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The Effectiveness of Brown Alga (*Sargassum polycystum*) Extract in Reducing the Lipoteichoic Acid Level of Enterococcus *faecalis* Bacteria Through Toll Like Receptor-2 Expression in Fibroblast Cell Culture

Andi Sumidarti*, Aries Chandra Trilaksana, Afniati Rachmuddin

Department of Conservative Dentistry, Faculty of Dentistry, Hasanuddin University, Indonesia

Abstract

To determine the effectiveness of brown algae extract (*Sargassum polycystum*) to reduce the lipoteichoic acid (LTA) level of *Enterococcus faecalis* bacteria through reducing Toll-Like Receptor-2 (TLR-2) expression in fibroblast cells. This study was an *in vitro* laboratory experiment with a post-test-only control group study design. The initial stage of this study was the production of brown alga (*Sargassum polycystum*) extract with concentrations of 0.5, 0.10, and 0.15 µg/ml. Each concentration of extract brown alga was given 10 µg/ml of LTA and inserted into cultured fibroblast cells, then the cultured cells were incubated in a carbon dioxide (CO_2) incubator for 24 hours. The amount of TLR-2 was then determined using the immunocytochemical method. The data were analyzed statistically using the Mann-Whitney test. The Mann-Whitney test showed significantly different results between treatments, with the 0.15 µg/ml concentration group having an average TLR-2 value in the fibroblast cell culture of 34.00 ± 1.73% significantly lower than the 0.05 µg/ml concentration group (mean 62.33 ± 8.08%) and 0.10 µg/ml concentration group (mean 62.00 ± 15.59). These results show that the treatment with a concentration of 0.15 µg/ml was effective in reducing the LTA level of *E. faecalis* bacteria through TLR-2 expression in fibroblast cell culture. The brown alga (*Sargassum polycystum*) extract used at a concentration of 0.15 µg/ml was effective in reducing the LTA level in *E. faecalis* bacteria through the expression of TLR-2 in fibroblast cells.
Keywords: Brown algal extract, Lipoteichoic acid, *E. faecalis*, TLR-2, fibroblast cells.

Introduction

Common causes of root canal treatment failure are directly or indirectly related to bacteria in the root canal system. Root canal treatment can be used as root canal disinfection drugs and can adapt to an environment where few nutrients are available [1].

One of the most common types of bacteria present in treated root canals is *Enterococcus faecalis*; this facultative fail even though all the basic principles of treatment have been followed [2]. Bacteria that survive in the treated root canal may be resistant to the medication anaerobic Gram-positive bacteria has natural properties that able it to survive despite the use of several intracanal medications and adapt to environmental conditions unfavorable for bacterial development [3]. This bacterium can metabolize various energy sources in a variety of environments, including high (alkaline) pH and extreme temperatures [4].

Persistent infection by *E. faecalis* can be caused by several virulence factors, including lipoteichoic acid (LTA), peptidoglycan, S aggregation substances, cytolysin, and lytic enzymes. LTA is a key factor in virulence because of its major role in the pathogenicity of colonization and invading host cells. It has been shown that *E. faecalis* can stimulate leukocytes to release certain inflammatory mediators and plays an important role in the formation of biofilm [5].

A wall of positive-Gram bacteria consists of a thick layer of peptidoglycan, in combination with teichoic acid and extracellular proteins. LTA binds to TLR-2, then stimulates leukocytes to release the mediators that play a role in inflammatory response and damage to periapical tissue [6].

The use of natural materials is increasing in the field of dentistry, for example, products from brown algae. Using methanol as a solvent, the brown alga *Sargassum polycystum* can yield

*Corresponding author e-mail: a_sumidarti@yahoo.co.; (Andi Sumidarti)
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extracts containing phenols, flavonoids, and saponins, which are believed to be active compounds with bacterial inhibition properties. [7] Brown algae contain protein, amino acids, and essential fatty acids; these include linoleic acid, which can function as an anti-inflammatory agent, regulate prostaglandin synthesis, and induce healing. In addition to active substances, protein, amino acids, and fatty acids, brown algae also contain iron (Fe) which plays a role in protein and collagen synthesis and in fibroblast proliferation [8].

