine chemo-resistence has been investigated in the role of thymidylate synthase transcript levels. *Imperata cylindrica* pure compound was tymidylate synthetase inhibitory potential agent. This study is explo protein interactions between *imperata cylindrica* pure compound and human thymidylate synthetase mutant variant. In silico docking studies were carried out using AutoDock 4.2. The results showed that flavonol-selected human thymidylate synthetase mutant binding energy (-5.61 to -4.91) when compared with standard (-4.01 to -3.82) and inhibition constant (77.59 μM to 250.28 μM) compared with standard (1.02 mM to -4.15 mM). *Imperata cylindrica* pure compound docking result has more efficient than Floxuridine.

Keywords: Insilico; *Imperata cylindrica*; Human thymidylate synthetase; Anti-cancer

1. Introduction

Gestational trophoblastic disease is a tumours with a wide range of biologic behaviour. Gestational trophoblastic disease refers to both the benign and malignant entities of the spectrum, include hydatidiform mole, invasive mole, choriocarcinoma, and placental trophoblastic tumour. The last three are termed gestational trophoblastic tumours, potentially fatal if untreated[1,2]. Choriocarcinoma is highly malignant tumor of trophoblastic cells. It is considered to be pertinent to molar pregnancy, normal or ectopic pregnancy and abortion[1]. Choriocarcinoma composed of two types of cells, syncytiotro-blasts and cytotrophoblasts. The syncytiotrophoblast is the differentiated hormone secreting component[3]. Choriocarcinoma, is even less common, affecting around 2 to 7 of every 100,000 pr ncies in the United States. Choriocarcinoma and other forms of gestational trophoblastic tumours are more common in many Asian and African countries. Overall, gestational trophoblastic tumors account for less than 1% of female reproductive system cancers[4] .

Several studies have demonstrated that cancer cells have a unique metabolism[5]. Metabolic changes in cancer cells are considered to be fundamental for the transformation of normal cells into ca r cells and are also responsible for the resistance to different types of chemotherapeutic drugs[6]. Among the different metabolic and signalling pathways that are altered in cancer cells, variations invthe expression and activity of several drug metabo ng enzymes play a critical role in drug resistance[7].

TS inhibitors are a new class of compounds that target TS, an enzyme in the folate metabolic pathways, necessary for thy midylate synthesis. By inhibiting TS, these agents decrease de novo thymidylate synthesis, which is necessary for DNA synthesis and repair. Contrary to prior empiric approaches to anticancer drug discovery, the development of new TS inhibitors was based on the targeted and specific design of new molecules and relied on extensive knowledge of the structure, conformation, and properties of TS and the mode of action

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of folate analogues[8].

Flavonoids are a group of more than 4000 polyphenolic compounds that occur naturally in foods of plant origin. These compounds possess a common phenylbenzopyrone structure (C6-C3-C6), and they are categorized according to the saturation level and opening of the central pyran ring, mainly into flavones, flavanols, isoflavones, flavonols, flavanones, and flavananonols[6]. These polyphenolic compounds display a remarkable spectrum of biological activities including those that might be able to influence processes that are dysregulated during cancer development. These include, for example, anti-allergic, anti-inflammatory, antioxidant, antimutagenic, antic-arcinogenic, and modulation of enzymatic activities[7].

They may therefore have beneficial health effects and can be considered possible chemopreventive or therapeutic agents against cancer. This review article will focus on the anticancer activity of flavonoids as well as their molecular mechanisms, since they are among the most promising anticancer agents.

2. Materials and Methodology

The purpose of current study is exploring protein interactions between *Imperata cylindrica* pure compound and human thymidylate synthetase mutant variant to exploit a potentially synergistic interaction to enhance anticancer efficacy. Out of all the entries for Human Thymidylate Synthase Complex (1JU6) were taken from RCSB protein data bank. The active site residues were found to be Arg50, Leu192, Cys195, His196, Gln214, Arg215, Ser216, Asp218, Asn226, His256, Tyr258. The selected mutant groub used in our experiment were single mutant (E23G, V84A, D110H, D250L), double mutant (T53S-Y258F), triple mutant (A17T, D11G, D254E) selected from the article The Journal of Biological Chemistry Vol. 277, No. 39, Issue of September 27, 2002.

Reeds were supplied from farmland in Malang, West Java, Indonesia. Manufacture of extracts and fractions include reeds dry powder was extracted by maceration using methanol at room temperature and the extract was then partitioned with n-hexana and then with ethyl acetate. The separation of ethyl acetate faction was done by gravitation column chromatography (thin layer chromatography) until one spot compound was obtained. Determination of isolated compound structure was done by Nuclear Magnetic Resonance spectroscopy (H-NMR and C-NMR). The result of analysis showed that the isolated compound was belonged to flavonoid group, which called 7,3',5'-trimetoxyflavonol.

We use floxuridine as standart ligand. The structures floxuridine and 7,3',5'-trimetoxyflavonol constructed using Swiss model and evaluated by PROCHECK. In silico docking studies were carried out using AutoDock 4.2, based on the Lamarckian genetic algorithm principle and PyMol. Three important parameters like binding energy, inhibition constant and intermolecular energy were determined.

3. Result and Disccusion

Human Thymidylate Synthase (TS) is an intracellular enzyme that provides the sole de novo source of thymidylate, making it a required enzyme in DNA biosynthesis with activity highest in proliferating cells. The methylation of deoxyuridine monophosphate to deoxythymidine monophosphate is an essential step in the formation of thymine nucleotides. This process is catalyzed by thymidylate synthase, a homodimer composed of two 30 kDa subunits. Human Thymidylate Synthase is also an important target for anticancer agents such as 5-fluorouracil. Thymidylate Synthase inhibitors are a new class of compounds that target TS. By inhibiting Thymidylate Synthase, these agents decrease thymidylate synthesis. Prior empiric approaches to anticancer drug discovery, the development of new TS inhibitors was based on the targeted and specific design molecules and relied on structure, conformation, and properties of TS[11].

Genetic variants of dTMP synthase have been reported. Heidelberger *et al*. reported a possible alteration in the substrate-binding site of dTMP synthase in Ehrlich ascites carcinoma cells, rendering the cells resistant to fluoro-pyrimidines. Similarly, resistance of a human lymphocytic leukemia cell line to FdUrd has been implicated in an alteration in dTMP synthase, causing the enzyme to have a lower affinity for nucleotides. Barbour *et al*. have suggested that a single amino acid substitution in an altered dTMP synthase enzyme from a human colon tumor cell line HCT116 may confer resistance on FdUrd. In the HCT116 enzyme, replacement of tyrosine by histidine at position 33, which is an evolutionarily conserved site, conferred reduced affinity on bot

In some patients, prolonged exposure to a single chemotherapeutic agent may lead to the development of resistance to multiple other structurally unrelated compounds. Chemotherapy resistance remains a major problem to the effective treatment of many tumor types. Resistance can occur prior to drug treatment or may develop over time following exposure. Several mechanisms, including alterations in drug pharmacokinetic and metabolism, modification of drug target expression or function, drug compartmentalization in cellular organelles, altered repair of drug-induced DNA damages, changes in apoptotic signaling pathways or expression of proteins directly affecting cellular drug transport are responsible of anticancer drug resistance[12].

Resistance to chemotherapy presents a major obstacle to attempt to improve the prognosis of patients with Gestational trophoblastic disease. Accordingly, it is important for the management of Gestational trophoblastic disease to elucidate the mechanisms of chemoresistance and to get over the resistance. Recently, the biological characteristics of Gestational trophoblastic disease have been molecular targeted agents including signal transduction inhibitors and anti proliferatif agent.

Women with high-risk Gestational trophoblastic disease present considerable difficulty in management and require combination chemotherapy with a selective use of surgery and radiotherapy. This group may include patients with metastases to the brain, liver, and gastrointestinal tract; complications such as massive bleeding may occur early in the course of the disease. These patients are also likely to develop drug resistance after prolonged chemotherapy[2].

The standard chemotherapy regimen for Gestational trophoblastic disease with high risk of metatastic process is EMA/CO11 (etoposide, dactinomycin, and methotrexate alternated at weekly intervals with vincristine and cyclophosphamide). Newlands *et al*. reported a five-year survival rate of 86%. Adverse prognostic variables are liver metastases and brain metastases. Gestational trophoblastic disease Drug resistance developed in 17% of patients, of whom 70% were salvaged with additional chemotherapy or surgery[2].

Tabel 1. Binding energy, Inhibition constant and Hidrogen bond parameter result

TS mutant	ΔG		*Ki*		Hidrogen Bond	
	Floxuridine	Flavonol	Floxuridine	Flavonol	Floxuridine	Flavonol
Single mutant						
E23G,	-3.70	-4.91	1.94 mM	250.28 μM	3	2
V84A,	-3.25	-5.61	4.15 mM	77.59 μM	2	2
D110E,	-4.01	-5.43	1.16 mM	104.09 μM	4	2
H250L	-3.61	-5.35	2.28 mM	120.68 μM	2	1
Double mutant						
T53S,Y258F	-4.08	-5.29	1.02 mM	131.96 μM	1	1
Triple mutant						
A17T, D11G,D254E	-3.70	-5.36	1.95 mM	117.85 μM	4	1

Automated docking was used to locate the appropriate binding orientations and conformations of Flavonol in the 1JU6 binding pocket. To perform the task, genetic algorithm implemented in AutoDock. In this docking simulation, semiflexible docking protocols were used, in which the protein structures were kept rigid and the TS being docked was kept flexible. Blind docking was performed using grid point value (X, Y and Z) of 100Å, The grid dimensions were, X=22,Y=22, Z=20, and spacing between the gird points was 0.375Å. All water molecules were removed from the original Protein Data Bank file. Polar hydrogen atoms were added and Kolllman charge, atomic solvation parameters and fragmental volumes were assigned to the protein. All torsions were allowed to rotate during docking.

Lamarckian genetic algorithm was selected for ligand conformational searching and default docking parameters were used. The best docked complexes based on the lowest binding energy were further analyzed for hydrogen bonding interactions and the binding energy of human thymidylate synthetase mutant and Flavonol was compared. Finally, the Protein-ligand complexes were analyzed using Discovery Studio Visualizer and PyMOL visualization tool. The same docking simulation approach was performed with other mutants.

The amino acid residues Arg50, Leu192, Cys195, His196, Gln214, Arg215, Ser216, Asp218, Asn226, His256, and Tyr258 were assigned as catalytic region as these residues were proven to be potentially active site. All mutant selected was not on active site, but its conformation make change on amino acid residue that make Van der waals bond. Docking simulation of 7,3',5'-trimetoxyflavonol into Human Thymidylate Synthetase selected mutant binding energy and inhibition constant result presented in Table 1. High result on amino acid residue that make van der wals bond was found at T53S,Y258F mutant from 2 to 3 bond, D110E mutant from 3 to 5 bond, H250L mutant from 3 to 4 bond.

Tabel 2. Amino acid residue that involved in VDW bond between TS mutant and Flavonol

TS mutant	VDW AA bond (TS – Flavonol)
Single mutant	
E23G,	Arg50*, Arg215*, Leu221, Gly222, Phe225, Asn226*, Tyr299
V84A,	Arg50*, Phe91, His196*, Gln214*, Asp218*, Asn226*, Val313
D110E,	Thr51, Cys195*, Leu198, Gln214*, Asp218*, Gly222, Phe225, Asn226*, Tyr258*
H250L	Glu87, Phe91, Cys195*, His196*, Gln214*, Asp218*, Ala312
Double mutant	
T53S,Y258F	Ile108, Trp109, Gln214*, Ser216*, Gly217, Asp218*, Gly222, Ala312
Triple mutant	
A17T, D11G, D254E	Glu87, Ile108,His196*, Gln214*, Asp218*, Gly222, Phe225.

*bond with TS mutant active site

Tabel 3. Amino acid residue that involved in VDW bond between TS and Floxuridine

TS mutant	VDW AA bond (TS – Floxuridin)
Single mutant	
E23G,	ARG50*, ASP218*, GLY222, ASN226*, TRY258*
V84A,	MET31, ASN112, CYS195*, GLN214*, SER216*, ASP218*, ASN226*, ALA312
D110E,	TRP109, ASN112, ILE118, GLN214*, ASP218*, LEU221, ASN226*
H250L	TRP109, ASN112, ASP218*, HIS256*, TYR258*, MET311, ASN112
Double mutant	
T53S,Y258F	GLU87, ILE108, TRP109, HIS196*, GLY222, ASN226*, LEU221
Triple mutant	
A17T, D11G, D254E	ASP49, ASP48, ARG50*, ARG215*, HIS256*, TYR258*

*bond with TS mutant active site

The V84A TS mutant-Flavonol has lowest binding energy of -5.61 Kcal/mol and lowest inhibitory constant (Ki) of 77.59 μM. The residues Met31, Asn112, Cys195, Gln214, Ser216, Asp218, Asn226, Ala312 interacted through 2 hydrogen bonds with Flavonol. The interaction was found to span within the chosen active site. Flavonol show interacted with Cys195, Gln214, Ser216, Asp218, Asn226 interacted with V84A TS mutant active site.

Interaction between VE23G TS mutant-Flavonol has highest binding energy of -4.91 Kcal/mol but its interaction still had lowest value compared with VE23G TS mutant-Floxuridine binding energy (-3.70 Kcal/mol). This VE23G TS mutant-Flavonol interaction binding energy resulted from Arg50, Arg215, Leu221, Gly222, Phe225, Asn226, Tyr299 van der waals bond (7 bond). It's more effective than VE23G TS mutant-Floxuridine which performed by 5 Van der waals bond (Arg50, Asp218, Gly222, Asn226, Try258).

Docking analysis show that all Flavonol-TS mutant interaction had low value of ΔG than Floxuridine-TS mutant. Sum of intermolecular energy and torsion energy was the binding energy. The negative and low value of ΔG indicated strong bonds, and demonstrated that flavonol was in a favorable conformation. Furthermore, the total inhibitory constant of V84A (-5.61 kcal/mol) was found to be lowest than other. The resulf also so that V84A mutant-Flavonol (-5.61 kcal/mol) had a better binding affinity than V84A mutant-Floxuridine in this analysis (-3.25 kcal/mol).

Hydrogen bonds formed between the compound and the protein contribute to the stability of the protein-ligand complexes, a large number of hydrogen bonds form more stable complexes. The results show that the D110E mutant-Floxuridine was stabilized by four hydrogen bonds. The active residues of the E23G, V84A, D110E, H250L mutant–Flavonol were also involved in two of hydrogen bonds formation, suggesting a stable complex. Therefore, in the study of protein-ligand binding mechanism, it was revealed that the triple mutant of human thymidylate synthetase has stronger interaction with floxuridine than other protein models (4 hydrogen bonds).

4. Conclusions

Imperata cylindrica pure compound-selected human thymidylate synthetase mutant docking result has more efficient than Floxuridine. These molecular docking analyses could lead to the further development of potent human thymidylate synthetase inhibitors for the treatment of Choriocarcinoma. Hence, this study demonstrates the importance of drug design studies and utility of computational tools in correlating experimental values with computational binding energy scores for the discovery of potential newer drug molecules against resistence.

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Loewe Additivity Analysis to Find Synergism Ratio Doxorubicin and Quercetin

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Abstract

Resistance to chemotherapeutic agent in cancer treatment like doxorubicin (DOX) is still being problem. The highest expression of efflux transporter P-glycoprotein (P-gp) is one of its resistance mechanisms. Quercetin (QUE) as antioxidant agent has been investigated in the ability on inhibiting this transporter. Mechanism of interaction between DOX and QUE was evaluated with the Loewe additivity methods including curve-shift analysis, combination index, and the universal response surface analysis. The cytotoxic effect in combination DOX and QUE was as concentration dependent. Interaction DOX and QUE on the cytotoxic test MCF-7 cell were most synergy at concentration DOX:QUE of 1:17.

Keywords: combination; interaction; synergic; cytotoxic; apoptosis

Nomenclature

ABC	ATP binding cassette	µM	micro-molar
CO_2	carbon-dioxide	M	Molar
CI	combination index	mg	Milligram
DMSO	dimethyl sulfoxide	g	gram
DOX	doxorubicin	kg	kilogram
EGFR	epidermal growth factor receptor	L	litre
E max	maximum effect	mL	millilitre
ER	estrogen receptor		
FasL	Fas/Fas ligand		
HSP	heat shock protein		
IC	inhibition concentration		
MDR-1	multiple drug resistance 1		
MTT	3-(4,5-dimethylthiazol-2-yl) -2.5 diphenyl tetra-zolium bromide		
P-gp	P-glycoprotein		
PKC	protein kinase C		
QUE	quercetin		

1. Introduction

Chemotherapy was used up to 98% in patients with breast cancer, and 63% of them use a combination with DOX [1]. However, the effectiveness in the administration of DOX has decreased due to resistance of cancer cell in apoptosis process. One of the mechanisms of cancer cell resistance to anticancer drugs DOX in particular was

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due to over-expression of MDR 1, which encoded a transporter protein P-gp. This was an ATP binding cassette transporter (ABC transporter) that played a role in extrusion (efflux) of hydrophobic anticancer drugs like DOX from cytoplasm of cancer cell[2].

