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CONTENTS

		Page
1.	Patient's expectation on communication performances community of Dental Health Services providers located in urban and rural area **Taufan Bramantoro** and **Ninuk Hariyani**	1–4
2.	Changes in setting time of alginate impression material with different water temperature **Decky J. Indrani** and **Niti Matram**	5–8
3.	Bactericidal and cytotoxic effects of *Erythrina fusca* leaves aquadest extract **Janti Sudiono, Ferry Sandra, Nadya Saputri Halim, Timotius Andi Kadrianto** and **Melinia**	9–13
4.	The role of inducible Nitric Oxide Synthase in teeth periapical lesions immunopathogenesis caused by *Enterococcus faecalis* **Tamara Yuanita, Latief Mooduto** and **Kuntaman**	14–17
5.	Antitumor activity of antisense oligonucleotide $p45^{Skp2}$ in soft palate carcinoma cell squamous in vitro **Supriatno, Sartari Entin Yuletnawati** and **Iwa Sutardjo Rus Sudarso**	18–22
6.	Evaluation of local muscle soreness treatment with anterior bite splint made of soft putty impression material **Harry Laksono** dan **Sherman Salim**	23–29
7.	Unidentified angular recurrent ulceration responsive to antiviral therapy **Rahmi Amtha** and **Siti Aliyah Pradono**	30–34
8.	The inhibition of malignant epithelial cells in mucosal injury in the oral cavity of strains by pomegranate fruit extract (*Punica granatum linn*) through Bcl-2 expression **Sri Hernawati**	35–38
9.	Shear strength of orthodontic bracket bonding with GIC bonding agent after the application of CPP-ACPF paste **Melisa Budipramana, Thalca Hamid** and **Sianiwati Goenharto**	39–44
10.	Hemolysin activities as virulence factor of *Enterococcus faecalis* isolated from saliva and periapical abscess (gene detection by PCR) **Dewa Ayu N.P.A, Sari Dewiyani** and **Dessy Sulistya Ashari**	45–49
11.	Effect of *Citrus aurantifolia swingle* essential oils on methyl mercaptan production of *Porphyromonas gingivalis* **Anindya Prima Yusinta, Ivan Arie Wahyudi** and **Anne Handrini Dewi**	50–54

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Research Report

Bactericidal and cytotoxic effects of *Erythrina fusca* leaves aquadest extract

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ABSTRACT

Background: *Empirically, Erythrina fusca has been used as traditional herb for its antibacterial and antiinflammation properties. Periodontal disease is one of the most oral infectious diseases with microorganism predominated as the contributing factors. Porphyromonas gingivalis (P. gingivalis) is one of the main bacteria pathogen found in periodontal diseases.* ***Purpose:*** *The purpose of this study was to examine the bactericidal effect of Erythrina fusca Leaves Aquadest Extract (EFLAE) at various concentrations on P. gingivalis and cytotoxic effect on fibroblast.* ***Methods:*** *Pure P. gingivalis was cultured in Brain Heart Infusion (BHI) medium for 24 hours with or without various concentrations of treatment of EFLAE. Calculation and statistical analysis of remaining bacteria were performed by inhibitory zone method to evaluate the EFLAE bactericidal effect and compared to chlorhexidine as positive control. To evaluate the cytotoxic effect, NIH 3T3 cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) containing of 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, pH 7.2, in 5% CO_2, and stored in humidified incubator under temperature 37^0 C. Cells were treated with/without various concentrations of EFLAE for 48 hours. The viable cells were then counted using 3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyl tetrazodium bromide (MTT) method.* ***Results:*** *EFLAE have bactericidal effect on P. gingivalis in a concentration dependent manner starting from 78%. The concentration of 90% EFLAE had stronger bactericidal effect (35.004 ± 1.546) than those of chlorhexidine as positive control (32.313 ± 1.619). One-way ANOVA showed significant bactericidal effect differences among concentrations of EFLAE and chlorhexidine ($p<0.05$) while Tuckey HSD test showed significant difference only between lower concentration of EFLAE (78%, 79%) and chlorhexidine. With the highest concentration of EFLAE (100%) applied in the bactericidal test, no cytotoxic effect of EFLAE on NIH 3T3 cells was detected.* ***Conclusion:*** *EFLAE could inhibit the growth of P. gingivalis in a concentration dependent manner, starting from 78%. There was no evidence of EFLAE's cytotoxic effect on fibroblast.*