Based on these known properties, brown alga (*Sargassum polycystum*) extract is considered a potential antibacterial agent which could be effective against *E. faecalis* and thereby reduce the failure rate of root canal treatment. Therefore, there is a need to test the effectiveness of this extract in reducing *E. faecalis* LTA levels through TLR-2 expression on fibroblast cells.

Materials and methods

Data collection

This study was an *in vitro* laboratory experiment with a post-test-only control group study design. The study was conducted during August 2018 in the Biopharmaca Laboratory of the Pharmacy Faculty, Hasanuddin University, Makassar, and the Integrated Research and Testing Laboratory (LPPT) at Gadjah Mada University, Yogyakarta. The materials used in this study were fibroblast cells, brown algal extract (*Sargassum polycystum*), and lipoteichoic acid (LTA) *Enterococcus faecalis* bacteria with ATCC 29212 code.

Extraction

The initial procedure was the production of an extract of the brown alga *Sargassum polycystum*. Seaweed obtained from Takalar Regency, South Sulawesi, was extracted using a maceration method. Maceration methods are an extraction step for plant material which coarsely powdered drug material, either leaves or stem bark or root bark is placed inside a container [9].

Incubation

The next preparation step was to obtain and culture fibroblast cells taken from a ligament attached to the mandibular first premolar teeth. A secondary culture was then made, and the cultured cells were subjected to test treatments. The fibroblast cell cultures were divided into six treatment groups based on the substances added. The additions to each treatment group were: group 1 (negative control) none; group 2 (positive control: antibiotics; group 3: LTA; group 4: brown algal extract (0.05 μg/ml) and LTA; group 5: brown algal extract (0.10 μg/ml) and LTA; group 6: brown algal extract (0.15 μg/ml) and LTA. There were three replicates of each treatment. The six groups were incubated for 24 hours in a CO_2 incubator.

Calculation of TLR-2

The final step was the calculation of the amount of TLR-2 through an immunocytochemical method using streptavidin-biotin-peroxidase labeled with streptavidin-biotin (D-bio Sys, USA). The results of the immunocytochemical test were classified according to the number of cells colored by the stain. The test was said to be positive if there was nuclear immunostaining with more than 5% of the cells stained. This immunocytochemical evaluation was carried out individually by a pathology consultant and researcher to obtain accurate results.

Data Analysis

The data obtained were subjected to statistical analysis performed in SPSS 17.00 for Windows 7. The Kruskal-Wallis test for normality was applied; if the data were not normally distributed, then the Mann-Whitney non-parametric test was applied to evaluate the significance of pairwise differences in the level of TLR-2 expression between the study groups. Statistical significance was evaluated at the 95% confidence limit ($\alpha = 0.05$).

Results

The TLR-2 expression data for each treatment group are presented as average numbers (mean ± standard deviation) in Table 1 and Figure 1. The data obtained on the effect of brown alga (*Sargassum plycystum*) extract in reducing Lipoteichoic acid (LTA) levels in *Enterococcus faecalis* bacteria through the expression of Toll-Like Receptor-2 (TLR-2) on fibroblast cells were not normally distributed. Therefore, the Kruskal Wallis test was applied. The p-value was 0.012 ($p < 0.05$), showing significant between-group differences, and post hoc Mann-Whitney pairwise tests were applied.

Table 1: TLR-2 expression on fibroblast cells culture after the administration of brown algae extract (*Sargassum polycystum*)

Treatment Groups	n	TLR-2 Expression (%) Mean	p
Concentration of 0.5	3	62.33 ± 8.08	
Concentration of 0.10	3	62.00 ± 15.59	
Concentration of 0.15	3	34.00 ± 1.73	
KS+	3	28.67 ± 5.13	
KS-	3	29.67 ± 3.06	0.012
KS+LTA	3	28.00 ± 5.00	

Normality test: * Kruskall Wallis ($p<0.05$), the data was normally distributed,
Post Hoc test: *Mann-Whitney test; $p<0.05$: significant