Various strategies had been explored to reduce the resistance of cancer cell. One of strategies was the use of compounds capable of inhibiting the efflux mechanism of anticancer out of cancer cell. Some drugs had been proven inhibit efflux mechanisms such as verapamil, cyclosporine, reserpine, quinidine, yohimbine, and tamoxifen[3]. However, these drugs were expensive and had side effects serious enough. One of the natural ingredients that were proven to inhibit the efflux of anticancer by P-gp transporter was QUE. QUE was a flavonoid contained in many foods such as apples, berries, grapes, garlic, tea, tomatoes, grains, nuts, gingko-biloba, hipericum perforatum (St. John's wort), and sambucus canadenesis[4].

QUE showed to inhibit cancer cell through inhibition of PKC[5], to suppress the activation of EGFR tyrosine kinase by inhibiting the process of fosforilization[6], and to inhibit cell growth by binding estrogen receptors[7]. In addition, QUE could increase the expression of p53, p21, and cyclin D1 but suppress cyclin D2, A, E[8]. QUE also induces apoptosis of cancer cell by inhibiting the synthesis of HSP, increased expression of FasL, annexin V laneling, and caspase-3 activity[9].This study evaluated the synergism ratio between DOX and QUE on MCF-7 cell. This evaluation was based on the activity of the combination of DOX and QUE on cytotoxicity and apoptosis of MCF-7 cell.

2. Methods

2.1 The chemical

DOX was in the preparation of liquid injection 10 mg/5ml, and quercetin hydrate 95%, brand sigma 337951. Concentrations of DOX and QUE dissolved in DMSO.

2.2 The cell line

This study used MCF-7 cell line to evaluate the interaction. MCF-7 was taken from a tank of liquid nitrogen and thawed in a water bath temperature of 37°C, and then centrifuged at 1200 rpm for 5 minutes. Supernatant was discarded and added medium grower with 5% FBS-containing in the culture flask. Under inverted microscope, alive cell appeared rounded, bright, and clear. The cells were incubated in 5% CO_2 37°C incubator. The number of cells needed to test the cytotoxicity MTT methods that use as many as 10,000 cells/mL.

2.3 The cytotoxic analysis

Cytotoxicity test performed by the method of MTT using a microplate with 96 wells. Each microplate well was filled with MCF-7 cell as many as 10,000 cells. Concentrations of DOX and QUE were made with 8 dose levels with a concentration of triplet 172.42: 86.21: 43.10: 22.55: 10.78: 5.39: 2.69 and 1.35 µM for DOX and 369.53: 184.76: 92.38: 46.19: 23.10, and 11.55 µM for QUE. Microplate was incubated for 24 hours in 5% CO_2 incubator at 37°C. After 24 hours incubation, each well added 0.1 mL MTT dilution. MTT was stopped with 10% SDS in 0.1 N HCl. The results of absorbance were obtained from the ELISA reader at a wavelength of 595 nm.

2.4 Analysis of synergism concentration

Interaction between DOX and QUE was analyzed using the fixed ratio method, where the DOX and QUE concentrations were present in fixed ratios of concentrations corresponding to the IC_{50} equivalents of single agents. The stock solutions contained 591.24; 259.62; and 147.81 μM QUE combined with 17.24 μM DOX, representing roughly DOX-to-QUE ratios of 1:34; 1:17; and 1:9. On the other hand, the stock solutions contained 73.91; 36.95; 18.48; and 9.24 μM QUE combined with 43.1 μM DOX ratios of 1:2; 1:0.8; 1:0.4; and 1:0.2. The cell was treated with serial dilutions (2-to 8-fold diluted) of the stock solutions. The controls were processed similarly but without drugs. The synergism ratio between DOX and QUE were evaluated with the Loewe additivity methods including curve-shift analysis, combination index, and the universal response surface analysis[10]. Analysis of combination index as calculated quantitatively between before and after the combination for each x% effect and equivalent to the IC_{50} of DOX and QUE. The effect and combination index was calculated by equation 1 and 2 respectively. Combination index equal to 1, less than, or greater than 1 indicated the interaction of the two drugs were additive, synergy, or antagonism respectively[11].

$$E = Emax \frac{C^n}{C_{50}{}^n + C^n} \quad (1)$$

$$\text{Combination index} = \frac{C_{D,X}}{IC_{X,D}} + \frac{C_{Q,X}}{IC_{X,Q}} \quad (2)$$

Universal response surface analysis was the additional analysis described by Greco *et al.* This method assumed that the concentration and effects on two-drug combination of a fixed relationship exists. Interaction parameter value of α was determined both drugs and calculated by equation 3. The interaction of these two drugs were additive if the value of α was zero, the value of α was negative or positive indicated synergism or antagonism respectively[10].

$$1 = \frac{C_{D,E}}{IC_{50,D}\left(\frac{E}{Emax-E}\right)^{1/n_D}} + \frac{C_{Q,E}}{IC_{50,Q}\left(\frac{E}{Emax-E}\right)^{1/n_D}} + \alpha \frac{C_{D,E}C_{Q,E}}{IC_{50,d}\,IC_{50,Q}\left(\frac{E}{Emax-E}\right)^{1/n_D+1/n_Q}} \quad (3)$$

Shift curve analysis was the last method to evaluate interaction. This curve provided the relationshipIC_{50} equivalent and effects. The effect was calculated by equation 4. The synergistic interaction was showed if the curve with a combination was in the left side of the curve of combination and called leftward shift. In the contrast, antagonist interaction if the curve of combination was being on the right called rightward shift[11].

$$\text{Combination effect} = \frac{Emax\left(\frac{C_{D,X}}{IC_{50,D}} + \frac{C_{Q,X}}{IC_{50,D}}\right)^{n\ combo}}{\left(\frac{C_{D,X}}{IC_{50,D}} + \frac{C_{Q,X}}{IC_{50,D}}\right)^{n\ combo} + IC_{50,combo}{}^{n\ combo}} \quad (4)$$

3. Results and discussion

3.1. Synergism analysis

In the present study, the concentrations of doxorubicin to inhibit growth of 50% MCF-7 cells (IC_{50}) was 21.47 µM. On the other hand, quercetin which had IC_{50} on MCF-7 cells of 103.1 µM. The interaction between DOX and QUE analysed by measuring index of combination, analysing the universal surface response, and comparing curve the single drug with the combination. Figure 1 summarized the results of analysis of combination index. At the high quercetin concentration, the CI values were generally below 1, indicating synergy. The most synergy was in combination DOX:QUE ratio of 1:17 where the CI of all concentration produced from 10% (CI=0.83) to 90% (CI=0.97) the maximum effect under 1. All of ratio combination consistently synergy in ratio concentration produced over 50% maximum effect (Table 1 and Fig.1).

Table 1. Result of combination index analysis

Ratio D:Q	CI ± SD								
	10	20	30	40	50	60	70	80	90
1:34	1.11 ± 0.0	0.99 ± 0.0	0.94 ± 0.0	0.90 ± 0.0	0.88 ± 0.0	0.86 ± 0.0	0.83 ± 0.0	0.81 ± 0.0	0.78 ± 0.0
1:17	0.83 ± 0.1	0.78 ± 0.1	0.77 ± 0.0	0.78 ± 0.0	0.80 ± 0.0	0.82 ± 0.0	0.85 ± 0.0	0.90 ± 0.0	0.97 ± 0.1
1:9	1.61 ± 0.2	1.13 ± 0.1	0.09 ± 0.1	0.75 ± 0.1	0.65 ± 0.0	0.57 ± 0.0	0.50 ± 0.0	0.44 ± 0.0	0.37 ± 0.0
1:2	5.47 ± 0.4	2.56 ± 0.3	1.62 ± 0.1	1.06 ± 0.1	0.72 ± 0.0	0.49 ± 0.0	0.33 ± 0.0	0.22 ± 0.0	0.12 ± 0.0
1:0.8	3.88 ± 0.2	2.16 ± 0.1	1.42 ± 0.1	0.99 ± 0.0	0.71 ± 0.0	0.51 ± 0.0	0.36 ± 0.0	0.25 ± 0.0	0.15 ± 0.0
1:0.4	4.19 ± 0.3	2.28 ± 0.1	1.46 ± 0.1	1.00 ± 0.0	0.70 ± 0.0	0.49 ± 0.0	0.33 ± 0.0	0.22 ± 0.0	0.12 ± 0.0
1:0.2	3.57 ± 0.3	2.01 ± 0.1	1.32 ± 0.1	0.92 ± 0.0	0.65 ± 0.0	0.46 ± 0.0	0.32 ± 0.0	0.21 ± 0.0	0.11 ± 0.0

D: doxorubicin; Q: quercetin; CI: combination index; SD: standard deviation

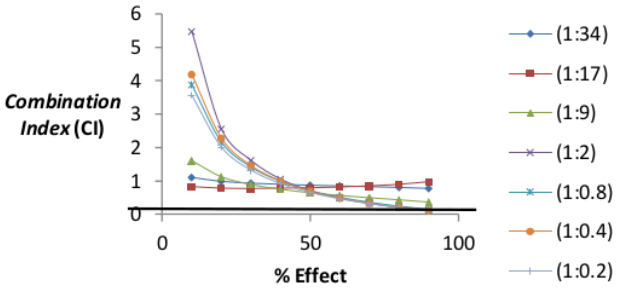

Fig. 1 Combination index curve. Less than 1 is synergy, equal to 1 is additive, and more than 1 is antagonist

Fig. 2 and Table 2 were obtained from analysis of DOX and QUE interaction with universal surface response method. The most synergy interaction was ratio 1:17 with the least negative α (-4.56) in 10% maximum effect, moreover, value of α in the ratio 1:17 was the only positive in 20% maximum effect, indicating synergy interaction. At the above of 50% effect maximum, all of ratio concentration showed synergy with a positive α.

Table 2. Result of universal surface response analysis at 10-90% maximum effect

Ratio D:Q	α ± SD								
	10	20	30	40	50	60	70	80	90
1:34	-12.70 ± 0.6	-1.45 ± 0.6	0.94 ± 0.5	1.45 ± 0.6	1.50 ± 0.3	1.35 ± 0.3	1.18 ± 0.2	1.08 ± 0.2	1.16 ± 0.2
1:17	-4.56 ± 5.5	4.86 ± 2.9	4.29 ± 1.3	2.90 ± 0.6	1.90 ± 0.2	1.12 ± 0.1	0.59 ± 0.1	0.26 ± 0.1	0.11 ± 0.1
1:9	-20.09 ± 0.5	-3.52 ± 1.2	1.20 ± 1.3	2.99 ± 1.1	3.70 ± 0.9	3.94 ± 0.7	3.96 ± 0.5	3.94 ± 0.4	4.01 ± 0.2
1:2	-29.54 ± 1.9	-12.38 ± 0.6	-5.20 ± 0.2	-0.57 ± 0.5	2.89 ± 0.5	5.88 ± 0.9	8.95 ± 1.1	12.91 ± 1.3	20.76 ± 1.8
1:0.8	-72.15 ± 1.9	-24.46 ± 0.3	-8.01 ± 0.4	-0.06 ± 0.5	4.42 ± 0.5	7.18 ± 0.4	9.06 ± 0.2	10.63 ± 0.1	12.85 ± 0.2
1:0.4	-133.14 ± 7.6	-45.80 ± 0.9	-15.26 ± 0.7	-0.28 ± 1.1	8.29 ± 1.1	13.70 ± 1.0	17.49 ± 0.9	20.76 ± 1.1	25.45 ± 1.8
1:0.2	-296.68 ± 17.3	-92.28 ± 1.4	-25.92 ± 1.9	4.26 ± 2.6	20.00 ± 2.5	28.79 ± 2.3	33.95 ± 2.3	37.47 ± 2.8	41.99 ± 4.1

D: doxorubicin; Q: quercetin; CI: combination index; SD: standard deviation

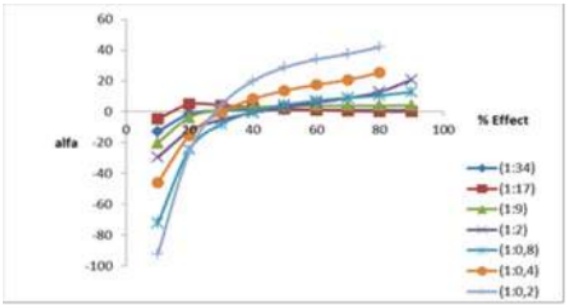

Fig. 2. Value of α of the combination doxorubicin and quercetin. The positive value is synergy, zero is the additive, and negative is antagonist.

The synergism interaction was also obtained by comparing IC_{50} equivalent each ratio with IC_{50} single concentration of doxorubicin and then plot to the curve as the nonlinear curve (Fig. 3 and Table 3). The IC_{50} equivalent indicated synergism interaction when IC_{50} equivalent of a ratio was above IC_{50} equivalent of single concentration DOX. The ratio of 1:17 was the only synergy interaction with IC_{50} equivalent of 0.53 compared in single DOX (0.52). All of ratio combination showed synergy interaction on concentration that produced above 50% maximum effect; consequently, all concentration-effect curves for various ratios in combination that caused effect above 50% were shift to the left of the curves for the single DOX. On the other hand, the curve on the right of the single DOX indicated antagonism interaction as low concentration as low effect. Figure 3 showed that the IC_{50} equivalent versus response curves for doxorubicin, quercetin, or their combinations in range of response from 10% to 90% the maximum effect.

Table 3. IC_{50} equivalent at combination of doxorubicin and quercetin on 10-90% maximum effect

Ratio	IC_{50} equivalent ± SD								
D:Q	10	20	30	40	50	60	70	80	90
1:34	0.04 ± 0.0	0.12 ± 0.0	0.27 ± 0.0	0.53 ± 0.1	1.00 ± 0.1	1.91 ± 0.1	3.87 ± 0.6	8.89 ± 2.5	28.79 ± 13.5
1:17	0.14 ± 0.0	0.27 ± 0.0	0.42 ± 0.0	0.61 ± 0.0	0.88 ± 0.0	1.26 ± 0.0	1.84 ± 0.1	2.89 ± 0.1	5.40 ± 0.2
1:9	0.09 ± 0.0	0.19 ± 0.0	0.32 ± 0.0	0.52 ± 0.0	0.80 ± 0.0	1.23 ± 0.0	1.97 ± 0.0	3.42 ± 0.2	7.34 ± 0.6
1:2	0.13 ± 0.0	0.23 ± 0.0	0.34 ± 0.0	0.47 ± 0.0	0.65 ± 0.0	0.89 ± 0.0	1.25 ± 0.1	1.85 ± 0.1	3.21 ± 0.6
1:0.8	0.28 ± 0.0	0.37 ± 0.0	0.49 ± 0.0	0.60 ± 0.0	0.72 ± 0.0	0.86 ± 0.0	1.05 ± 0.1	1.32 ± 0.1	1.82 ± 0.1
1:0.4	0.18 ± 0.0	0.29 ± 0.0	0.41 ± 0.0	0.54 ± 0.0	0.71 ± 0.0	0.93 ± 0.0	1.25 ± 0.0	1.75 ± 0.0	2.81 ± 0.0
1:0.2	0.18 ± 0.0	0.29 ± 0.0	0.40 ± 0.0	0.54 ± 0.0	0.70 ± 0.0	0.91 ± 0.0	1.21 ± 0.0	1.68 ± 0.0	2.66 ± 0.1

D: doxorubicin; Q: quercetin; CI: combination index; SD: standard deviation

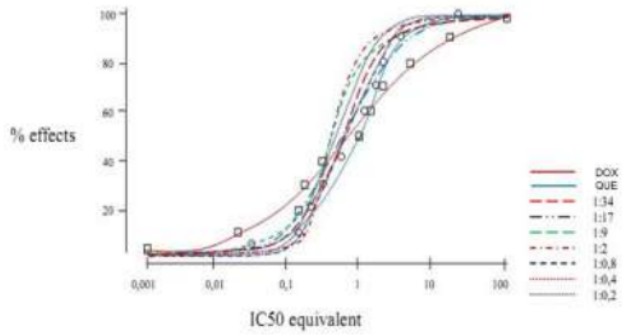

Fig. 3. Curve shift analysis of the interaction of doxorubicin and quercetin

The Loewe additivity analysis informed that interaction between DOX and QUE had range effects 10-90% effects from antagonism to synergism effects. Ratio combination 1:17 had no antagonism effect in CI and universal surface analysis but not in curve shift analysis. Synergism interaction between DOX and QUE in the three methods analysis of the Loewe additivity in the cytotoxicity effects was showed in the Table 4.

Table 4. Synergism of the interaction DOX and QUE in the cytotoxicity effects

Ratio	Analysis	Cytotoxicity effects (%)								
		10	20	30	40	50	60	70	80	90
1:34	Combination index	A	A	AD	AD	S	S	S	S	S
	Curve shift	A	A	A	AD	S	S	S	S	S
	Unversal surface	A	A	S	S	S	S	S	S	S
1:17	CI	AD	S	S	S	S	S	S	S	AD
	Curve shift	A	A	A	AD	S	S	S	S	S
	Unversal surface	AD	S	S	S	S	S	S	S	AD
1:9	CI	A	A	AD	S	S	S	S	S	S
	Curve shift	A	A	A	AD	S	S	S	S	S
	Unversal surface	A	A	AD	S	S	S	S	S	S
1:2	CI	A	A	A	AD	S	S	S	S	S
	Curve shift	A	A	A	AD	S	S	S	S	S
	Unversal surface	A	A	A	A	S	S	S	S	S
1:0.8	CI	A	A	A	AD	S	S	S	S	S
	Curve shift	A	A	A	AD	S	S	S	S	S
	Unversal surface	A	A	A	AD	S	S	S	S	S
1:0.4	CI	A	A	A	AD	S	S	S	S	S
	Curve shift	A	A	A	AD	S	S	S	S	S
	Unversal surface	A	A	A	AD	S	S	S	S	S
1:0.2	CI	A	A	A	AD	S	S	S	S	S
	Curve shift	A	A	A	AD	S	S	S	S	S
	Unversal surface	A	A	A	S	S	S	S	S	S

CI: combination index; A: antagonism; AD: Additive; S: Synergy

3.2. Apoptosis effect in synergism concentration

Apoptosis test was performed in the ratio DOX and QUE which in the most synergy ratio of 1:17. In the apoptosis test, MCF-7 cells was observed under flouresence microscope after double staining and showed in Fig. 4.