Key words: *EFLAE, bactericidal, citotoxicity*

ABSTRAK

Latar belakang: *Erythrina fusca telah digunakan secara empiris sebagai tanaman obat tradisional untuk khasiat antibakteri dan antiradang. Penyakit periodontal merupakan salah satu penyakit infeksi mulut terbanyak dengan mikroorganisme sebagai faktor kontributor utama. Porphyromonas gingivalis (P. gingivalis) merupakan salah satu bakteri patogen utama yang ditemukan pada penyakit periodontal.* ***Tujuan:*** *Tujuan penelitian ini untuk mengamati efek bakterisid terhadap P. gingivalis dan efek sitotoksik terhadap sel fibroblast dari beberapa konsentrasi ekstrak akuades daun Erythrina fusca (EFLAE).* ***Metode:*** *P. gingivalis murni dikultur pada medium Brain Heart Infusion (BHI) selama 24 jam dengan atau tanpa pemberian beberapa konsentrasi EFLAE. Perhitungan dan analisis statistik terhadap bakteri yang masih hidup dilakukan dengan metode zona hambat untuk mengevaluasi efek bakterisid EFLAE dibandingkan dengan chlorhexidine sebagai kontrol positif. Untuk mengevaluasi efek sitotoksik, digunakan kultur sel NIH 3T3 pada medium Dulbecco's Modification of Eagle's Medium (DMEM) yang berisi fetal bovine serum (FBS) 10% dan penicillin-streptomycin 1%, pH 7.2, dalam CO_2 5%, dan diinkubasi pada suhu 37° C. Sel diberi perlakuan dengan atau tanpa beberapa konsentrasi EFLAE selama 48 jam, kemudian sel yang masih hidup dihitung menggunakan metode 3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyl tetrazodium bromide (MTT).* ***Hasil:*** *EFLAE mempunyai efek bakterisid terhadap P. gingivalis mengikuti kenaikan konsentrasinya dimulai dari 78%. Pada konsentrasi 90%, EFLAE menunjukkan efek bakterisid lebih kuat (35.004 ± 1.546) dibandingkan dengan chlorhexidine (32.313 ±*

1.619) sebagai kontrol positif ANOVA-1 jalan menunjukkan perbedaan efek bakterisid yang bermakna di antara beberapa konsentrasi EFLAE dan chlorhexidine (p<0.05) sedangkan uji Tuckey HSD menunjukkan perbedaan bermakna hanya ditemukan antara konsentrasi EFLAE yang lebih rendah (78%, 79%) dengan chlorhexidine. Efek sitotoksik terhadap sel NIH 3T3 tidak terdeteksi pada pemberian konsentrasi tertinggi EFLAE (100%) yang telah diaplikasikan pada uji bakterisid. ***Kesimpulan:*** *EFLAE dapat menghambat pertumbuhan P. gingivalis sesuai dengan konsentrasinya dimulai dari 78%. Tidak ada efek sitotoksik EFLAE terhadap sel fibroblast.*

Kata kunci: *EFLAE, bakterisid, sitotoksisitas*

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INTRODUCTION

Erythrina fusca (E. Fusca) is the most widespread species in the genus available wild in both the Old and New World tropics. In asia and oceania it occurs along coasts and rivers planted throughout the humid tropic. *E. fusca* is found from sea level up to 200 m altitude, within a wide range of rainfall pattern, from 1,200 mm to over 3,000 mm annually, with or without seasonal distribution. *E. fusca* has many functions and been used by several countries; as in Indonesia, the scraped inner bark is used for poulticing fresh wounds.[1] Prior study of ethanol extract of E.fusca showed inhibitory effect of cyclooxygenase 2 (COX2).[2] In Vietnam, the bark is used to treat toothache. The young leaves are eaten as a vegetable in Java, Bali, and Guatemala.[1]