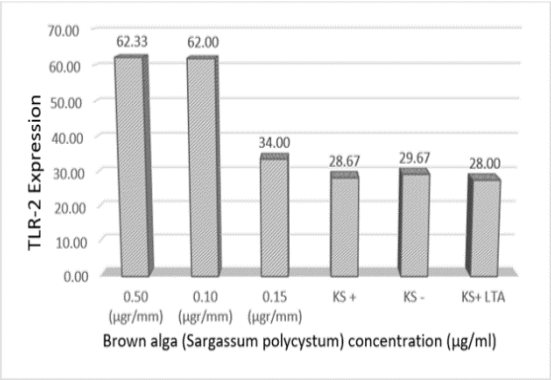

Figure 1: TLR-2 expression in fibroblast cells culture after the administration of brown algae extract (*Sargassum polycystum*)

The results of the post hoc Mann-Whitney pairwise tests are shown in Tables 2-4. The differences between TLR-2 expression in the 0.05 µg/ml algal extract concentration group and all other treatments (Table 2) were significant ($p < 0.05$) except with the 0.10 µg/ml algal extract treatment ($p > 0.05$).

Table 2: Comparison of TLR-2 expression between the 0.5 µg/ml concentration group and other treatment groups

Treatment group	Comparison	n	TLR-2 Expression (%) Mean	p
Concentration of 0.5	Concentration of 0.10	3	0.33	0.957
	Concentration of 0.15	3	28.33	0.000
	SG+	3	33.67	0.000
	SG-	3	32.67	0.000
	SG + LTA	3	34.33	0.000

*Post Hoc test: * Mann Whitney test; p<0.05: significant*

The treatment group with 0.10 µg/ml algal extract was significantly different ($p < 0.05$) from the 0.15 µg/ml concentration treatment and all non-algal extract treatments (Table 3).

Table 3: Comparison of TLR-2 expression between the 0.10 µg/ml concentration group and other treatment groups

Treatment group	Comparison	n	TLR-2 Expression (%) Mean	p
Concentration of 0.10	Concentration of 0.15	3	28.00	0.000
	SG+	3	33.33	0.000
	SG-	3	32.33	0.000
	SG + LTA	3	34.00	0.000

*Post Hoc test: * Mann Whitney test; p<0.05: significant*

Table 4: Comparison between the TLR-2 expression in the 0.15 µg/ml concentration group and other treatment groups

Treatment group	Comparison	n	TLR-2 Expression (%) Mean	p
Concentration of 0.15	SG+	3	5.33	0.394
	SG-	3	4.33	0.487
	SG + LTA	3	6.00	0.339

*Post Hoc test: * Mann Whitney test; p<0.05: significant*

Discussion

Persistent endodontic infections are caused by bacteria that play a role in primary or secondary infection and are able to survive intracanal antimicrobial procedures and periods of nutrient deficiency in the treated root canal. [10],[11] Anaerobic Gram-positive or facultative Gram-positive bacteria that are often detected include *Pseudoramibacter alactolyticus, Lactobacillus, Parvimonas micra Enterococcus faecalis, Olsenella uli,* and bacteria in the genera *Streptococcus, Actinomyces,* and *Propionibacterium*. This supports claims that Gram-positive bacterial are more resistant to antimicrobial treatment and are able to adapt to environmental conditions in treated root canals even with medication. *E. faecalis* is the species most often detected in teeth that have received root canal treatment, with a prevalence up to 90% in both culture and non-culture studies [10],[11].

While *E. faecalis* has several virulence factors, Lipoteichoic acid (LTA) plays an especially important role because it acts as an adhesion molecule that binds to host cells through their lipid content, enabling the colonization and invasion of host cells. In addition, LTA has functions that are important for the growth and survival of Gram-positive bacteria [12]. The LTA from *E. faecalis* is known to inhibit the mechanism of periapical bone repair by reducing the proliferation of osteoblast-like cells and stimulating apoptosis. [5,6].

This study observed the *E. faecalis* LTA level in fibroblast cells through TLR-2 expression because LTA is a part of pathogen-associated molecular patterns (PAMPs) that bind specifically to TLR-2 on the surface of fibroblast cells. The bonds between LTA and TLR-2 stimulate the expression of receptor regulators, namely NF-κβ (Nuclear Factor Kappa Beta), which then stimulates these receptor regulators to release pro-inflammatory cytokines such as TNF-α (Tumor Necrotizing Factor Alpha), IL-1β (Interleukin-1 Beta), and IL-8 (Interleukin-8)[13]. The cells used in this study were fibroblast cells derived from Human Periodontal Ligament Fibroblasts (HPDLFs). These cells play an important role in the host's defense response. The exposure of fibroblast to PAMPs, in

this case, LTA from *E. faecalis*, can stimulate the release of pro-inflammatory cytokines [14].