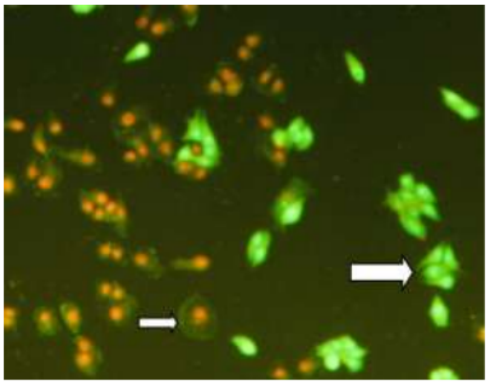

Fig. 4. Apoptosis effect on MCF-7 in the combination DOX and QUE with acridine orange and ethidium bromide staining. A thick arrow indicated viable cells and a thin arrow showed apoptotic cell.

The percentage of apoptotic MCF-7 cell in concentration of combination ratio 1:17 is no significance difference with either single IC_{50} DOX (p=0.783) and IC_{50} QUE (p=0.869). However, amount of apoptotic cells in single concentration DOX or QUE in ratio of 1:17 was decrease significance statistically from in combination ratio 1:17. Amount of apoptotic cells showed in Table 5.

Table 5. Percentage of apoptotic cells

Drugs	Concentrations (μM)	Apoptotic cell (%) ± SD	P
Control	0	0	
DOX	21	50.11 ± 1.95	0.783
DOX (1:17)	3.74	28.22 ± 2.46	0.001*
QUE	103	50.33 ± 0.67	0.869
QUE (1:17)	64.04	39.22 ± 2.14	0.010*
DOX:QUE(1:17)	3.74:64.04	50.67 ± 3.76	Ref

*statistically significance difference (p<0.005)

The report explored interaction between DOX and QUE on cytotoxic effect on MCF-7 human breast cancer cell line with different methods. Quercetin IC_{50} of less than 147.81 µM quercetin was considered that quercetin can be as anti-proliferative from natural derivatives[12].

Test cytotoxic activity of DOX and the combination with QUE on MCF-7 cells with the MTT assay method were conducted to analyse the evaluation of the interaction of DOX and QUE. Concentration ratio of DOX and QUE in combination was based on the exploration on 7 ratios of 1:34; 1:17; 1:9; 1:2; 1:0.8; 1:0.4; 1:0.2. Test the interaction between DOX and QUE performed on the combination index, universal surface analysis, and curve shift analysis[10]. This study was also proved the basic concept about the relationship concentration and effect as the occupational theory, which the higher concentration, the higher effect[13]. However, each range of concentration in combination had interaction likely antagonist, additive, or synergy as this study found.

The first method to test the interaction of DOX and QUE on cytotoxicity of MCF-7 cells were analysed by the combination index method. It was a quantitative calculation of IC_{50} between the concentration of single and combination concentration. This method was to know the interaction of DOX and QUE at 10% to 90% maximum effect of inhibitory on MCF-7 cells. Combination index analysis results showed that the interaction DOX and QUE was synergy at concentrations that caused effects above 50% maximum effect. CI decreased followed by increasing in effect. CI in ratio 1:17 is the most synergy because this ratio was the first having CI under 1 in the lowest concentration.

The evaluating the interaction of DOX and QUE in MCF-7 cell cytotoxicity with response surface method was an additional analysis and introduced by Greco *et al.* In this method the interaction parameters of both drugs was determined by the value of alpha (α). The calculation of the value of α was based on equations derived from equation of the hill. A value around 10-30% at a concentration ratio of the maximum effect showed an antagonistic interaction, at 40% maximum effect showed an additive interaction, and above 50% maximum effect showed synergistic interaction. Ratio 1:17 had also the most synergy compared the others.

The last method was the curve shift analysis. Each curve of the combination compared to the curve DOX and QUE in single dose. The whole curve to the effects on the entire concentration ratio showed the consistent of results. Antagonistic and synergic interaction respectively occurred at concentrations causing effects combination of less than 30% and above 50% of maximum effect. It was characterized by curve combinations of DOX on the leftward. The most synergistic effect obtained from the data ratio of DOX:QUE on 1:17.

Overall, the interaction mechanism was on the concentration or dose dependent. Antagonistic interactions were occurred at low concentrations, ie concentrations that produced effects below 30% maximum effect. Synergistic interaction occurred at high concentrations, the concentrations that produced effects above 50% maximum effect. The observations of combination index, the universal response surface analysis and curve shift analysis were compared with each other. The result of all method consistently agreed that the ratio DOX and QUE of 1:17 on the inhibition of MCF-7 cells was the most synergy. Synergistic interaction between QUE and DOX occurred through the mechanism of competition for P-gp transporter. Doxorubicin was a P-gp substrate and increased the activity of P-gp H types, whereas QUE inhibited P-gp by binding to H type so that the efflux by P-gp was inhibited. The inhibition of P-gp activity by QUE could increase the concentration of DOX in cancer cells[12]. According to the analysis of combination index analysis, analysis of universal surface response, and the shift curve analysis showed that the antagonistic interaction occurred at concentrations that pose a combination of effects below 30% maximum effect. On the low effects, the concentrations of quercetin doxorubicin were also low because both drugs were dose dependent. At low concentrations, QUE had a proliferative effect on MCF-7 cells through stimulation of the ER. At low concentrations of QUE increased the number of free estrogen receptors in the absence of quercetin-binding. Thus, growth factors can trigger stimulation that result in signal transduction was occurred[14]. Mechanism QUE and DOX in estrogen receptors still need depth exploration.

4. Conclusions

The interaction between DOX, as chemotherapy in cancer, with QUE, as P-glycoprotein blocker, in cytotoxic effect in MCF-7 cells was as concentration dependent. This interaction was synergy when the concentration of quercetin was higher, but in the lower concentration of quercetin could cause an antagonistic interaction. The most synergy interaction DOX and QUE was in ratio 1:17. Furthermore, this ratio had been tasted in the apoptosis evaluation result in no significance difference between combination and single drug producing 50% apoptotic cell in MCF-7.

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Preventive Effect of *Marsilea crenata* Leaves and Stalks Juice Againts Urolithiasis in RATS (*Rattus norvegicus Wistar Strain*)

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Abstract

Urolithiasis frequently as an impact of dry food given regularly, keeping animals so the movement becomes restricted, lack of water intake, by microbial infection. *Marsilea crenata*contains some compounds have function as diuretic, antioxidant, antiinflammatory. The research purpose was to determine potency of the *M. crenata* leaves and stalks juice to prevent urolithiasis invivo. Research was true experimental, by complete random design. Males *Rattusnovergicus*, divided into: negative control; positive controlurolithisis (given 0.75% ethylene glycol/EG, 2% ammonium chloride/AC, 2 ml/ day, for 10 days); four groups of preventive controls (given different dose juice of *M. crenata* and 0.75% EG + 2% AC). Parameters: urine quality; proinflammatory cytokines; free radicals; kidney and gall blader histopathologic. Results, *M. crenata* leaves and stalks juice increased urine quality and SOD, decreased IL-1β, TNF-α and MDA, and prevented kidney and gall blader from tissue damage. Concluded, *M. crenata* leaves and stalks juice have preventive function against urolithiasis in rats animal model.

Keywords: preventive; urolithiasis; *Marsilea crenata*

1. Introduction

Urolithiasis is a pet animals common disease, particularly occur in dogs and cats. It is caused by deposit crystal in urinary tract and sediment urine minerals. Urolithiasis caused *feline lower urinary tract disease* and *idiopathic cystitis*.

The Ohio State University Veterinary Hospital evaluates 109 cats with stranguria and 15 cats of them are urolithiasis[1]. *Waltham Centre for Pet Nutrition* reported, the newest case of *feline lower urinary tract disease* is 0.5-1% /year from cat population in Europe and South America.

The common crystal is calcium oxalate (CaOx) and magnesium ammonium phosphate (MAP). Treatment of urolithiasis done by administration drugs and using catheter. But after treatment the urolithiasis can be occur again. Sparkes and Philippe[2] reported that 20-50% of urolithiasis in cat and dog are relapse, if no preventive properly.

Nowadays some traditional herbs, such as kumis kucing (*Orthosiphon aristatus*), blue grapes fruit (*Vitis vinifera L.*), and tempuyung leaves (*Sonchus arvensis*) contain substances that play as preventive agent against infection or disease. Water semanggi (*M. crenata*) is one of wild plant suspected contain active chemical substances able to destroy crystal urolithiasis.

Juice of leaves and stalks *M. creanata* contain chemical active substances suspected can prevent urolithiasis. These substances are: K^+ ions and flavonoid have diuretics function, as antioxidant, antiinflammation; alkaloid and polyphenolas antioxidant.

In this research, was used white rats (*R. norvegicus*) Wistar strain as urolithiasis animal model. These animals were inducing with combination of 0.75% EG and 2% AC. Base on Jagannath research[3], administration this

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combination for ten days can induce forming of CaOx crystals. Research objective is juice of leaves and stalks water semanggi *(M. crenata*) can be used to prevent urolithiasis in white rats (*R. norvegicus*) Wistar strain? Research purposes is to prove the ability of water semanggi (*M. crenata*) to prevent urolithiasis in white rats (*R. norvegicus*) Wistar strain, base on: urine quality, level of inflammatory cytokines, level of free radicals, and kidney and gall bladder histopathology. Research usefulness is base for next research about the toxicity effect of *M. crenata* and base for herbs improvement as alternative drugs.

2. Methods

2.1. Sample

Inclusion criteria: Health male rats, 175-200 gr BW, and 12 weeks old. The animal was obtained from Unit Pengembangan Hewan Percobaan (UPHP) Gadjah Mada University, and had certificated by The Ethical Committee Medical Research of Medical Faculty Brawijaya University (No. 421/EC/KEPK/07/2013). Number of samples: $p\,(n-1) \geq 15$[4]. The animal were adapted for 7 days before treatment (Lina *et al*, 2003) and placed in metabolic cage.

2.2. Variables

Dependent variable: urine quality, inflammatory cytokines, free radical, and kidney and gallbladder histopathology. Independent variable: *M. crenata*, EG, AC. Control variable: homogeneity of the animal model, diet, *M. Crenata*.

2.3. Dose juice of leaves and stalksM. crenata

The doses are 5%, 10%, 20%, 40% (modified Purwono, 2010)[5].

2.4. Dose of ethylene glycol (EG) and ammonium chloride (AC)

Combination of 0.75% EG and 2% AC was used to induce urolithiasis (modified Anggraeni, 2013)[6].

2.5. Juice of leaves and stalks M. crenata

The herb was obtained from UPT. Matrika Medika Batu, East Java, Indonesia. The juice was made using fruit juicer without dilution, so the concentration is 100%.

2.6. Treatment

Table 1. Groups of treatment

Group	Treatment
Negative control	Normal condition (normal diet)
Positive control	Normal diet + 0.75% EG + 2% AC
Treatment 1	Normal diet + 5% juice *M. crenata* + 0.75% EG + 2% AC
Treatment 2	Normal diet + 10% juice *M. crenata* + 0.75% EG + 2% AC
Treatment 3	Normal diet + 20% juice *M. crenata* + 0.75% EG + 2% AC
Treatment 4	Normal diet + 40% juice *M. crenata* + 0.75% EG + 2% AC

The treatment was done for 10 days orally using stomach sonde.

2.7. Urine sampling

The urine was collected at 11th of day[5]. Volume, pH, specific gravity, bilirubin were analysed using Urine Analyser (Sacher *et al*., 2002).Urine sediments were examined using Gandasoebrata method[7].

2.8. Level of TNF-α and IL-1β

Euthanasia the animal models were done by cervical dislocation. The serum was collected for ELISA.

2.9. Level of MDA and SOD

The kidney MDA and the serum SOD were measured by spectrophotometry method (Laboratory of Pharmacology, Medicine Faculty, Brawijaya University, 2013).

2.10. Histopathology of kidney and gallbladder

The tissues were stained using haematoxylin-eosin.

2.11. Data analysis

The data was analysed using one-way ANOVA and Kruskall-Wallis with $\alpha = 0,05$.

3. Results and discussion

3.1. Urine examination
3.1.1. Urine pH

Gandasoebrata (1992)[7], normal rat pH urine is 4.6-8.5. Urine pH group with *M. crenata* 5%, 10%, 20%, 40% increase significantly (Table 2). It is may cause by K^+ ions in leaves and stalks of *M. crenata,* and the highest at the 40%. Huang, *et al*. (2006)[8], explained that combination 0.75% EC and 2% AC caused chronic hyperoxaluric, with base urine. Base pH urine can promote forming of crystals CaOx in urine. Parmar *et al*. (2012)[9], in CaOx urolithiasis was found at normal until base. Different with uric acid urolithiasis, causing by crystals of uric acid, occur in acid urine[10].

Table 2. pH urine

Group	Mean ± SD pH urine
Negative control	7.13 ± 0.25^{a}
Positive control	7.50 ± 0.41^{ab}
5% of *M. crenata*	7.63 ± 0.25^{ab}
10% of *M. crenata*	7.63 ± 0.25^{ab}
20% of *M. crenata*	7.75 ± 0.29^{ab}
40% of *M. crenata*	7.88 ± 0.25^{b}

Excess of K^+ ions in body will be eliminating via urine and sweat[29]. K^+ ions are strong base increasing urine pH animal model[11]. Increasing of the urine pH insignificantly at group with *M. crenata*, and unable influenced kidney function in regulation of H^+ and HCO^{3-} body fluid animal models urolithiasis. Guyton and Hall (1997)[12], base and acid urine regulation by the kidney is a one of important body mechanism to stabilizing body pH. Kidney controls the urine acid-base by releasing through urine and depends on acid-base extracellular body fluid. The role of this control is by secretion of hydrogen ions, reabsorbs bicarbonate ions and production of new bicarbonate ions. Factors influencing acid secretion by kidney are changes of K^+ ions, CO_2 intracellular pressure, level of anhydrase carbonate and some hormone such as aldosterone, steroid adrenocortex, angiotensin II[13].

3.1.2. Specific gravity

Kidney damage induced by combination EG and AC decrease urine specific gravity in male rats[14,15,16], specific gravity human and animal urine under 1,015 (± 1,010) indicated strong kidney tissue damages. Its may caused by alternation of reabsorption function the kidney at proximal tubules, henle loop, distal tubules, or colectivus tubules.

Administration of juice leaves and stalks *M. Crenata* induced increasing significantly the specific gravity the urine. Specific gravity depends on urine indigrient and volume. In this research suggested influence by fragments calcium and oxalate in the urine.

3.1.3. Urine bilirubin

Bilirubin is form of haemoglobin degradation product in reticuloendothelial. Bilirubin absent in healthy animals urine, but find in individual with urinary tract disease, liver disease, and may associated with other body system[17].

In this research the juice of leaves and stalks *M. crenata* has effect reduces level bilirubin in the urine, that indicated the urinary tract tissues damage was decrease or with other body system associated with bilirubin production.

3.1.4. Urine CaOx sediments

Juice of leaves and stalks of *M. crenata* can destruct urine CaOx crystals. It is may caused by diuresis effect of the juices. It showed by decreasing of urine sediments significantly (Fig. 3).

In this research, in urine also found struvite crystals in each group of treatment. Struvite crystals may present normally in some animal as body waste metabolic product[18]. No uric acid and cystin crystals in urine. Crystal uric acid just find in acid urine[19], and cystinin autosomal recessive individual (Orson, 2006). Crystal CaOx≤5- may normal in healthy animals[7].

Urine sediment examination is one of important laboratory diagnosis for urinary tract diseases. K^+ and Na^+ ions balance kidney electrolytes. K^+ ions from *M. crenata* juices bind to oxalate acid or uric acid, then was dissolved the crystals be acid substances that water-soluble. Crystal fragments will go out the body with urine. The reaction[20]:

$$2K^+ + CaC_2O_4 \rightarrow K_2C_2O_4 + Ca^{2+}$$

CaC_2O_4 or oxalate stone sediments are water-soluble. K ions inhibit combining of Ca^+ ions with carbonate, oxalate or uric and until Ca^+ become soluble[20].

Juice of leaves and stalks *M. crenata* also contain flavonoid. CaOx crystal may combine with –OH of flavonoid and form Ca-flavonoid complexes, it may cause more water-soluble and easily go out when urination. Flavonoid also plays as diuretic substance[21].

3.2. Level of TNF-α and IL-1β

By one-way ANOVA and Tukey, α= 0.05, the level of inflammatory cytokines (TNF-α and IL-1β) decreased following the increasing of juice *M. crenata* administration (Table 3).