The first compounds isolated from Erythrina were alkaloids. Subsequently, homoerythrina alkaloids were investigated for their anti-cancer activity**.** Recently, research involving Erythrina has focused on other chemical effects, primarily the antimicrobial action of Erythrina lectins and the enzymology of proteinase inhibitors isolated from Erythrina. However there was no research about its effect on periodontal disease as one of the most prevalent oral diseases in Indonesia therefore this research was conducted**.**

The incidence of periodontal disease reached 70% in entire population of the world, including Indonesia, especially in elderly.[3] Periodontal disease is an infectious-typed disease which can be caused by local factor as well as systemic factor. Commonly, the main cause of periodontal disease is local factor, which is caused by bacteria and afterwards is aggravated with the existence of systemic factor. The main pathogenic bacteria which cause the periodontal disease is *Pophyromonas gingivalis* (*P. gingivalis*), This bacteria has the ability to infect the periodontal ligament; which in early stage starts with infection of the gum (gingivitis) and continue to chronic infection which involve all the periodontal ligament (periodontitis).[4,5]

Phytochemistry test on E. fusca leaves aquadest extract (EFLAE) in Balai Penelitian Tanaman Obat dan Aromatik (BALITTRO), Bogor (2010), showed that EFLAE contains alkaloid, glycoside, saponin, tanin, triterphenoid and steroid. The strongest compounds found in EFLAE are alkaloid and glycoside. Alkaloids have the ability as anti-bacterial agent. Tanin has also shown potential as antibacterial agent.[6,7] and other previous research concluded that triterphenoid and saponin worked as antibacterial agent.[8]

These knowledge become the foundation for the implementation of this scientific research about the bactericidal effect of EFLAE on *P. gingivalis.* Concerning to its bactericidal potency, the cytotoxic effect of this natural biomaterial need to be evaluated to know whether EFLAE biocompatible to be applied on oral mucosa. Cytotoxic test was conducted on NIH3T3 cells as fibroblast is one of the important structures of oral mucosa.[9] Therefore the purpose of this study was to examine the bactericidal effect on *P. gingivalis* and cytotoxic effect on fibroblast of EFLAE at various concentration.

MATERIALS AND METHODS

This study is an experimental laboratory research**.** Extraction of *E. fusca* leaves were performed by maceration tehnique using aquadest to find out gel form extract.[10] *E. fusca* leaves (50 mg) were dried for 5 days, grinded, diluted in aquadest (500 mL) for 24 hours, refined, then evaporated with rotary evaporator 40° C up to gel formation of EFLAE. Dimethyl sulfoxide (DMSO) used in this study as polar aprotic solvent that dissolves both polar and nonpolar compounds and is miscible in a wide range of organic solvents as well as water. This study used *serial dilution* method to get various concentration of EFLAE.

Pure *P. gingivalis* was cultured in Brain Heart Infusion (BHI) medium for 24 hours in 37° C humidified incubator, with/without various concentrations treatment. Observation and calculation of remaining bacteria were performed by inhibitory zone method to evaluate EFLAE bactericidal effect and compared to chlorhexidine as positive control since up to know chlorhexidine is accepted as gold standard of periodontal treatment**.**[11] The results were then analyzed using One Way Anova with $\alpha = 0.05$. The bactericidal test showed that bactericidal effect occured up to concentration 80% of EFLAE while the concentration of 70% showed negative result therefore the concentration treatment diluted into lower concentration gradually which were 80%, 79%, 78%, 77%, 76%, 75%, 74%, 73%, 72%, 71%, and 70% in order to find out the minimum bactericidal effect concentration.