Recent research shows that LTA stimulates the inflammatory response through TLR2-MyD88-dependent mechanisms. LTA is a ligand to TLR-2, easily bound to TLR-2 compared to other TLRs in infected pulp because more positive TLR-2 macrophages are found in the inflamed pulp. The interaction between LTA and TLR-2 activates intracellular messengers, such as MyD88 (Myeloid differentiation primary response 88) and MAPKs (Mitogen-activated protein kinases) [5].

Microscopic examination results in this study showed the presence of brown cell membranes in TLR-2 expression in the treatment group. These observations are in line with the results of Putu et al. (2010) [15], who observed the effectiveness of probiotics in reducing TLR-2 expression induced by *E. faecalis* bacterial lipopolysaccharides and also showed the presence of brown cell membranes as a representation of TLR-2 expression under microscopic examination.

In this study, the use of LTA from *E. faecalis* bacteria with a concentration of 10µg to release TLR-2 in fibroblast cells is in line with a study conducted by Wang et al. (2015) [5] which measured TNF-α through the expression of NF-κβ induced by lipoteichoic acid from *E. faecalis* bacteria in macrophages, showing a dose-dependent increase in pro-inflammatory cytokines such as NF-κβ which stimulated by TLR-2 with the concentration of 10µg/ml of LTA. The results show that the administration of brown alga (*Sargassum polycystum*) extract had an effect; there was a decrease in *E. faecalis* LTA levels through TLR-2 expression in fibroblast cells. There was a significant decrease in TLR-2 expression in cells exposed to 10µg /ml LTA. When the concentration of brown alga (*Sargassum polycystum*) extract increased, the level of TLR-2 expression decreased. TLR-2 was around 62% in the cells exposed to extract concentrations of 0.05 µg/ml and 0.10 µg/ml, but 34% in the group of cells exposed to the algal extract at a concentration of 0.15 µg/ml.

The significant decrease in the amount of TLR-2 expression is thought to be due to the presence of active compounds, such as phenols, flavonoids, and saponins, in the brown alga (*Sargassum polycystum*) extract. The activity of phenol compounds can suppress the growth of Gram-positive bacteria because of the ability of these compounds to penetrate bacterial cell walls. Phenol compounds include fat-soluble compounds. The phenol group is capable of damaging cell membranes, activating enzymes, and denaturing proteins so that the bacterial cell wall is damaged due to decreased permeability [16].

According to Cushnie and Lamb (2006) [17], several types of flavonoid compounds have greater activity against Gram-positive bacteria than Gram-negative bacteria. Mechanisms through which flavonoid compounds can inhibit bacterial growth include: inhibiting the synthesis of nucleic acids; inhibiting cytoplasmic membrane function; disrupting cellular membrane permeability and damaging membrane function; and inhibiting energy metabolism in bacteria.

The results of this study are consonant with theories on the mechanisms by which saponin compounds contained in natural materials work on phosphate groups in the cell phospholipid membrane and enter the cells, which could explain the inhibitory/bacteriostatic effect of saponin compounds [18], [19]. This action results in loss of membrane semi-permeability and leakage of nitrogen elements, enabling the compounds to enter the cell, denaturing protein and damaging cell membranes, while the anionic compounds cause major damage to the cell membrane lipoprotein framework.

Increasing the concentration of brown alga (*Sargassum polycystum*) extract, which contains active materials including phenols, flavonoids, and saponins, has been shown to be effective in inhibiting *E. faecalis* bacteria through its action on bacterial cell walls.

Conclusion

From the results of this study, it can be concluded that an extract of the brown alga *Sargassum polycystum* at a concentration of 0.15 µg/ml is effective in reducing the LTA level of *Enterococcus faecalis* bacteria through TLR-2 expression in fibroblast cells. The concentration of 0.05 µg/ml and 0.10 µg/ml of *Sargassum polycystum is not effective* in reducing the LTA level of *Enterococcus faecalis* bacteria through TLR-2 expression in fibroblast cells.

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