Table 3. Level of TNF-α

Group	Mean ± SD TNF-α (µg/ml)	Mean ± SD IL-1β (µg/ml)
Negative control	42.75 ± 0.65[a]	0.95 ± 0.03[a]
Positive control	95.63 ± 4.21[d]	2.07 ± 0.06[d]
5% of *M. crenata*	77.38 ± 1.89[c]	1.67 ± 0.04[c]
10% of *M. crenata*	73.88 ± 1.70[c]	1.66 ± 0.03[c]
20% of *M. crenata*	65.13 ± 0.85[b]	1.23 ± 0.03[b]
40% of *M. crenata*	47.50 ± 1.68[a]	1.17 ± 0.02[b]

Concentration 5% and 10% juice *M. crenata* showed have same effect on TNF-α level. The highest TNF-α is at 40%. Jagannath, *et al*[3], combination of 2% EC and 0.75% AC in 10 days induce increasing of urine calcium and oxalate, and CaOx crystals. The crystals caused ureter obstruction lead to urolithiasis. It's trigger inflammation responses sign by high inflammatory cytokines, TNF-α and IL-1β.

Flavonoid as antiinflammation induced decreasing the production and releasing of TNF-α and IL-1β by immune cells. These responses are influenced by inhibition and inactivation effect to *protein tyrosine kinase* (PTK) p56[22], and blocked transcription and translation process of cytokines[23].

3.3. Level of MDA and SOD

The result showed that the MDA level decreased after administration of *M. crenata,* oppositely to the SOD level (Table 4).

Table 4. Level of MDA and SOD

Group	Mean ± SD MDA (ng/ml)	Mean± SD SOD (u/ml)
Negative control	125.88 ± 6.57[a]	14.40 ± 0.28[d]
Positive control	197.13 ± 9.44[e]	9.97 ± 0.70[a]
5% of *M. crenata*	179.00 ± 6.12[d]	10.50 ± 0.37[a]
10% of *M. crenata*	169.00 ± 13.07[c]	11.73 ± 0.52[b]
20% of *M. crenata*	151.50 ± 6.12[b]	13.12 ± 0.50[c]
40% of *M. crenata*	125.25 ± 3.23[a]	14.18 ± 0.10[d]

Urolithiasis patients cannot urine normally. This condition induces generation of reactive oxygen species

(ROS). ROS is very reactive, causing tissue injury and failure of cells function. ROS activate nuclear factor kappa B (NF-kB) and *IkB kinase* (IKK) regulators. IKK is a complex of enzyme can induce cellular inflammation responses. IKK induce forming of IkBα, which bind to NF-kB as mediator of cells proliferation, inflammation responses, and apoptosis factors[24].

High-level ROS in the body tissues cause oxidative stress may induce increasing of lipid metabolic product, malonyldyaldehide (MDA). High level of MDA is one of predictor cellular tissues damage by free radicals[25].

Flavonoid in juice of leaves and stalks of *M. crenata* has antioxidant and antiinflammation functions. As antioxidant, it has either directly or indirectly effect. Directly, by donor its hydrogen ions to neutralized toxic effect of ROS. Flavonoid also acts as chelating agent of O-in nitrite peroxide (ONOO-)[26]. Indirectly, by enhancing of endogenous antioxidant gene through some mechanisms, such as activation of *nuclear related factor 2* (Nrf2) that induce synthesis of endogenous antioxidant enzyme gene of *superoxide dismutase* (SOD). SOD play as free radicals scavenger[27,28].

3.4. Histopathology of kidney and gallbladder

The tissue damage was scored and analyzed statistically using Kruskall-Wallis. Results showed, that the kidney and gallbladder damages decrease significantly after administration juice leaves and stalks *M. crenata*. Combination 0.75% EG and 2% AC induce urilithiasis, showed by kidney and gallbladder tissues damage, including inflammation, hemorrhagic, lipid degeneration, necrosis, and edema (Fig. 1).

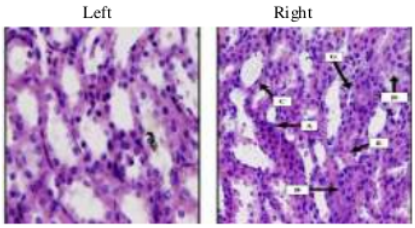

Fig. 1. Left: normal kidney tubule; Right, positive control A. Inflammation; B. Hemorrhage; C. Crystals; D. Lipid degeneration; E. Necrosis; G. Edema (400x magnification)

Tissue damages may be caused by toxic effect of EG and AC, inflammation process, free radicals, and traumatic effect of urolithiasis. Higher concentration of the juice cause lower tissue damage. Effect antioxidant and antiinflammation of juices leaves and stalks *M. crenata* prevent progression of tissue damage. The highest tissue damage is control positive, and the lowest is group 15% *M. crenata* (Fig. 2 and 3).

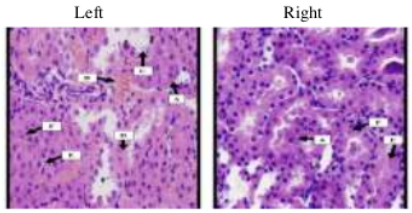

Fig 2. Left, Kidney tubules; 5% *M. crenata*: A. Inflammation; B. Hemorrhage; C. Crystals; D. Lipid degeneration; E. Necrosis; F. Hydropic degeneration; Right, kidney tubule, 10% *M. crenata*: A. Inflammation; B. Necrosis; F. Hydropic degeneration) (400x magnification)

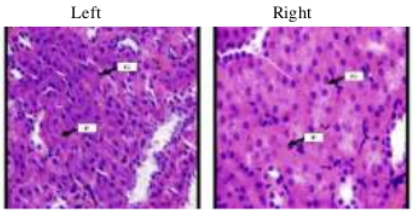

Fig 3. Left, kidney tubule, 20% *M. crenata*. E. Necrosis, F. Hydropic degeneration; Right, kidney tubule, 40% *M. crenata*. E. Necrosis, F. Hydropic degeneration (400x magnification)

4. Conclusion

From this research, can be concluded that juice of leaves and stalks *M. crenata* have prevention effect against in *R. Novergicus* animal model.

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Pathogenecity and Molecular Studies of Zoonotic Disease Caused *Edwardsiella tarda*

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Abstract

The aim of study was to determine a) pathogenecity of *Edwardsiella tarda* isolates from different regions and type of fish, b) genetic variation of *Edwardsiella tarda* based on molecular study. Isolates of *Edwardsiella tarda* were taken from Tilapia (Yogyakarta), *catfish* (Semarang), imported Tortoise (Brazil), goldfish (Pontianak). All isolates were extracted, amplified in the SSU rRNA-16S and sequenced for phylogenetic tree. Redness lesions began to appear on the second day post infection. Three isolates of *Edwardsiella tarda* from Pontianak, Jambi, Yogyakarta was same strain originating from fish, where as the isolates from turtle was same strain as isolates from human origin.

Keywords: Phylogenetic tree; *Edwarsiella tarda*; Tilapia

1. Introduction

Edwardsiella tarda caused *Edwardsiollosis/Emphisematous Putrevactive Disease of Catfish* (EPDC) or *Edwardsiella Septicaemia* (ES). *Edwardsiollosis* was a primer disease at catfish aquaculture in America and transmitted to Europe, Japan, Taiwan, Thailand, Singapore, and Malaysia[7]. In Indonesia, *Edwardsiolosis* has been reported from island of Java, Sumatra and Kalimantan.

Edwardsiella tarda was a bacterium enteric type, could be transmitted horizontally among fish and could become a carrier in the water and mud[9]. *E. tarda* could be alive in freshwater and marine, spread to reptiles, frog, lobster, pig, and human. Mortality in *channel catfish* was low (5%), however, it could increase to 50% since they were moved to agriculture[10].

Edwardsiella tarda was isolated 75% from samples water pool of catfish, 64% from samples mud pool of catfish and 100% from frog, turtles and crayfish from crayfish's pool. This condition showed that *E. tarda* was a microflora at catfish's pool and the present of this bacteria makes the potential of fish disease still there[3].
Edwardsiellosis can be diagnosed with someway: 1) Isolation and identifiacation causative agent *E. tarda*[3] 2). Immune reaction with ELISA 3). Immunohistochemistry on infected organs[6] and 4). Molecular with PCR methods[2].

Edwardsiella tarda was isolated from *Tilapia* fish and infected water by PCR with hemolycin gene application. Samples from liver and intestine of fish showed clearly band beside infected water[2]. Detection *of E. tarda in oyster road fi*sh directly by using PCR provide an accurate and fast results using a comparator *E. ictaluri* and *Vibrio* sp. as a negative control[1].

E. tarda infections in humans are generally transmitted through fecal contamination of human, food and water contaminated with these bacteria called fecal-oral transmission. *E. tarda* strain on the fish obtained transmission process only between fish[5].

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2. Methods

Edwardsiella tarda have been isolated and identified from carp (Pontianak), tilapia (Yogyakarta), catfish (Semarang), turtles (Brazil) and the ATCC isolates from Singapore. All isolates carried revirulence prior to treatment. Revirulence done by infecting carp intraperitoneally with 0.1 cc of each isolate *E. tarda* were 107 cells/ml. All goldfish autopsied, reisolated and reidentified against *E. tarda*.

Molecular test to isolate performed by PCR method which consists of the extraction of DNA with Qiagen kit and 16S rRNA amplification. Primers used are: Forward Eta 2-351 (5'-TAG TGT AGG GAA GGA GGA-3'). Reverse Edwsp-780r (TAG 5'CTC CTT CTT AGT GCC-3 ') and from human Eta 1-363 F (5'-GTG TCC GCA GTG TTA ATA-3'). Reaction amplification using Intron Kit and running on condition predenaturation 94°C for 2 minutes, denaturation at a temperature of 94°C for 1 minute, annealing at a temperature of 51°C for 1 minute, extension at a temperature of 72°C for 30 seconds and post-extension at a temperature of 72°C for 5 minutes, with amplification reactions are run as many as 26 cycles[1]. Electrophoresed amplification product by means of PCR products of 8 ml was added 2 ml of loading dye is checked by 1.5% agarose gel at 100 V for 30-45 minutes. Amplification results showed good bands and sharply by 50 ml done sequensing DNA purification and Biotechnology Laboratory, PT. Wilmar, Cikarang, West Java. Sequencing results of 16S rRNA in the area of statistical analysis from multiple sequence alignment program Clustal W version 1.8 and proceed with the neighbor-joining method and maximum parsimony method to get the highest yield phylogenetic tree[8].

3. Results and discussion

Results of the isolation and identification of bacteria showed that four isolates are the same as the results *Edwardsiella tarda* ATCC. Results identify the characteristics shown in Table 1.

Table 1. Results of identification characteristics of five isolates *Edwardsiella tarda*

Media	ATCC	Pontianak Carp	Semarang Catfish	Jogja Tilapia	Brazilia turtles
Gram test	-	-	-	-	-
Oxidase	+	+	+	+	+
Catalase	+	+	+	+	+
Motility	+	+	+	+	+
Indole	+	+	+	+	+
H_2S in TSIA	+	+	+	+	+
Voges-proskover	+	+	+	+	+
Urea hydrolysis	-	-	-	-	-
Lysine decarboxylase	+	+	+	+	+
Ornithine decarboxylase	-	-	-	-	-
Gelatin hydrolysis	+	+	+	+	+
Gas of glucose	+	+	+	+	+
Acid of Glucose	+	+	+	+	+
Acid of Lactose	+	+	+	+	+
Acid of Sucrose S	+	+	+	+	+

Result rRNA sequencing of five isolates of *E. tarda* in small areas subunit (SSU) 16S looked for variations in genotype (Fig. 1).

```
#ETD                GTA TAT --A TTT ATC TTT TGT TTT CTC TTT  [ 30]
#KK                 ACT TAC CGA AAA AAC ACC GGG CTA CTC CGT  [ 30]
#LL                 AAT AAT AAA AAA AAA AAA GAA CGC AAG AGG  [ 30]
#E2                 ACC TAC AGA AGA AGC ACC GGC TAA CTC CGT  [ 30]
#ETP                ACC TAC AGA AGA AGC ACC GGC TAA CTC CGT  [ 30]
#Edwardsiella_tarda ACC TAC AGA AGA AGC ACC GGC TAA CTC CGT  [ 30]

#ETD                TTT TTC TCC GAT CTT CCC TCT GGA TGC GTC  [ 60]
#KK                 GCC AGC AGC CGC GGT AAT ACG GAG GGT GCA  [ 60]
#LL                 CAG AGG AGG GGA TAA AAT AAG AAG AAC GTA  [ 60]
#E2                 GCC AGC AGC CGC GGT AAT ACG GAG GGT GCA  [ 60]
#ETP                GCC AGC AGC CGC GGT AAT ACG GAG GGT GCA  [ 60]
#Edwardsiella_tarda GCC AGC AGC CGC GGT AAT ACG GAG GGT GCA  [ 60]

#ETD                CCA GTT AA- GCT GGT GTT CTT T-- --C AAT  [ 90]
#KK                 AGC GTT A-- ATC TGA ATT ACT GGG CGT AAA  [ 90]
#LL                 TAA GAA AAT AAC CAC AGC AAA CAA AAG AAA  [ 90]
#E2                 AGC GTT AT- ATC GGA ATT ACT GGG CGT AAA  [ 90]
#ETP                AGC GTT A-- ATC GGA ATT ACT GGG CGT AAA  [ 90]
#Edwardsiella_tarda AGC GTT A-- ATC GGA ATT ACT GGG CGT AAA  [ 90]

#ETD                TTA CCA ATC GCT GCT GTC TTA CTC CCT TAA  [120]
#KK                 GCG CAC GCA GGC GGT TTG TTA ATA ATT GGA  [120]
#LL                 ATT TTT ATT TCT TTC CCT TTA CGC CCC CAA  [120]
#E2                 GCG CAC GCA GGC GGT TTG TTA A-- GTT GGA  [120]
#ETP                GCG CAC GCA GGC GGT TTG TTA A-- GTT GGA  [120]
#Edwardsiella_tarda GCG CAC GCA GGC GGT TTG TTA A-- GTT GGA  [120]

#ETD                TTC GTA AAT --G CTG CTC CTC CTA TGG CCT  [150]
#KK                 TGT GAA ATC --C CCG GGC TTA ACC TGG GAA  [150]
#LL                 ACA ACA ACT TAC CCA AAA CTC ATT TTA TTA  [150]
#E2                 TGT GAA ATC --C CCG GGC TTA ACC TGG GAA  [150]
#ETP                TGT GAA ATC --C CCG GGC TTA ACC TGG GAA  [150]
#Edwardsiella_tarda TGT GAA ATC --C CCG GGC TTA ACC TGG GAA  [150]

#ETD                      GGT GTT GCT AGT ATT TGC AGG TGT TAC  [177]
#KK                       CTG GAT CCA AGA CTG G-C AAG CTA CAG  [177]
#LL                       AAA TTT ACG AAA TCG CAC AAC AGT ACA  [177]
#E2                       CTG CAT CCA AGA CTG GGC AAG CTA CAG  [177]
#ETP                      CTG CAT CCA AGA CTG GGC AAG CTA CAG  [177]
#Edwardsiella_tarda       CTG CAT CCA AGA CTG G-C AAG CTA GAG  [177]
```

Fig. 1. The sequence results rRNA sequencing 5 isolates of *E. tarda* on 16S SSU area.

Maximum parsimony analysis results indicate that the 3 isolates *E. tarda* of Pontianak, Yogyakarta, Jambi one cluster with *E.tarda* derived from Genbank with a validity of 99%. *E. tarda* isolates of turtles Brazil one cluster with isolates ATCC with the number of validity 93% (Fig. 2).

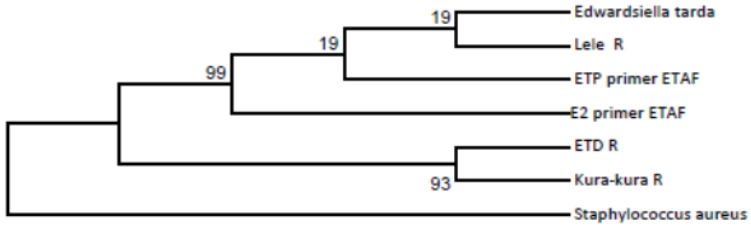

Fig. 2. Result of analysis with maximum parsimony bootsrap 1000x resampling of rRNA sequencing results at 5 isolates *E. tarda* 16S area.

Neighbor-joining analysis results showed that three isolates of *E. tarda* from Pontianak, Yogyakarta, Jambi very closely related to *E. tarda* derived from GenBank with validity of 99%. While *E. tarda* isolates of turtles Brazil a relative with ATCC isolate the validity figure of 95% (Fig. 3).

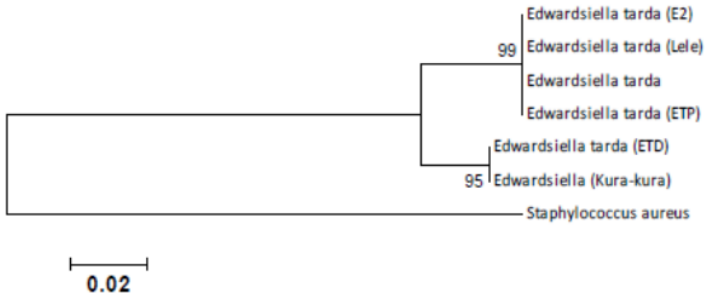

Fig. 3. Result of neighbor-joining analysis of the result of rRNA sequencing 5 isolates *E. tarda* on the area 16S.