NIH3T3 cells were cultured in 100 µL Dulbecco's Modification of Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin using the 96 wells-plate, pH 7.2, in 5% CO_2 37° C humidified incubator.[14] Hemacytometer with trypan blue staining was used to count the number of viable cells. The viable cells is unstaining cells. Viable cell per mL is equal to average viable cell count per square into dilution factor into 10^4. Dilution factor is total volume sample and diluting liquid divided by volume of sample. Percentage cell viability is total viable cells (unstain cells) divided by total cells (viable and dead cells) into 100. Total viable cell/ sampel is viable cell per mL into the original volume of fluid from which the cell sample was removed. Volume of media needed is number of cells needed divided by total number of viable cells into 1.000. The number of cells used in this cytotoxicity test was 2.000 cells. Cells were treated with/ without various concentrations of EFLAE (0%, 10%, 100%) for 48 hours. The viable cells were then counted by 3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyl tetrazodium bromide (MTT) assay method using the standard curve formula. This assay based on the changes of tetrazodium salt. MTT will transmute formazan in mitochondria. The formazan's concentration, purple in colour can be determined by spectrophotometry. Formazan crystal which was formed will be dissolved by the addition of acid isopropanol. The absorbance was then evaluated using Elisa plate reader with wave length (λ) of 570nm.[12] The cells absorbance was in liniar with viability.

RESULTS

EFLAE used in this study was in gel form, brownish-green in colour and solid consistency. Bactericidal effect of EFLAE occured starting from concentration 78% of EFLAE and having tendencies to increase and reached its peak on 80% (Figure 1).

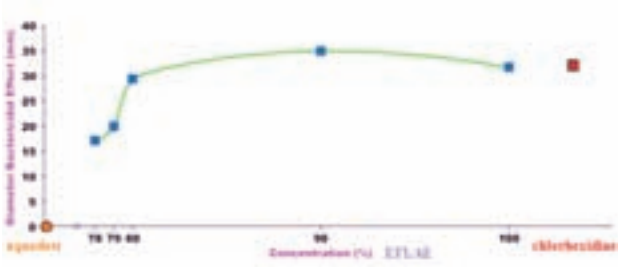

Figure 1. Diameter of bactericidal effect of EFLAE in various concentrations (blue); aquadest (orange); and chlorhexidine (red). This line diagram showed that bactericidal effect of EFLAE (blue dot) tends to increase following increase concentration while aquadest showed no bactericidal effect and chlorhexidine showed bactericidal effect equal as 100% EFLAE.

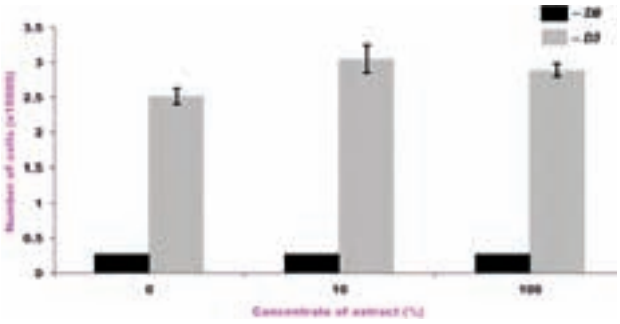

Figure 3. Number of NIH3T3 cells with various concentrations of EFLAE at the time of seeding (*D0*) and day 3 (*D3*). On day 3, the number of NIH3T3 cells with 10% and 100% of EFLAE were slightly higher than control (0%).

EFLAE has bactericidal effect on *P. gingivalis* in a concentration dependent manner starting from 78%. The concentration of 90% EFLAE had stronger bactericidal effect (35.004±1.546) than those of chlorhexidine as positive control (32.313±1.619). No significant difference between the concentration of 100% and 80% EFLAE with those of chlorhexidine as positive control. One-way ANOVA showed significant bactericidal effect differences among concentrations of EFLAE and Chlorhexidine ($p<0.05$) while Tuckey HSD test showed significant difference only between low concentration of EFLAE (78%, 79%) and chlorhexidine (Figure 1).

Various quantitities of NIH3T3 cells were cultured in DMEM using 96 wells-plate for 24 hours to find out standard curve. The test was conducted in three consecutive weeks using ELISA plate reader, with $\lambda = 570$ nm to find out the absorbance of the cells; in which a formula of standard curve acquired. NIH3T3 cells with the same quantity (2.000 cells) were treated with various concentrations (0%, 10%, 100%) of EFLAE. The test was done in three consecutive weeks (week 1, 2, and 3).

Afterward, the result of the cell's absorbance was substituted to the formula of standard curve to calculate the number of viable cells. Number of viable NIH3T3 cells which were cultured in DMEM with/without various concentrations of EFLAE showed that EFLAE did not induce cytotoxicity on NIH3T3 cells (Figure 2). On day 3, the number of NIH3T3 cells with 10% and 100% of EFLAE were slightly higher than those in control (0%). ANOVA test showed significant difference between groups ($p=0.00<0.05$). However, Tuckey HSD test showed that on day 3, there was no significant difference number of viable cells between control and 100% ($p=0.256>0.05$) and between 10% and 100% ($p=0.080>0.05$), while there was significant difference of viable cells number between control and 10% of EFLAE group ($p=0.005<0.05$) (Figure 3).

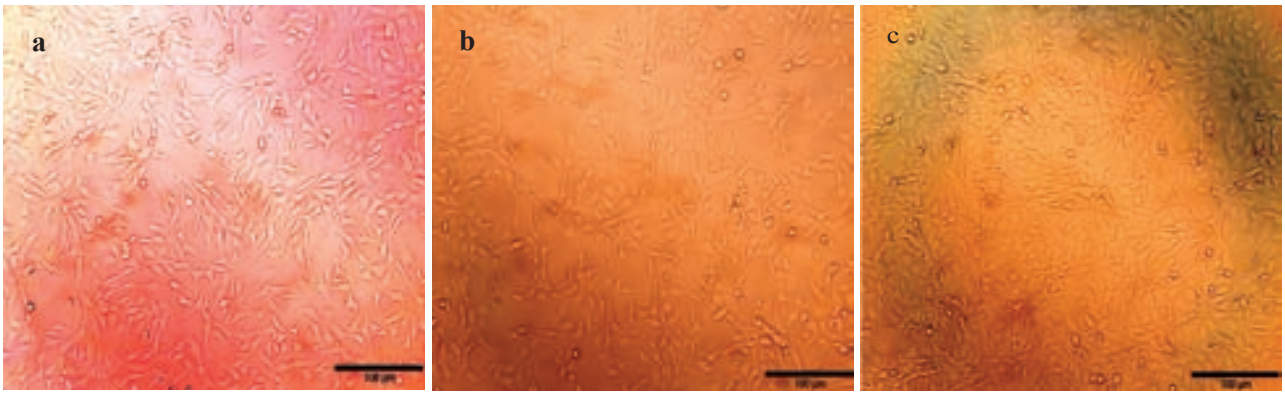

Figure 2. EFLAE did not induce cytotoxicity on NIH3T3 cells. NIH3T3 cells which were cultured in 96 wells-plate for 24 h then treated by 0% (a), 10% (b) and 100% (c) EFLAE for 48 h. Pictures were captured under light microscope on day three. Black bar = 100 µm. The viable cells determine based on the microscopic feature refer to no cytopathic effect (CPE). Maginification (40x10).

DISCUSSION

This study used aquadest to find out Erythrina fusca leaves extract (EFLAE) with consideration that aquadest is not influenced in phytochemical compound of *Erythrina fusca* leaves compared to those of other solvent such as ethanol, chloroform or methanol. Beside the result of phytochemical test of EFLAE consisted of potential compounds as also found by other studies such as alkaloid, glycoside, saponin, tannins, triterpenoids, and steroids. Another reason, the exploration of this aquadest extract can be used orally at the future as its simply soluble in gastrointestinal tract that be absorbed fastly. Moreover, this type of extract can be directly applied on the oral mucosa.