Isolates from turtles allegedly obtained from human feces. *Edwardsiellosis* can infect humans that cause hemorrhagic enteritis, disease transmission can occur from pregnant mothers to babies born to infected humans or *Edwardsiella tarda* when performing activities in contaminated waters[4]. *Edwardsiellosis* in humans generally show very serious consequences such as hemorrhagic enteritis, inflammation of the kidneys compared *Edwardsiellosis* in fish[5]. This is consistent with the phylogenetic tree obtained shows isolates of turtles are in a cluster with isolates from humans (ATCC).

The second method is to analyze *Edwardsiella tarda* maximum parsimony and neighbor-joining showed similar results, among others, the formation of two clusters are clusters of fish coming from Pontianak, Yogyakarta and Semarang are in one cluster, while isolates turtles are a cluster with ATCC isolates from humans.

Conclusion

Three isolates of *E. tarda* is the same strain derived from fish compared to isolates of *E. tarda* of turtles Brazil and ATCC isolates derived from human phylogenetic tree.

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Biological Toxicity and Identification of Extract of n-Hexane, Chloroform, Ethyl Acetate and Ethanol from *Chlorella pyrenoidosa* Against Larvae of *Artemia salina*

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61
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Abstract

Research on the identification and biological toxicity of some extracts from microalgae *Chlorella pyrenoidosa* had carried out. The biomass of microalgae *Chlorella pyrenoidosa* was extracted using n-hexane, chloroform, ethyl acetate, and ethanol by way of soxhletation. Toxicity of biological using Brine Shrimp Letalyti Test against *Artemia salina* Leach. Results showed that extract of ethyl acetate, n-hexane, chloroform and ethanol of Chlorella pyrenoidosa had been potentialy toxic against *Artemia salina* Leach with value of LC50 respectively 10.256 µg/ml, 30.130 µg/ml, 80.723 µg/ml, and 129.717 µg/ml. Ethyl acetate extract of *Chlorella pyrenoidosa* potential as raw material for anti-tumor or cancer because it has an LC_{50} value by category of highly toxic and compounds contained in the ethyl acetate extract is Eucalyptol, Trans-caryophyllene, and limonene.

Keywords: Toxicity; identification; n-hexana; chloroform; ethyl acetate; ethanol; C. *pyrenoidosa*; *A. salina L.*

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1. Introduction

Chlorella is one type of Chlorophyceae that contain bioactive compounds that nourish the protein content of about 60% dry weight[1]. In addition, Chlorella also contains unsaturated fatty acids such as Ecosapentanoic Acid (EPA) and Decosaexaenoic Acid (DHA), saturated fatty acids, amino acids, nucleic acids, enzymes, carotenoids, chlorophyll a and chlorophyll b[2]. Therefore, Chlorella can be developed as food, industrial raw materials, cosmetics, pharmaceutical and health. *Chlorella pyrenoidosa* is one type of microalgae potential as anticancer, antitumor, improve the immune system, and antibacterial. Chlorella also contains beta-carotene which is believed to boost the immune system and anti-cholesterol, efficacious prevent the calcification of blood vessel walls and as an antioxidant[3]. One of the method of extraction to obtain bioactive compounds of *C. pyrenoidosa* is soxhletation. The principle of filtering the soxhletation is repeated in order to obtain perfect results, and the solvent used is relatively less. Extraction was done using organic solvents with different polarity level.

The first extracted using n-hexane and then chloroform which aims to attract non-polar compounds such as terpenoids and lipids. After that, followed by ethyl acetate and ethanol which aims to separate the compound which is more polar compounds, such as proteins, xanthon, flavonoid and polyphenols. However, it often does not produce a perfect separation of compounds-compounds extracted. Biological toxicity test is one method used to determine the level of toxicity of a substance with the help of test animals. Animal testing is *Artemia salina* leach. *Artemia salina* Leach used because it has a thin skin and sensitive to the environment, so that substances can easily enter the body by diffusion so that Artemia will die if these substances are toxic, life faster, easier bred and the price is cheap. Principles of bioactive component toxicity test is always toxic if given in high doses and into drugs at low doses. One method of biological toxicity testing is BSLT (Brine Shrimp Lethality Test), which is a test and a screening to determine whether a compound has activity biology and pharmacological with significant the level of trust 95%[4].

The toxicity of the extract caused by the compounds contained in these materials. Therefore, the identification of compounds by GC-MS (Gas Chromatography-Mass Spectrometer). This method has high sensitivity, so as to separate the various compounds are mixed with each other and are able to analyze various compounds although the level / low concentrations[5]. Based on the above, then this research aimed to determine the biological activity of some extracts of biomass *Chlorella pyrenoidosa* against *Artemia salina* Leach and identification of compounds by using GC-MS.

2. Methods

2.1. Cultivation of Microalgae Chlorella pyrenoidosa

Chlorella pyrenoidosa cultivated in medium commercial technique consisting of trisodium phosphate (0.3 g/L), ammonium sulfate (0.8 g/L), Gandasil-D (1 g/L) and urea (1 g/L). Cultivation is done on a 1 liter bottle with a light intensity of 2500 lux. Aeration flowed continuously and pH 7. Growth of microalgae using the spectrophotometric method of turbidimetry at a wavelength of 680 nm.

2.2. Extraction of Biomass from Chlorella pyrenoidosa

25 g of dry biomass *C. pyrenoidosa* was extracted with soxhletation method using successive solvent is n-hexane, chloroform, ethyl acetate and ethanol. Extract obtained and evaporated using an evaporator until thick, then dried using waterbath at 50°C.

2.3. Identification of Compounds by Gas Chromatography-Mass Spectrometer (GC-MS)

Type of GC-MS ie Agilent Technologies 5973 N Gas Chromatograph with Auto Sampler and 5873 chemstation Mass Selective Detector and a data system. Capillary column (innowax), Length: 60 m Width: 0.25 mm, film thickness 0.25 mm, temperature of injector: 2900C, Temperature of detector: 2900C, temperature of program: 900C (150/min)-2900C (20 min), carrier gas: Helium 1 ml/min, Constant Flow injection Volume: 1 mL split (ratio 50: 1).

2.4. Toxicity tests with the BSLT (Brine Shrimp Letality Test)

20 mg egg of *Artemia salina* Leach hatched in a container that has been of water sea/synthetic sea water and irradiated with fluorescent lamp. After 24 hours, nauplii were transferred to other places, and 24 hours later the nauplii can be used as test animals. The concentration of the test solution used was 1000, 100 and 10 ppm. Concentration is made with three replications, then into each vial entered 3 mL of seawater and 10 tails nauplii and add sea water to 5 ml. Observations were made after 24 hours by counting the number of dead and life

larvae. Measurement of mortality (%) larvae of *Artemia salina* is calculated with the following formula: accumulation of dead divided by the accumulation of life plus the accumulation of dead multiplied by 100%.

LC_{50} is the concentration of the test solution which can cause death in 50% of experimental animals. Calculating of LC_{50} using regression equation (Y = a + bX) with log D as the x- axis and y-axis as persen of mortality. An active or toxic substance expressed when the LC_{50} value <1000 mg/ml.

3. Result and discussion

3.1. Extraction biomass dry Chlorella pyrenoidosa with n-hexane, chloroform, ethyl acetate and ethanol with soxhletation.

According Voight (1994), the process of withdrawal of material (extraction) occurs by the flow of solvent into the fuel cell which causes the protoplasm swell, and the cell content in the material to be dissolved according to solubility. High dissolving power associated with the polar solvent and polar materials are extracted[6].

The initial step of this study is to perform the extraction of the biomass *Chlorella pyrenoidosa* using successive solvent ie n-hexane, chloroform, ethyl acetate and ethanol. The use of some types of solvents aims to attract non-polar organic compounds of up to polar. Extraction is done by soxhlation, because the sample can extracted with perfect and it is done repeatedly, amount of solvent which is used less, rapid, number of samples required a little and, organic solvents can take organic compounds repeatedly.

Extracts produced from 20 g of dry biomass of *C. pyrenoidosa* was 0.83 g of n-hexane extract, chloroform extract 2.90 g, 3.25 g ethyl acetate extract and extract of 5.15 g ethanol. The highest extract obtained from ethanol, because ethanol is a universal solvent that can dissolve many compounds. While the smallest dry extract produced by the n-hexane is a mixture of volatile liquid hydrocarbons (Table 1).

Table 1. The results of the extraction of biomass from *Chlorella pyrenoidosa*

No	Solvents	Weight of Extract (g)
1	*n*-Hexane	0.83
2	Chloroform	2.90
3	Ethyl Acetate	3.25
4	Ethanol	5.15

3.2. Identification extract of Chlorella pyrenoidosa has potential toxicity using GC-MS (Gas Chromatography-Mass Spectrometer).

According to analysis by GC-MS, compounds having a percent quality/similarity more than 90 on the n-hexane extracts are compounds of fatty acids is 44.07%, n-hexane is a non-polar solvent that can only attract non polar compounds. While the smallest fatty acid compounds produced by ethanol amounted to 4.92%. The highest terpenoids compounds produced on the ethyl acetate extract are 93.4% while the lowest is produced by extract of n-hexane are 2.02%. The largest alkane compounds is produced by chloroform in the amount of 2.98% while the lowest alkane compounds is produced by n-hexane of 2.51%. Largest alkene compounds is produced by n-hexane that is equal to 7.69%, while the lowest alkene compounds is produced by chloroform of 3.52%. While the phenolic compounds found only in n-hexane of 0.20% (Table 2).

Alkanes are saturated aliphatic hydrocarbon compound which binds all single carbon chain. Usefulness of alkanes: for example methane as fuel for cooking, and carbon black (ink, paint, polish, tires). While, alkenes are unsaturated hydrocarbons in the carbon chain are double bonds. Usefulness of alkenes such as ethene: used as a raw material for making plastic polythene (PE). Propene: used to make plastics, synthetic rubber. Can be used as an anesthetic (mixed with O_2).

Table 2. Results of gc-ms analysis of extract n-hexane, chloroform, ethyl acetate and ethanol from *Chlorella pyrenoidosa*

Coumponds	BM	Molecule formula	Extract							
			n-hexana		Chloroform		Ethyl Acetate		Ethanol	
			RT	Area %	RT	Area %	RT	Area %	RT	Area %
Fatty acids										
Isopropyl Miristate	270.45	$C_{17}H_{34}O_2$	16.25	7.47	-	-	-	-	-	-
Hexadecanoic acid	256.42	$C_{16}H_{23}O_2$	17.05	4.39	17.06	4.99	-	-	17.36	4.92
Octadecenoic acid	284.48	$C_{19}H_{38}O_2$	18.70	1.38	-	-	-	-	-	-
9-octadecenoic acid	282.46	$C_{18}H_{34}O_2$	18.54	4.16	18.54	4.82	-	-	-	-
9,12-Octadecadien acid	280.44	$C_{19}H_{34}O_2$	18.90	2.48	-	-	-	-	-	-
9,12,15-Octadecatrien	278.43	$C_{18}H_{30}O_2$	18.96	4.48	-	-	-	-	-	-
Di-(2-ethylhexyl) Phthalate	390.55	$C_{24}H_{38}O_4$	24.06	19.71	-	-	-	-	-	-
Contents			44.07%		9.81%		-		4.92%	
Terpenoid										
Azulene	128.17	$C_{10}H_8$	11.22	0.29	11.18	1.68	-	-	-	-
Eucalyptol	154.249	$C_{10}H_{18}O$	-	-	-	-	3.52	86.47	-	-
Limonene	136.24	$C_{10}H_{16}$	-	-	-	-	5.11	2.84	-	-
Trans-aryophyllene	204.36	$C_{15}H_{24}$	-	-	-	-	7.02	4.09	-	-
Neophytadiene	278	$C_{20}H_{38}$	16.41	1.73	16.42	13.75	-	-	16.76	3.81
Contents			2.02%		15.43%		93.4%		3.81%	
Alkana										
Pentadecane	305.33	$C_{15}H_{32}$	13.70	0.17	13.71	0.56	-	-	-	-
Hexadecane	226.44	$C_{16}H_{34}$	14.52	0.43	-	-	-	-	-	-
Heptadecane	240.46	$C_{17}H_{36}$	15.30	0.46	-	-	-	-	-	-
Octadecane	254.49	$C_{18}H_{38}$	16.06	1.02	13.71	0.56	-	-	-	-
Tetradecane	198.39	$C_{14}H_{30}$	-	-	12.83	0.46	-	-	-	-
Eicosane	282.55	$C_{20}H_{42}$	14.52	0.43	14.53	1.40	-	-	-	-
Contents			2.51%		2,98%		-		-	
Alkena										
Hexadecene	224.43	$C_{16}H_{32}$	14.47	0.26	-	-	-	-	-	-
Octadecene	252.48	$C_{18}H_{36}$	16.02	1.09	-	-	-	-	-	-
Nonadecene	266.50	$C_{19}H_{38}$	21.48	1.97	17.58	3.52	-	-	-	-
Eicosene	280.53	$C_{20}H_{40}$	17.57	4.37	-	-	-	-	-	-
Contents			7.69%		3.52%		-		-	
Fenol										
Phenol,2,4-bis (1,1-dimethylethyl)	588.95	$C_{14}H_{22}O$	13.95	0.20	-	-	-	-	-	-
Contents			1.93%		-		-		-	

3.3. Toxicity test of extract n-hexane, chloroform, ethyl acetate and ethanol from Chlorella pyenoidosa with bslt (Brine Shrimp Letality Test)

BSLT method using larval shrimp *Artemia salina* Leach as test animals is one method which is widely used for the search of new anticancer compounds derived from plants or microorganisms such as microalgae. The results of toxicity tests with this method has been shown to have a correlation with power cytotoxic anticancer compounds. In addition, this method is also easy to do, inexpensive, fast and accurate enough[4].

Otherwise extract has the ability or biological activity when generating high mortality based on its LC_{50} value. Meaning LC_{50} is the concentration of how the extract can be lethal to 50% of the test organisms, such as the larva *Artemia salina* (brine shrimp). The smaller the value of the LC_{50}, the greater the ability of its toxicity. An extract is considered highly toxic when the LC_{50} value of below 30 ppm, is considered toxic if it has a value of 30-1000 ppm LC_{50} and considered not toxic if LC_{50} values above 1000 ppm. The toxicity level to give meaning to its activity as a potential anticancer drug. The smaller value of LC_{50}, the compounds are more toxic and more potential as an anticancer compound[4].

In Table 1 is the result of the percentade of mortality of *A. salina* L. against extract of n-hexane, chloroform, ethyl acetate, and ethanol from *Chlorella pyrenoidosa* with BSLT method. Based on these results, will be obtained molarity percentage and for calculating of LC_{50} of each extract used the calculations of probit analysis

using SPSS 13 for calculating the LC_{50} of each extract were tested. LC_{50} results can be seen in Table 3.

Table 3. Test calculations result of BSLT extract n-hexane, chloroform, ethyl acetate and ethanol from *Chlorella pyrenoidosa*

Samples	Dose (ppm)	Log D (x)	Dead	Life	Accumulated dead	Accumulated life	% mortality (y)	LC_{50} ppm
Ethyl Acetate	1000	3	27	3	68	3	95.774	10.256
	100	2	23	7	41	10	80.392	
	10	1	18	12	18	19	48.648	
n-hexane	1000	3	22	8	56	8	87.5	30.130
	100	2	18	12	34	20	62.962	
	10	1	16	14	16	26	38.095	
Chloroform	1000	3	24	6	48	6	88.888	80.723
	100	2	15	15	24	21	53.333	
	10	1	9	21	9	42	17.647	
Ethanol	1000	3	10	20	42	20	67.741	129.717
	100	2	9	21	32	41	43.835	
	10	1	23	7	23	48	32.394	

In Table 3 shown extracts of n-hexane at a concentration of 10 ppm resulted in percentase of mortality ie 38.095%, a concentration of 100 ppm resulted in percentase of mortality ie 62.962%, and a concentration of 1000 ppm resulted in percentase of mortality ie 87.5%, so that value of LC_{50} of n-hexane extract is 30.130 ppm, therefore that the n-hexane extracts are included in the category of toxic.

The percentage of mortality of larvae *Artemia salina* on extract of chloroform with concentrations of 10 ppm is 17.647%, at a concentration of 100 ppm is 53.333%, and a concentration of 1000 ppm is 88.888%. LC_{50} value of chloroform extract is 80.723 ppm, so the chloroform extracts are included in the category of toxic.

At the ethyl acetate extract, showing the percentage of mortality of larvae *Artemia salina* respectively 10 ppm is 48.648%, a concentration of 100 ppm is 80.392%, and a concentration of 1000 ppm is 95.774%. LC_{50} values of ethyl acetate extract of 10.256 ppm, based on the value of LC_{50} ethyl acetate extracts are included the category of highly toxic because it is below 30 ppm.

Further, the percentage of mortality in the ethanol extract in a row on the extract concentration 10 ug/ml namely 32.394%, a concentration of 100 ppm is 43.835%, and a concentration of 1000 ppm is 67.741%. LC_{50} value of the ethanol extract is 129.717 ppm, so the ethanol extract was included in the category of toxic. Base on the result, showing that the greater the dose of the extract concentration, the mortality of larvae *Artemia salina* is also getting bigger. This is in accordance with Harbone (1978), that the higher the concentration of the extract, the toxic also higher. Mortality of the extract caused by the toxic properties of the compounds contained in the extract[5]. Results of control with seawater (mortality 0%), it indicates that larvae that die due to toxic compounds in the extract, not because of other factors.