In Indonesia, there is not much scientific research about EFLAE as phytotherapeutic agents especially against *P. gingivalis* as one of the most pathogen bacteriae of periodontal disease. Outside of Indonesia such as in sub-Saharan Africa, Erythrina species is sources of lead compounds or new class of phytotherapeutic agents for fighting against major public health (MDR infections, cancer, diabetes, obesity). Some phytochemicals (vogelin B, vogelin C, isowightcone, abyssinin II, derrone) were demonstrated as the active principles as antibacterials, antifungals, antiplasmodials and inhibitors of enzyme borne diseases (protein tyrosine phosphatase (PTP) inhibitor/ PTP1B, HIV protease).[13] In Japan and Thailand, there was also some studies about the phytotherapeutic agents of Erythrina.[14–16]

This study found that 80%, 90% and 100% of EFLAE has bactericidal effects on *P. gingivalis* as strong as those of Chlorhexidine and the 90% concentration of EFLAE had stronger bactericidal effect than those of chlorhexidine which was in general accepted as commercial antibacterial dental medicine. However, the optimum concentration of EFLAE was at 80% because no significant increase in bactericidal effect after this concentration. Indeed, the 80% of EFLAE can be explored to be used as dental medication in the future.

Bactericidal effect of EFLAE were suspected from some of the components found in EFLAE such as alkaloid, tannins, and saponin.[6–8] Phytochemistry test done in this study showed alkaloid is a highest percentage component of EFLAE. The result of this study assumed that alkaloid play a role in resulting the bactericidal effect on *P. gingivalis* as stated in previous research that alkaloid act as antibacterial agent, by means of disrupting the compiler's component of peptidoglycan bacterial cell therefore the cell wall is not fully formed, resulting in apoptosis of the cell.[8] Beside that, alkaloid is known as the compound to be a DNA intercalator and an inhibitor of DNA synthesis through topoisomerase inhibition.[17–19] In order to ensure this mechanism, further research need to be conducted. There were several cytotoxicity studies on Erythrina species. The cytotoxicity study of Innok *et al*.[20] about flavanoids and pterocarpans compound in the bark of *Erythrini fusca* (which was also found in the leaves at this phytochemistry study) revealed that three new isomeric flavanones, fuscaflavanones A; six known flavanones, lupinifolin; lonchocarpol A; phaseollidin showed moderate to weak activity against KB, BC and NCI-H187 cells, whereas fuscaflavanones A(2) exhibited only weak activity against KB cells. Another cytotoxicity study of *Erythrina stricta* roots and *Erythrina subumbrans* stems extracts by Rukachaisirikul *et al*.[16] stated that erybraedin A (2) showed the highest activity against the NCI-H187 and BC cells (IC50 2.1 and 2.9 microg/mL, respectively), whereas erysubin F exhibited the highest activity against the KB cells (IC50 4.5 microg/mL).

Cytotoxic test in this study was done to examine EFLAE's cytotoxic effect on fibroblast cells. The result of this test showed that the highest concentration of EFLAE (100%) did not induce cytotoxicity of cells. However the lower concentration (10%) of EFLAE showed increase viability of cells compared to those of control.

Based on the bactericidal test and citotoxicity test of EFLAE, this study suggested to develop 80% EFLAE as a traditional herbs in gel state for periodontal disease since this concentration does not show cytotoxic effect but urges the growth of cells result in recuperation and proliferation of cells beside its optimum bactericidal property. In conclusion, EFLAE could inhibit the growth of *P. gingivalis* in a concentration dependent manner starting from 78%. There was no evidence of EFLAE's cytotoxic effect on fibroblast.

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