Meyer *et al*. (1982) explained that the activities of the toxicity of an extract in BSLT if the extract can cause the death of 50% of larvae at a concentration of less than 1000 ppm[4]. The most toxic extracts can be seen from the ability to cause the death of test animals larger with increasingly smaller concentrations. This shows that the most toxic extract is an extract of ethyl acetate with 10.256 ppm value, followed by n-hexane extract is 30,130 ppm, then the chloroform extracts namely 80.27 ppm and ethanol extract is 129.717 ppm.

Molarity percentage of *A. salina* in ethyl acetate extract showed more toxic LC_{50} value (10.256 ppm) due semipolar compounds dissolved in ethyl acetate extract having a smaller size so that the extract easier penetration into the cell membrane through the process of diffusion in the tails (tail) *A. salina* which has hydrophobic properties on a phospholipid bilayer. This causes to more rapid cell damage, so the cell will die. Besides, it is also because of the class of terpenoid compounds in the ethyl acetate extract were very large compared with other compounds, namely 93.4%, consisting of eucalyptol, limonene and trans-caryophyllene. This is according to research Indrayani *et al*. (2006), which suggests that the terpenoid compounds contained in extracts of leaves *Stachyterpheta jamaicensis* L. Vahl are toxic to larvae of *Artemia salina* Leach and have anticancer activity[7]. Several studies report stated that eucalyptol has been used to treat rhinitis bronchitis, chronic sinusitis, and also for the treatment of asthma, anti-inflammatory, inhibits tumor production[8]. Research in vivo (bioassays) shows limonene inhibits the growth of liver cancer, lung adenomas, and gastric tumors[9]. Tans-caryophyllene has been used for anti-inflammatory, antioxidant, antibiotic, local anesthetic activity and anti-cancer[10].

4. Conclusions

Extract ethyl acetate, n-hexane, chloroform and ethanol from *Chlorella pyrenoidosa* has toxic properties because the LC_{50} value is less than 1000 ppm. The ethyl acetate extract of *Chlorella pyrenoidosa* potential as anticancer because it has a LC_{50} value less than 30 ppm is equal to 10.256 ppm. Results of identification of compounds of extract of Ethyl acetate from *Chlorella pyrenoidosa* is Eucalyptol, Trans-caryophyllene, and limonene.

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Beta Glucan from *Saccharomyces cerevisiae* Derived from the Local Ragi and Its Potential as Antioxidants

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Abstract

Four strains of *S. cerevisiae* have been isolated from the local ragi named *S. cerevisiae* RT03, RT12, RT14 and SAF and it was cultured respectively on GYP medium at 30°C. β-Glucan extraction using acid-base neutralization method. Analysis of functional groups was carried by FTIR. Levels of β-glucan equivalents glucose most of 6.9042% acquired by *S. cerevisiae* RT03 with 3.7065% of protein content. Beta glucan from local ragi tested its potential as antiradical DPPH. The results indicate that β-glucan from *S. cerevisiae* SAF has the highest antiradical DPPH activity with IC_{50} of 74.5 μg.ml^{-1}, followed RT12, RT03 and RT14 with IC_{50} respectively 105, 5; 145.71 and 186.1 μg.ml^{-1}. Fractionated by column chromatography showed stronger antiradical DPPH.

Keywords: Beta Glucan; *Saccharomyces cerevisiae*; antiradical DPPH

1. Introduction

Yeast is a well known microorganism that is used in biotechnology since ancient times. Therefore it is a good source of β-glucan (homopolymer of glucose). The cell wall of *Saccharomyces cerevisiae* consists of approximately 29-64% β-glucans, 31% mannans, 13% proteins, 9% lipids, and 1-2% chitin[1,2]. However, the exact structure and composition of the yeast cell wall depends strongly on the cultivation conditions. The β-glucan component in the *Saccharomyces cerevisiae* cell wall, with the function of maintaining the rigidity and shape of the cell, is often named simply glucan or yeast glucan. That polysaccharide consists mainly of a linear central backbone of D-glucose linked in the β-(1→3) position with glucose side branches (β-(1→6)-linkage) of various sizes, which occurs at different intervals along the central backbone[3]. Triple helical multimer provides structure and support to the yeast cell wall. The native structure of β-glucans as well as their biological activities could be changed during isolation if harsh procedures are applied. Primary structure, solubility, degree of branching and molecular weight, as well as the charge of their polymers and structure in aqueous media, are responsible for biological activity of β-glucan.

Based on the structure, β-Glucan is a polysaccharide consisting of thousands of glucose units. The units of glucose on β-glucans are linked with β-glycosidic bond to form a polymer (Fig. 1).

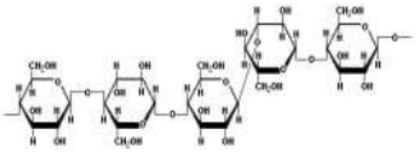

Fig.1 Structure of Beta Glucan.

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In recent years evidence suggested that biopolymers (like β-glucan) also exhibit antioxidative activity[4]. The exploration of antioxidants is of great interest because they are able to protect living organisms from the attack of reactive oxygen species (ROS), and in this way to decrease the risk of several degenerative diseases[5,6]. *Saccharomyces cerevisiae* contains several endogenous substances which act as antioxidants[7,8].

In many living organisms, oxidation is essential for the production of energy to accelerate biological processes. However, when the mechanism of oxidation becomes unbalanced by factors such as aging, deterioration of physiological functions may occur resulting in diseases such as cancer, rheumatoid arthritis, and atherosclerosis[9]. Thus, maintenance of equilibrium between free radical production and antioxidant defenses (enzymatic and nonenzymatic) is an essential condition for normal organism functioning. Antioxidant supplements or foods containing antioxidants may be used to help the human body reduce oxidative damage[10]. Antioxidants are compounds that can delay or inhibit the mechanism of oxidation by blocking the initiation or propagation of oxidizing chain reactions. Several commercial synthetic antioxidants are widely used in food industry such as butylatedhyroxyanisole (BHA), butylatedhydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ)[11]. However, the utilization of these synthetic antioxidants becomes restricted due to their toxicity and carcinogenicity effects to the liver. Therefore, it is necessary to investigate and produce more effective natural antioxidants that are less harmful to human body. If it were to be shown that β-glucan, a natural component of grain, exerts antioxidant activity, the utility of β-glucan as a polymeric excipient for supplement or food additive would increase further. However, the antioxidant activity of β-glucan has not been extensively investigated.

In several regions in Indonesia, yeast used for fermentation of foods such as ice guess, lamang tapai, and also wine which is typical regional food or drink. Beta glucan derived from yeast isolated from sources of food and drinks. This research is focused to produce β-glucans from *S. cerevisiae* isolated from three local samples of yeast. The parameters measured are dry weight of β-Glucan crude (mg), characterization of β-glucans with Fourier Transform Infra Red (FTIR), β-Glucan levels equivalent glucose and protein levels were analyzed by UV-Vis spectrophotometer. For determination of radical scavenging activity of β-Glucan crude and β-Glucan fractionation results by column chromatography by using DPPH.

2. Methods

2.1. Microorganism and Media

Saccharomyces cerevisiae were isolated from four samples locally derived from Padang (RT03), Tasikmalaya (RT12), Pekalongan (RT14), and commercial instant yeast (SAF) as a comparison. The cultures were maintained on the medium of potato dextrose agar (PDA) at 4°C. The microorganisms were subcultured at regular intervals (2 months) to maintain viability. The inoculum was prepared by growing *S. cerevisiae* RT03, RT12, RT14 and SAF in Erlenmeyer flasks containing 50 mL of the GYP medium (w/v): yeast extract 1%, 2% peptone, 2% glucose on a rotary shaker at 150 rpm, for two days, at 25°C.

2.2. β-glucan production

Cultivation was carried out in Erlenmeyer flasks containing 300 mL of the GYP medium was inoculated with 2% (v/v) of the inoculum. The inoculum culture were RT03, RT12, RT14 and SAF cultivated triplicate for 6 days on a rotary shaker at 150 rpm at 25°C and cultivated for 6 days.

2.3. Extraction Beta Glucan (Alkali-acid method)

Beta glucan were isolated from the cell wall of *S. cerevisiae* by a combination method of Williams *et al*[12] and Hunter *et al*[13]. Briefly, the cell wall of *S. cerevisiae* was dispersed in 0.75 M (3%) sodium hydroxide (NaOH), and extracted in water bath at 75°C for 2 hours. The suspension was cooled to room temperature and centrifuged (4000 rpm, 10 min), the resulting pellet collected and this material then washed three times using 0.5 L distilled water. The residue was then mixed with 0.2 L of 0.75 M NaOH, and extracted in boiling water for 3 hours. Distilled water was then added to the cooled suspension, the pH adjusted to 7 with HCl, and the supernatant after centrifugation discarded. This water wash was repeated until the residue became white and flocculent, and finally the pellet was washed with absolute ethanol until the supernatant became colorless. Particulate glucan was obtained by drying the washed pellets by oven vaccum at 40°C[12,13].

2.4. FTIR analysis of β-glucan

β-Glucan were analyzed by FTIR-JASCO 4100 Spectrophotometer. IR spectra of the compounds were recorded for 400-4000 cm^{-1} in KBr pellet using a FT-IR spectrophotometer.

2.5. β-glucan assay

β-glucan hydrolysis was added to aqueous 50% H_2SO_4 solution so that β-glucan concentration was 2 mg/ml. Hydrolysis was held for 24 h. Then solution was neutralized with 2 M NaOH aqueous solution (5 mL 2 M NaOH for 1 mL β-glucan hydrolysis solution with 50% H_2SO_4). Glucose monomers concentration in solutions was determined using phenol sulphuric acid method DuBois *et al*[14]. Protein concentration analysis was determined using Lowry protein assay[15].

2.6. Glucose Analysis with Phenol–Sulfuric Acid method[14]

A 2 mL aliquot of a carbohydrate solution is mixed with 1 mL of 5% aqueous solution of phenol in a test tube. Subsequently, 5 mL of concentrated sulfuric acid is added rapidly to the mixture. After allowing the test tubes to stand for 10 min, they are vortexed for 30 second and placed for 20 minutes in a water bath at room temperature for color development. Then, light absorption at 490 nm is recorded on a spectrophotometer. Reference solution is prepared in identical manner as above, except that the 2 mL aliquot of carbohydrate is replaced by water. The 5% phenol in water (w/w) was prepared immediately before the measurements.

2.7. Protein Analysis by Lowry method[15]

An amount of 0.25 mL of test solution was diluted with distilled water to 0.5 mL; 0.25 mL of 1 N NaOH was added and boiled for 20 minutes; 1.25 mL of solution of reagent D (50 ml Na_2CO_3 5% , 1 mL $CuSO_4.5H_2O$ 1%, 1 ml of potassium tatrat 2%) was added, shaken in order to be homogeneous, and allowed to stand for ten minutes; 0.25 mL of Folin Ciocalteu was added that so blue color appeared, allowed to stand for 30 minutes, absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 750 nm.

2.8. Determination of β-Glucan level as equivalent of Glucose and Protein Content Resulted from Fractionation

Fractionation performed by column chromatography using silica gel absorbent and eluent consisting of a mixture of n-butanol, ethanol , water (5 : 5 : 4 , v/v). The levels of β–Glucan as equivalents of glucose and protein levels of fractions resulted were analyzed as the assay prior to fractionation.

2.9. Determination of antioxidant activity Scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical[16]

The DPPH radical scavenging activity was evaluated using the method of Yon and Lewis (2002). DPPH radical solution (0.004%, w/v) in methanol was prepared. A volume of 2 mL of DPPH in methanol was added to 2 mL of sample, well vortexed and incubated for 30 min in dark room at room temperature. Absorbance of each sample at 517 nm was measured using UV-Visible spectrophotometer (Shimadzu). Methanol was used as a blank, while DPPH solution in methanol served as control. Vitamine E (Sigma) was used for comparison. The antioxidant activity was expressed as percentage of DPPH activity calculated as:

$$\text{DPPH activity (\%)} = \frac{\text{Absorbance of blank} - \text{absorbance of sample}}{\text{Absorbance of blank}} \text{ x } 100$$

Calculating IC_{50} using linear regression $y = a + bx$

Whereas : $y = 50$ a = intercept

$x = IC_{50}$ b = slope/tilt

r = correlation coefficient

The value of IC_{50} (Inhibition Concentration 50%) indicated that concentrations of β- glucan could inhibit 50% of the activity of free radicals (DPPH). β−Glucan is declared to be potentially antioxidant if the value of IC_{50} was less than 200 pg/ml[17] .

3. Results and discussion

3.1. Dry weight of beta glucan crude

The use of four isolates of *S. cerevisiae* produced distinctive dry weight of β-glucan. The highest result obtained by isolates of *S. cerevisiae* RT 12 at 2.970 $g.L^{-1}$, whereas the lowest was obtained by isolates of *S. cerevisiae* RT03 at 0.428 $g.L^{-1}$ (Table 1). Differences in the production of β-Glucan in four isolates of *S. cerevisiae* are due to the differences in genetic trait. The genetic differences can affect the result of fermentation of *S. cerevisiae*

Table 1. Dry weight of β-glucan crude from 4 different strains of *Saccharomyces cerevisiae* (gL^{-1})

No	Sample *S. cerevisiae*	dry weight of β-glucan
1	RT03	0.428
2	RT12	2.970
3	RT14	2.380
4	SAF	2.000

3.2. β-Glucan crude Analysis by FTIR

FTIR results showed that β-glucans crude from four isolates of *S. cerevisiae* have functional groups according to the structure of the β-glucans standards from Takeda-Japan. The FT-IR spectra of the glucans are shown in Fig. 2, were obtained functional group of –OH (alcohol), -C-C-C- (alkane), and -R-O-R (ether) (Table 2).

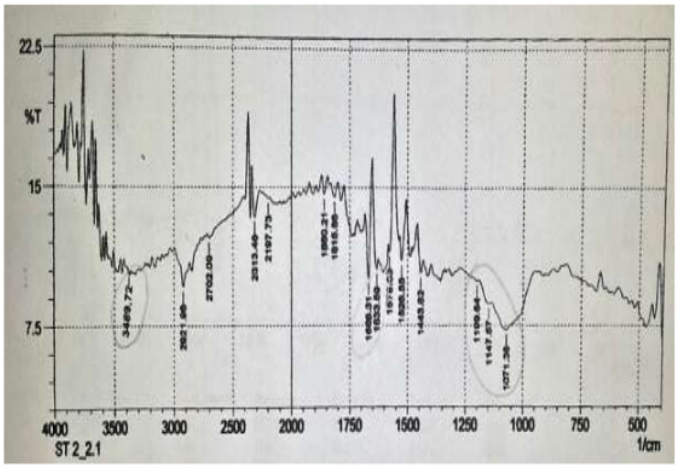

Fig. 2. FTIR spectra of beta 1,3-glucan (Takeda- Japan)

Table 2. Analysis of functional groups of β-glucans crude from 4 isolates of *S. cerevisiae* by FTIR

Functional Groups	Wave number Literature (cm^{-1})[17]	Wave number sample (cm^{-1})				
		S. cerevisiae				
		Std of β Glucan	RT 03	RT 12	RT 14	SAF
stretch –OH	3000 – 3700	3173.65	3489.72	3456.20	3357.84	3279.73
				3332.76		
stretch -C-C-C	1680-1600	1608.52	1633.59	1657.70	1638.42	1663.49
		1668.31	1668.31		1628.77	
stretch -R-O-R	1050-1260	1075.24	1071.38	1133.10	1080.06	1157.21
		1155.28	1147.57	1157.21	1153.35	
		1228.57	1199.64			

3.3. Levels of β-Glucan as equivalent of glucose and protein

Phenol–Sulfuric Acid method, this is the most widely used colorimetric method for determination of carbohydrate concentration in aqueous solutions[14]. The basic principle of this method is that carbohydrates, when dehydrated by reaction with concentrated sulfuric acid, produce furfural derivatives. Further reaction between furfural derivatives and phenol develops detectible color.

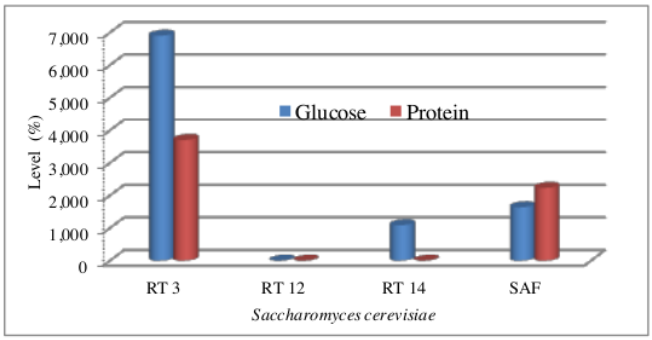

Fig 3. Levels of β-Glucan equivalent glucose and protein levels of 4 isolates *S. cerevisiae* with Spectrophotometer UV-Vis λ 490 nm

3.4. β -Glucan levels as equivalent of glucose

Assay was conducting by using Phenol-Sulfuric Acid. The analysis is done because glucose is the monomer of β-Glucan. The four different *S. cerevisiae* isolates produce different glucose levels, it can be caused by genetic factors of the strain and the influence of environmental factors both physically and chemically. Each strain requires specific conditions for optimum growth. The highest results of the analysis of the levels of β-Glucan glucose as equivalents obtained by *S. cerevisiae* RT03 *isolates* at 6.9042%, while the levels of β-Glucan lowest glucose as equivalents obtained by *S. cerevisiae* RT12 isolates at 0.7855% (Table 3). The results of statistical analysis of β-Glucan level as equivalent of glucose showed that *S. cerevisiae* RT03 highly distinctive to the other isolates.

3.5. Protein Content

The Lowry method for total protein determination is one of the most common colorimetric assays performed by biochemists. This procedure lies in the reactivity of the peptide bonds with the Cu^{2+} under alkaline conditions and the subsequent reduction of the Folin-Ciocalteau phosphomolybdic phosphotungstic acid to heteropoly molybdenum blue by the copper-catalyzed oxidation of aromatic acids. The Lowry method is sensitive to low concentrations of protein. The major disadvantage of the Lowry method is the narrow pH range within which it is accurate. However, in this experiment very small volumes of sample are used which have little or no effect on pH of the reaction mixture. A variety of compounds (some amino acid derivatives, zwitter ionic and non ionic buffers, drugs, lipids, sugars, salts, nucleic acids, sulphydryl reagents, ammonium ions and thiol compounds) can interfere with the Lowry procedure[15].

The protein content was analyzed for *S. cerevisiae* cell walls contained β-Glucan in the form of complex compounds bound as proteoglucan. Protein content in the crude β-Glucan is expected to be as low as possible for applications in the field of food and pharmaceuticals. It is projected to avoid the consumer become allergic to a particular protein. Nitrogen source in the fermentation medium was used for protein synthesis in the cell. The existence of the cell absorption of this nitrogen source causes the protein content in the media increasingly reduce along with the length of fermentation time. The analysis results showed that the highest protein content was obtained by isolates of *S. cerevisiae* RT03 at 3.7065%, while the lowest was obtained by isolates of *S. cerevisiae* RT12 at 0.4432% (Table 3). The statistical analysis showed that the protein content of the isolates of *S. cerevisiae* RT12 and RT14 are not significantly different, while RT03 and SAF showed significant difference (Table 3) .

3.6. Ratio of β-Glucan levels as equivalent of Glucose: the protein content of the highest fractionation results

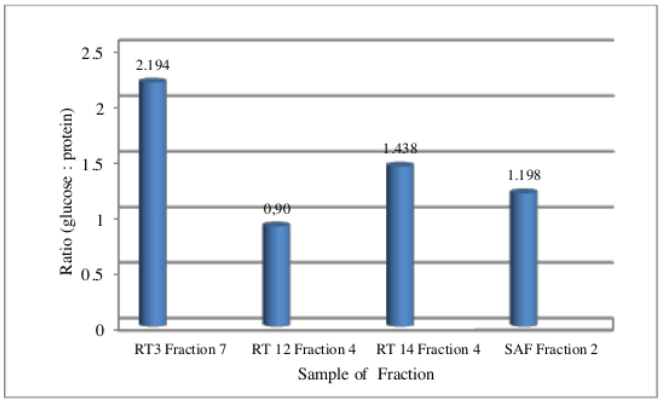

Fig. 4. Ratio of β-Glucan levels as equivalent of glucose: the protein content of the four isolates *S. cerevisiae* fractionation results

Fractionation was conducted to obtain a more pure extract with a ratio of β-Glucan levels as equivalent of glucose to the higher protein content compared to the protein content before fractionation. Fractionation was performed using column chromatography. The β-Glucan levels as equivalent of glucose of the samples column results were then analyzed with the phenol sulfate method and protein content was analyzed by Lowry method. The analysis showed that the ratio of levels of β-Glucan as equivalent of glucose: the highest protein content was produced by isolates of *S. cerevisiae* RT03 (fraction 7) of 1.1348, while the ratio of levels of β-Glucan as equivalent of glucose: the lowest protein content was produced by isolates of *S. cerevisiae* RT14 (fraction 4) of 0.8247. Levels β-Glucan as equivalent of glucose and protein levels of β-Glucan compound after fractionated lowered. It is due to the components of other compounds contained in crude β-Glucan was eliminated in the process of fractionation using column chromatography.

3.7. Antioxidant activity of β-glucan from 4 isolates of S. cerevisiae by DPPH free radical scavenging assay.

Fractionation of β-glucan each sample of four isolates of *S. cerevisiae* using column chromatography obtained 10 fractions. Then, three fractions were chosen based on the highest ratio of glucose and protein level for testing the antioxidant potential. Next, one fraction, that is potential as an anti-radical DPPH to be compared with the test results crude β-glucan, was chosen. Analysis was performed using UV-Vis spectrophotometer at λ 517 nm, which is the highest uptake of compounds DPPH. DPPH solution was purple in methanol, purple color changed to yellow when reacted with antioxidants. DPPH color reduction occured because of the existence of compounds that can provide radical hydrogen to DPPH radicals, so they were reduced to DPPH-H (1,1-diphenyl-2-picrylhydrazyl-hydrate) and became more stable. It is seen when measuring absorption by UV-Vis spectrophotometer i.e. the solution poured into the cuvette turned into a reddish yellow. Experiments were performed using vitamin E as a positive control.

The measurement results show that vitamin E has IC_{50} value of 33.55 μg /ml, RT03, RT12, RT14 β-glucan and SAF consecutively 145.71; 105.5; 186.1; and 74.5 μg /ml. The result of β-glucan extract test compared to fractionated samples was that RT03 β-glucan fraction -7 generated IC_{50} value of 122.7µg /ml, RT12 β-glucan fraction -4 produced 99.4 pg/ml, RT14 β-glucan fraction-4 produced 155.85 µg/ml, β-glucan fraction SAF-2 fraction 55.9 µg/ml. Beta glucan sample fractionation by column chromatography showed increased activity of DPPH radical, as shown by the declining value of IC_{50}. Fractionated sample extract produced more pure β-glucan because separation had been done by column chromatography. Based on the results obtained, all test samples as potentially anti-radical DPPH IC_{50} because the values were below 200 μg/ml. Observations results can be seen in Table 4 and 5. The highest antioxidant potential was produced by vitamin E as a positive control followed by sample of β-glucan *S. cerevisiae* successively SAF, RT12, RT03 and RT14.

Table 3 . DPPH radical scavenging activity and IC_{50} value of beta glucan *S. cerevisiae* (RT03, RT12, RT14, and SAF) and positive control (Vitamin E)

Sample Code	Concentration (μg/ml)	Inhibition (%)	Linear Equations Y= a + bx	IC_{50} (μg/ml)
RT03	50 75 100	45.716 46.853 47.989	50 =43.443 + 0.045 x r = 0.999	145.71
RT12	50 75 100	41.870 46.066 49.038	50 = 34.906 + 0.143 x r = 0.995	105.5
RT14	50 75 100	45.804 46.590 47.377	50 = 44.230 + 0.031 x r = 0.999	186.1
SAF	50 75 100	45.45 50.34 54.45	50 = 36.58 + 0.18 x r = 0.998	74.5
Vitamin E (Positive Control)	50 75 100	50.586 52.209 52.840	50 = 48.497 + 0.045 x r = 0.969	33.55

Table 4. DPPH radical scavenging activity and IC_{50} value of beta glucan *S. cerevisiae* (RT03, RT12, RT14, and SAF) fractionation results.

Sample Code	Fraction	Concentration (μg/ml)	Inhibition (%)	Linear Equations Y= a + bx	IC_{50} (μg/ml)
RT03	7	12.5 25 50	17.30 25.96 28.93	50 =15.64 + 0.28 x r = 0.891	122.7
RT12	4	12.5 25 50	12.41 14.24 28.58	50 = 5.24 + 0.45 x r = 0.973	99.4
RT14	4	12.5 25 50	10.57 13.72 20.89	50 = 6.98 + 0.28 x r = 0.999	155.8
SAF	2	12.5 25 50	13.81 28.84 44.31	50 = 6.07 + 0.78 x r = 0.983	55.90

4. Conclusions

The highest β-Glucan Dry Weight was obtained by isolates of *S. cerevisiae* RT12 at 111.3 mg, whereas the lowest was obtained by isolates of *S. cerevisiae* RT03 at 14.4 mg. Characterization of crude β-Glucan from four isolates of *S. cerevisiae* using FTIR showed that each of the four isolates had its functional group that was similar to the standard β-Glucan compounds. Isolate *S. cerevisiae* RT03 originally from Payakumbuh West Sumatra-Indonesia, gave the best results with levels of β-Glucan as equivalents of glucose amounted to 6.9042% and 3.7065% for protein content. Samples of β-Glucan *S. cerevisiae* RT03 resulted from fractionation namely Fraction 7 had the highest levels ratio of β-Glucan as equivalent of glucose: the highest protein at 1.1348. Samples of β-Glucan *S. cerevisiae* SAF had the lowest IC_{50} value indicating the highest potential ant radical DPPH. The fractionation of the four sample of β-glucan *S. cerevisiae* had IC_{50} values lower than β-Glucan crude of *S. cerevisiae*.

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Anti-tuberculosis Activity of Chloroform and Methanol Extracts of *Michelia champaca* L. Stem Bark against *Mycobacterium tuberculosis* MDR

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Abstract

Michelia champaca L. stem bark of Magnoliaceae, was traditionally used to treat cough based on Ayurveda. This research was conducted to evaluate the anti-tuberculosis activity of chloroform and methanol extract of *M. champaca* against *Mycobacterium tuberculosis* MDR. Extraction was conducted by successive extraction with *n*-hexane, chloroform and methanol. Anti-tuberculosis activity test was done by using proportion method with Lowenstein Jensen medium and extract concentration of 1, 10 and 100 mg/mL then incubated in 5% CO_2 incubator at 37°C for six weeks. Bacterial colonies were observed from 3rd to 6th week. Anti-tuberculosis activity was determined by inhibition of extract against *M. tuberculosis*, which was calculated by comparing amount of colonies of treatment group to control. TLC Densitometry of extracts showed that terpenoids and flavonoids were detected in chloroform and methanol extracts. Extracts used in this study were active as anti-tuberculosis with the inhibition against *M. tuberculosis* MDR above 90% at extract concentration of 10 and 100 mg/mL

Keywords: Michelia champaca L.; terpenoid; flavonoid; anti-tuberculosis; *Mycobacterium tuberculosis* MDR; extracts

1. Introduction

The incidence of tuberculosis has been significantly increasing in the last decade around the world. In 2012, there are 730.000 cases with a mortality rate of 27%[12]. Increasing cases of tuberculosis caused by various factors, such as treatment failure, drop out of treatment, improper treatment, patients with HIV infection, and poor drug absorption that resulted in the appearance of resistance of *Mycobacterium tuberculosis* to current anti-tuberculosis drugs. In Indonesia, tuberculosis still becomes health problem, both in terms of mortality, diagnosis and treatment. According to the WHO Global Report 2012, Indonesia was ranked ninth out of 27 countries with the highest burden of multi-drug resistant (MDR) tuberculosis in the world[11]. MDR tuberculosis is estimated at 1.9% of all new cases and 12% of the cases of tuberculosis with re-treatment[12]. The problem of drug resistance is pushing the need for appropriate strategies in controlling MDR tuberculosis.

Exploring the potential of plants used in traditional medicine is one of important strategy that has been conducting. *Michelia champaca* which is commonly known as Cempaka Kuning is traditionally used as cough remedy. The traditional usage of this plant is commonly as infusion. Several pharmacological activities of this plant have been reported. Ariantari *et al*. (2013), has been reported for the antimalarial activity against *Plasmodium falciparum* 3D7 of n-hexane, chloroform and methanol extract of *M. champaca* stembark. Methanol extract of leaves, seeds, stem and root barks, stem and root heartwoods of *M. champaca* is also reported for its pharmacological activity as antimicrobial[6]. Flowers extract of *M. champaca* revealed antidiabetic activity in alloxan induced diabetes model[5], antiinflamatoryactivity[1] and wound healing activity in Wistar rats[9]. In present study we investigate the anti-tuberculosis activity of chloroform and methanol extract of *M. champaca* stembark against *M. tuberculosis* MDR.

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2. Methods

2.1. Plant material

Stembark was collected in September 2012, from Pupuan Tabanan Bali-Indonesia. Plant specimen has been identified at Kebun Raya Eka Karya, Bedugul, Tabanan-Indonesia.

2.2. Bacteria

M. tuberculosis MDR was derived from Clinical Microbiology Department, Sanglah General Hospital, Denpasar, Bali-Indonesia.

2.3. Procedures

2.3.1. Extraction

Coarsely dried *M. champaca* stembark powdered (1 kg) was extracted with n-hexane (10 L) to remove resins and fatty acid. Residu then extracted successively with chloroform (10 L) and methanol (10 L) to obtain chloroform extract (13.33 g) and methanol extract (25.83 g).

TLC Densitometry of extract was done using Silica gel GF_{254} as stationary phase and chloroform : methanol (95.0 : 5.0) as mobile phase. Ammonia and 10% sulfuric acid was used as spray reagent.

2.3.2. Anti-tuberculosis Activity Assay

Anti-tuberculosis activity assay was done by using proportion method refer to method previously reported by Gupta *et al*. (2010). Addition of extracts into Lowenstein-Jensen medium to get serial concentration of extract of 1, 10, and 100 mg/mL. Control received 1% of dimethylsulfoxide. *M. Tuberculosis* MDR inoculated on this medium then incubated in 5% CO_2 incubator, 37°C for 6 weeks. Colonies growth as colony forming unit (cfu) was observed three times a week starting from 3rd until 6th week. Anti-tuberculosis activity of extract was determined from percentage of inhibition of extract against *M. Tuberculosis* growth, which was calculated by comparing colonies growth in treatment group to control.

3. Results and discussion

Chromatogram profile of *M. champaca* stembark extract showed the presence of flavonoids (rf 0.78) and terpenoids both in chloroform and methanol extracts. Ammonia is used to detect the presence of the flavonoids and 10% sulphuric acid is used to detect the presence of terpenoids. Spot with rf 0.78 both in chloroform and methanol extract showed yellow fluorescent under 366 nm UV light and color changes to yellow orange after sprayed with ammonia indicated the presence of flavonoid. Refer to Markham (1988), the possibility of this flavonoid could be a flavonol 3-OH-free with/without free 5-OH or a dihidroflavonol. This spot has different color intensity in chloroform and methanol extracts. Chloroform extract showed more intensive color spot than methanol extract. The AUC value of this yellow spot (rf 0.78) in chloroform extract (62.97%) also higher than spot in methanol extract (60.11%). According to Markham (1988), flavonoid spectrum consists of two maxima in the range of 240-285 nm (Band II) and 300-550 nm (Band I). Spectrum of spot rf 0.78 of chloroform extract revealed maxima wavelength of 277 nm (Band II) and the shoulder peak wavelength of 324 nm (Band I). Compared to UV spectrum of flavonoid class proposed by Markham (1988), UV spectrum of spot rf 0.78 of chloroform and methanol extract are similar to dihydroflavonol. Terpenoids form a pink to purple spot after being sprayed with 10% sulphuric acid. Spot with rf 0.90 both in chloroform and methanol extract are belonged to terpenoids. The result of anti-tuberculosis activity of these extract was showed in Table 1.

Inhibition of both chloroform and methanol extracts of *M. champaca* with concentration of 1 mg/mL against *M. tuberculosis* MDR was under 90%. The increasing concentration of extracts (10 and 100 mg/mL) give higher inhibition against *M. tuberculosis* MDR with inhibition above 90%. According Gupta *et al*. (2010), extract was active as anti-tuberculosis when its inhibition above 90%. Therefore, chloroform and methanol extracts of *M. champaca* were active and potential as new agent for anti-tuberculosis. Flavonoids and terpenoids might be contributed for its activity.

Table 1. Anti-tuberculosis activity of chloroform and methanol extract of *M. champaca*

No. observation		Inhibition of extract against *M. tuberculosis* MDR (%)					
		Concentration of chloroform extract (mg/mL)			Concentration of methanol extract (mg/mL)		
		1	10	100	1	10	100
3rd week	1	25.00	100	100	12.50	100	100
	2	34.62	100	100	19.23	100	100
	3	37.25	100	100	43.14	100	100
4th week	4	37.14	100	100	38.57	100	100
	5	41.38	97.70	100	37.93	100	100
	6	41.75	98.06	100	33.01	100	100
5th week	7	42.74	97.44	100	23.93	100	100
	8	42.50	96.67	100	22.50	100	100
	9	42.40	96.00	100	17.60	100	100
6th week	10	41.73	95.28	100	18.11	100	100
	11	42.42	92.42	100	19.70	100	100
	12	39.85	91.73	100	18.05	100	100

Several studies also reported anti-tuberculosis activity of flavonoids and terpenoids. Sesquiterpene lactone partenolida and costunolida from *Magnolia grandiflora* and *Magnolia virginiana* (Magnoliaceae) were reported to have anti-tuberculosis activity against *M. tuberculosis* H37Rv with MIC of 16 and 32 μg/mL[3]. Dihidroflavonol, laburnetin, isolated from *Ficuschlamydocarpa* and *Ficuscordata* exhibited anti-tuberculosis activity with MIC values of 4.88 μg/mL[7]. Flavanones isolated from *Chromolaenaodorata* also revealed anti-tuberculosis activity with MIC values of 174.8 µM[10].

4. Conclusions

Chloroform and methanol extracts of *M. Champaca* stembark is active as anti-tuberculosis. Further researches are needed to isolate active phytochemical constituents as anti-tuberculosis from chloroform and methanol extracts of *M. champaca* stembark.

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Determination of free Prostate of Specific Antigen (fPSA) in Normal Patients Using The Immunoradiometricassay (IRMA) PSA Kit Developed in Center for Radioisotopes and Radiopharmaceuticals Technology National Nuclear Energy Agency

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Abstract

Prostate cancer is a cancer in the male reproduction system that can spread to the bones and lymph nodes. Prostate cancer is a very frequent cause of mortality of about 2-3% in men. One of the prevention of prostate cancer is done by early detection screening which is recommended for men over 40 years old. PSA is an enzyme released by prostate gland that can be determined by IRMA or ELISA method. Center for Radioisotopes and Radiopharmaceuticals Technology has developed a reagent kit in the form of IRMA PSA tracer, PSA standard and coated tube that gave the NSB of 0.9% and %B/T 19.57. The values met the requirements of a good kit (%NSB<2% and %B/T>10%). The standard calibration curve showed a high level of confidence of linear equation Y=0.2346X+0.922, with the value of R^2 0.998. The purpose of this study is to determine the sensitivity, spesificity and accuracy of PSA IRMA kit developed in the CRRT by determination of PSA level of the NNEA employees (normal patients) compared with the result using the PSA IRMA kit of commercial product. The results showed that the sensitivity, specificity and accuracy are 11.76%, 94.80% and 85.96% respectively.

Keywords: prostate cancer; PSA; IRMA; 125I; diagnosis.

1. Introduction

Prostate cancer is the most malignant among other specific urogenital systems on males. This type of cancer can grow and spread to the surrounding lymph nodes and in its late stadium it might affect the nearest or farthest organs resulting in the bone destruction. The prostate cancer attributes to 2-3 percent of male deaths. The cancer cells can spread faster due to the influence of testosteron hormone and can be impeded by the removal of testis by injecting estrogen hormone. In some cases the mestatasis cancer to the bone can be cured in months or years by removing testis through the estrogen hormone therapy or both. After the treatment the metastasis will degenerate and the spreading to bones disappears. The treatment does not completely stop the prostate cancer but it can slow down and reduce the bone pains significantly[1]. One of the methods in the prevention of prostate cancer is by early detection through screening, recommended for males starting at the age of 40. PSA is an enzyme produced by the prostate gland, which content can be measured through either IRMA or ELISA method[2].

The IRMA method uses a binding technique between antigen and radionuclide labeled antibody. The antigen will bind parts of the antibody, the free antibody is then removed by adding solid antigens, and radioactive labeled antibody bound to antigen in liquid is then measured. The detected radioactivity is equivalent to the number of antigens. The technique is highly suitable for determining tumor markers in the complex matrix serum and the content of which is highly varied. This assay technique is based on the reaction between antigen (Ag) found in the sample or standard (tumor marker) with the excessive radioiodinated antibody (Ab*) which

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forms antigen-antibody complex (Ag-Ab*). Therefore, the higher the content of tumor marker (Ag), the higher the antigen-antibody complex, thereby producing the higher radioactive count[3].

Enzyme Linked Immunosorbent Assay (ELISA) is used to determine antibody. In this respect, the antigen is firstly tied to solid substance then the antibody of interest is added. Next, the enzyme marked antigen such as peroxidase and phosphatase is added. Then, chromogenic substrate is added which produces color change when it reacts with the enzyme. The change of color occurs in line with the number of tied enzymes and also in line with the antibody being searched. In the ELISA test, the more stable but less sensitive reagent compared to RIA/IRMA reagent is used[4,5].

Prostate Specific Antigen is a glycoprotein with molecular weight of 34,000 dalton produced mainly by epitel cells that coat asinus and prostate gland tract. At a normal state, only a little amount of PSA gets into the blood circulation but if inflammation or damage occurs at the prostate tract, the PSA content in the blood increases. The increase in the PSA content may be resulted from prostate cancer or benign prostatic hyperplasia (BPH). The blood PSA is found in free condition (free PSA) and most of it is tied by protein (complexed PSA/c-PSA). In the case of prostate cancer the increase of c-PSA is more dominant compared to the free PSA concentration, whereas in the BPH case the free PSA is more dominant. For males of over 60 years old, the result of PSA measure can be misleading, as to whether it is caused by BPH or prostate cancer; therefore, it is advisable to conduct an examination when the ratio of free PSA/PSA-total or ratio of c-PSA/PSA total, particularly for those whose total PSA content is between 2.6-10 ng/mL. The result of interpretation is as follows: if the ratio of free-PSA/PSA total <10%, it is predicted as prostate cancer, 10%-25% as BPH, >25% as BPH. PSA examination is conducted for the purpose of screening (PSA total), diagnosis (PSA total and ratio of free-PSA/PSA-total or ratio of c-PSA/PSA-total), for disease and treatment monitoring after the removal of prostrate[6].

The treatment procedure for prostate cancer at the early stadium is by conducting observation, then in the next stadium it is advisable to remove prostate glands (radical prostatectomy) or radiation therapy, and at the further metastasis stadium the hormoneal treatment has to be made to reduce or get rid of androgen hormoneal influence to the prostrate tissue. Other important action is to check the PSA content regularly for the post radical porstatectomy patients and hormoneal treatment to check relapses[8,12,13,15,18].

In 2012 the CRRT successfully developed IRMA PSA reagent kit in the form of PSA tracer, coated tube and the standard with %NSB of 1.26 and %B/T of 14.12 to meet the proper kit requirements (%NSB<2% and %B/T>10%) under standardised calibration curve that suggested the particularly strong relation shown by R^2 0.9938 value obtained from the equation of linear regression line Y=0.1247X + 1.7099[9].

In 2013 the IRMA PSA kit assay was optimised, including reagents and reaction condition so that optimum condition was reached by standardised volume composition of 50 uL, successive (tracer) activity of 50000 cpm and successive (tracer) volume of 25 uL as well as optimum reaction with the incubation period of 18 hours and incubation temperature of 25°C. The pereaction and reaction condition produce immunology results of %B/T and %NSB of 21.67% and 0.26% respectively to meet the proper kit requirements[10]. Using the optimum condition and reagent, validation of IRMA PSA was conducted by determining the detection limit, accuracy (intra and inter assay), assay performance and kit stability with the following results: detection limits to enable % CV of 14.43, intra assay precision performed by one operator who conducted assay for 8 times by involving QC so that %CV of 14.43 was obtained, inter assay accuracy was performed by eight operators who conducted an assay by involving QC to get the result %CV of 14.95[11].

The objective of this study is to determine sensitivity, specificity and accuracy of CRRT PSA IRMA kits, using commercial kit as gold standard (IRMA PSA, Izotop kit) which is applied to normal patients among the NNEA employees at the PUSPIPTEK Serpong area.

2. Methods

The material used in this study were monoclonal antibody PSA type M86806 to produce PSA tracer, monoclonal antibody PSA type M66280M to produce coated tube (CT) PSA, a calibatror grade type A01238H to produce a standardised PSA, $Na^{125}I$, chloramine-T, buffer Na_2CO_3 0.05 M pH 9.6, Bovine Serum Albumine (BSA), commercial IRMA PSA kits (Izotop, Hungary) and other chemicals. Equipment used in the study includes Gamma Management System (GMS), Gamma Mini Assay, micropipette, mixing tool (multimix and vortex), electrophoresis and shaker.

2.1 The production of PSA Tracer

The PSA tracer was made using MAb type M86806M with the amount of 75μg/25μL placed in the iodination tube, added with 5 μL of phosphate buffer 0.25 M pH 7.4 and 2 μL of $Na^{125}I$ (activity ± 1000 μCi) and 5 μL of Chloramine-T (0.5 mg in 1 mL phosphate buffer 0.25 M pH 7.4). Next, the mixture is stirred for about 90 second using vortex. Then 10 μL of $Na_2S_2O_5$ solution (0.5 mg $Na_2S_2O_5$ in 1 mL phosphate buffer 0.25 M pH 7.4) and 100 μL of KI solution (10 mg KI and 3 mg BSA dissolved in phosphate buffer 0.25 M pH 7.4) were added, after that, the mixture was stirred using vortex for one minute. The result was purified using sephadex G-25 superfine column which had been conditioned with phosphate buffer 0.05 M pH 7.4 and saturated with 1 mL of BSA 5% solution. The anti PSA monoclonal antibody labeled with ^{125}I (hereafter

referred to as tracer) was eluted from sephadex G-25 column using buffer solution of phosphate 0.05 M pH 7.4 and the eluate fraction of 500 uL were collected in the reaction tubes to make 25 fractions. The radioactivity of each eluate fraction was measured by Gamma Mini Assay. The radiochemical purity of the fraction with highest radioactivity was measured using electrophoresis with Whatman 1 paper as so lid phase and barbital buffer 0.05 M pH 8.6 as mobile phase, eluted for one hour at 300 volt, 500 mA[9].

2.2 The production of PSA antibody monoclonal coated tube

The production of coated tube was carried out using MAb PSA type M66280M and Na_2CO_3 0.05 M pH 9.6. MAb of 250 μL as a coating solution was put into a starred base tube and incubated overnight at a room temperature (25ºC). The initial washing stage was made using buffer solution of Na_2CO_3 0.05 M pH 9.6 containing Tween-20 0.05% resulted in 500 μL volume. The resulted washing tube was blocked with Na_2CO_3 0.05 M pH 9.6 containing BSA 8% and 0.05% NaN_3, and then incubated overnight at room temperature (25ºC). Then the tube was washed with 500 uL of buffer containing Tween-20 0.05% once, and dried for 8 hours[9].

2.3 The production of standardised PSA solution[9]

The concentration of standardized PSA solutions to be prepared were 0, 0.5, 1, 2, 4, 8, 16, 40 and 80 ng/mL. The standardised PSA solution was made using Human PSA calibrator grade (Biodesign, USA) of 18 μL dissolved with 20000 μL of phosphate buffer 0.025 M pH 7.4 containing BSA 5% and 0.1% NaN_3 as the main solution, with the dilution as shown in Table 1.

Table 1. Volume comparison in the production of standardised PSA solution

No	Concentrated standardised PSA solution (ng/mL)	Volume of Human PSA main solution (μL)	Phosphate Buffer Solution 0.025 M pH 7.4 containing BSA 5% and NaN_3 0.1% (μL)
1	0	10000	0
2	0.5	5	9995
3	1	10	9990
4	2	20	9980
5	4	40	9960
6	8	80	9920
7	16	160	9840
8	40	400	9600
9	80	8000	9200

2.3 Immunology Testing

Eighteen antibody PSA starred based polystyrene reaction tubes (coated tubes, CT) were numbered consecutively (1, 2, 3 etc). A total of 50 μL of PSA standardised solution of 0, 0.5, 1, 2, 4, 8, 16, 40 and 80 ng/mL are added to the respective coated tube/CT accordingly based on the consecutive numbers, added with 25 μL of PSA-^{125}I with the radioactivity of ≈50.000 cpm, and then homogenised with vortex, incubated for 18 hours at room temperature by stirring it using a shaker. The liquid was then removed and the CT was washed with 500 μL of washing buffer once, then decanted and dried. The remaining radioactivity in the tube was measured with GMS for one minute[10].

2.4 Sample testing with the IRMA PSA CRRT

Eighteen antibody PSA starred based polystyrene reaction tubes (coated tubes, CT) were numbered consecutively (1, 2, 3 etc). A total of 50 μL of PSA standardised solution of 0, 0.5, 1, 2, 4, 8, 16, 40 and 80 ng/mL were added to the respective coated tube/CT accordingly based on the consecutive numbers, added with 25 μL of PSA-^{125}I with the radioactivity of ≈50.000 cpm, and then homogenised with vortex, incubated for 18 hours at room temperature by stirring it using a shaker. The liquid was then removed and the CT was washed with 500 μL of washing buffer once, then decanted and dried. The remaining radioactivity in the tube was measured with GMS for one minute[10].

2.5 Sample testing with the Izotop IRMA PSA kit (Hungary)

Eighteen antibody PSA starred based polystyrene reaction tubes (coated tubes, CT) were numbered consecutively (1, 2, 3 etc). A total of 50 μL of PSA standardised solution of 0, 0.5, 2, 10, 50 ng/mL and control and sample are added respective coated tube/CT accordingly based on the consecutive numbers, added with 200 μL of PSA-^{125}I with the radioactivity of ≈200.000 cpm, and then homogenised with vortex, incubated for 2 hours at room temperature by stirring it using a shaker. The liquid was then removed and the CT was washed with 1000 μL of washing buffer once, then decanted and dried. The remaining radioactivity in the tube was measured with GMS for one minute[10].

Table 3. Calculation of sensitivity, specificity and accuracy [14]

Comparative Standard Kit			
	3	-	Total
+	a	b	a+b
-	c	d	c+d
Total	a+c	b+d	a+b+c+d

$$Sensitivity = \frac{a}{a+c} X\ 100\%$$

$$Specificity = \frac{d}{b+d} X\ 100\%$$

$$Accuracy = \frac{a+d}{a+b+c+d} X\ 100\%$$

Note :
(+) : positive result; (-) : negative result
a : number of samples which give positive result using both kits (the CRRT and commercial kit)
b : number of samples which give negative result using commercial kits but positive result using the CRRT kit
c : number of samples which give positive result using commercial kits but negative result using the CRRT kit
d : number of samples which give negative result using both kits (the CRRT and commercial kit)

3. Results and discussion

The performance of the CRRT's IRMA free PSA assay was determined using the standardized PSA 0, 0.5, 1, 2, 4, 8, 16, and 80 ng/mL and they are shown results in Fig 1 below.

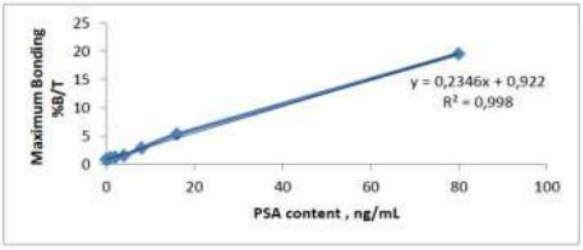

Fig 1. Performance result of the CRRT's IRMA free PSA kit with %NSB of 0.9 and %B/T of 19.57% with value R^2 0.998

The result of assay performance obtained has met the good kit requirements since the values of %NSB<2% and %B/T>10%.

The calibration curves of the free PSA content measured in normal patients using the CRRT's standardised free PSA and Izotop kits are shown in Fig 1 and Fig 2 respectively.

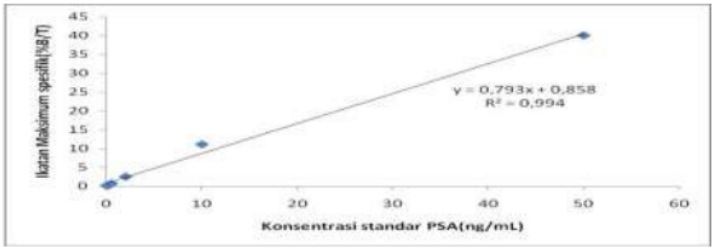

Fig 2. Calibration of the Izotop standardized IRMA PSA kit, with 0.2% NSB and 40.0% B/T

The determination of free PSA content using the CRRT's IRMA free PSA CRRT kit and commercial IRMA free PSA kit (Izotop, Hungary) was applied to 171 samples of the NNEA normal patients at Serpong nuclear area joined by 7 centers consisting the of CRRT (52 persons), Center for Informatica and Nuclear Strategic Zone Utilization, CINSZU (30 persons), Center for Nuclear Fuel Technology, CNFT (25 persons), Center for Nuclear Facilities Engineering, CNFE (23 persons), Radioactive Waste Technology Center, RWTC (18 persons), Center for Multipurpose Reactor, CMPR (12 persons), Center for Nuclear Reactor Technology and Safety, CNRTS (9 persons) and others (13 persons), of males over 40 years old. The blood sample was prepared and tested using the CRRT's IRMA PSA kit (local) and the Izotop IRMA PSA kit (commercial kit) which resulted in 154 negative samples (true negative) meaning normal, two abnormal positive samples (true positive), 15 false negative samples and eight false positive as shown in Table 3. After calculation of data, the sensitivity of

11.67%, specificity of 94.80% and accuracy of 85.96% were obtained. The sensitivity was still low, because the CRRT IRMA PSA kit reading was low compared to Izotop IRMA PSA kit; therefore, 15 samples were read negative although the IRMA PSA Izotop showed positive results in the same samples; consequently, a further study is required to increase sensitivity. The low sensitivity is probably resulted from the instability of the developed IRMA PSA kit component and the decreased efficiency of GMS detector which has been over 10 years old. Hence it might result in the less sensitive readings which lead in a larger significant gap between false positive and false negative. It is therefore necessary to conduct calibration of the existing Gamma counter equipment to make the component more stable.

4. Conclusion

The processed data of the PSA content in normal patients of National Nuclear Energy Agency employees using the CRRT IRMA PSA kit compared to the commercial IRMA PSA kit (Izotop) results in the value of sensitivity 11.76%, specificity 94.80% and accuracy 85.96%. It indicates that the local product conformed with the specification.

Suggestion

It is necessary to conduct further study to increase the stability of the developed IRMA PSA kit and clinical test to determine the free PSA content on the prostate cancer patients and benign prostrate hyperlasia from the hospital. It is also advisable to recount values of normal patients to verify the sensitivity value, and to compare both kits using spearmen or Wilcoxon rank test.

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