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Research Report

The role of Kuniran (*U. moluccensis*) and Gurami (*O. goramy*) fish thorns and scales in increasing salivary leukocyte and monocyte cells viability against *Streptococcus mutans*

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ABSTRACT

Background: *Kuniran thorns and Gurami fish scales are rich in protein and minerals such as dentin believed to increase cell viability against Streptococcus mutans (S. mutans) that causes dental caries. These, in turn, can cause systemic diseases if left untreated.* ***Purpose:*** *This study aims to analyze the influence of Kuniran thorns and Gurami fishes scales on the viability of monocytes and salivary leukocytes against S. mutans.* ***Methods:*** *Monocytes and leukocytes salivary cells were placed on a microtiter plate and treated according to the nature of each group. This study comprised the following groups: control group: untreated; S. mutans group: induced by S. Mutans; Gurami thorn group: thorns + S. Mutans; Gurami scales group: scales + S. Mutans; Kuniran thorn group: thorns + S. Mutans; Kuniran scales group: scales + S. mutans. Viability analysis involved staining with Tripan Blue. Furthermore, the number of viable cells (white) was calculated under an inverted microscope at 200 times magnification from five fields of view. Data was analyzed by means of an ANOVA test followed by LSD test.* ***Results:*** *The ANOVA and LSD tests confirmed significant differences (0.01<P). Kuniran thorns and Gurami fish scales increased the viability of monocytes and salivary leukocytes, but not significantly. The content of flavonoids, amino acids, omega 3, omega 6 and antioxidants increased leukocyte metabolism, thereby increasing cell resistance to S. mutans infection.* ***Conclusion:*** *Kuniran thorns (U. moluccensis) and Gurami (O. goramy) fish scales increase the viability of salivary leukocyte and monocyte cells against Streptococcus mutans.*

Keywords: *Osphronemus goramy; scales; Streptococcus mutans; thorns; Upeneus moluccensis; viability*

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INTRODUCTION

In 2018, the National Basic Health Research Department (Riskesdas) stated that the rate of dental and oral problems stood at 57.6%. Dental caries remain an intractable national problem in Indonesia which, according to Riskesdas figures for 2013 and 2018, had reached the level of 4-5 teeth per person, while the international standard stood at 2.5 teeth.[1] *Streptococcus mutans (S. mutans)* is a bacterium constituting the main cause of dental caries whose spread can lead to systemic diseases. Consequently, researchers are increasing their understanding of its presence and studying the pathogenesis of dental caries together with their systemic expansion. There is evidence that bacteria (*S. mutans*) originating in dental caries play a role in the occurrence of endocarditis [2] since they are found in the coronary atherosclerotic plaque of patients who suffer fatal heart attacks.[3] Since monocytes and salivary leukocytes play a role in the spread of S. mutans from the mouth throughout the systems of the body, the importance of

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the role of leukocytes in saliva and monocytes in blood in the spread of S. mutans is evident. On the other hand, prevention and treatment of dental caries can be achieved by employing natural resources readily available in Indonesia. Two such resources are fish thorns and scales which, since they are regarded as waste, remain under-utilised. However, such materials must be compatible.[4] In Jember, Kuniran (*Upeneus moluccensis)* and Gurami (*Osphronemus goramy*) are two of the five most common fish species[5] with the latter representing an important national aquaculture commodity.[6] Therefore, this study seeks to analyze the viability of Kuniran thorns and Gurami fish scales in monocytes and salivary leukocytes acting against *S. mutans*.

Researchers have previously proved that fish thorns and scales consist of proteins that supposedly act as immunomodulators because they contain omega 3, omega 6, flavonoid, various amino acids (alanine, leucine-isoleucine, valine, arginine, methionine, proline, glutamic acid, histidine, lysine, glycine, serine and tyrosine) which are believed to prevent dental caries. A number of researchers have confirmed that fish waste such as scales, thorns and skin represent a potential source of collagen that is crucial to tissue formation and organ function, while also being involved in various functional expressions of cells.[4,7–9] Scientists from Nanyang Technological University (NTU) in Singapore have demonstrated that collagen derived from fish scales is highly effective across a variety of biomedical applications such as wound healing.[10] Research conducted by Zhou *et al.* (2013), strongly suggests that fish scales containing collagen can increase blood vessel and lymph formation, thereby promoting tissue repair and regeneration.[11] However, the findings of these studies suggest that the viability of the scales and thorns of Gurami and Kuniran fish remains unproven. It is known that Gurami (fresh water) and Kuniran (salt water) are the types of fish most frequently consumed by the population of Jember, East Java, Indonesia.

Cell viability testing is central to drug discovery studies.[12] Determination of cell viability is the most commonly employed method of assessing the impact of various types of stressors in both toxicity and industrial microbiology studies. Viability is defined as the percentage of living cells within the entire population.[13] Therefore, this study was intended to analyze the effects of thorns and scales from Gurami and Kuniran fish on the viability of salivary leukocyte and monocyte cells after induction of *S. mutans*.

MATERIALS AND METHODS

The research, approved by the Ethical Committee, Faculty of Dentistry, University of Jember (079/UN.25.8/KEPK/DL/2018), was conducted between April and September 2018. The materials comprised the following: peripheral blood, *S. mutans* from the Microbiology Laboratory, Faculty of Dentistry, University of Jember; Ficoll-hypaque (Sigma); HBSS/Hank's Balanced Salt Solution (Gibco); RPMI medium (Roswell Park Memorial Institute/Gibco); Immunostaining KIT (Daco); PBS/Phosphate Bufer Saline (Sigma); DAB/Diaminobenzidine (Daco); HRP/horseradish peroxidase (Daco) and fungizone (Gibco).

The fish thorns/scales were placed in an alumunium pan once the water temperature had reached 80°C and boiled for 60 minutes, before being dehydrated in a drying oven for 48 hours at 65°C, mashed using a grinder mill and sieved through a 100 mesh filter. ±1g of the dry sample was subsequently weighed and dissolved using PBS at a ratio of 1:1.[14]

Blood and saliva were collected from healthy individuals (males deemed to be free of systemic disease and caries who were teetotal non-smokers). Saliva was collected by means of the subject spitting onto a dish placed on ice. The blood and saliva was then carefully layered with histopaque and ficollpaque to avoid their mixing, before being centrifugated at 1,900 rpm for 30 minutes at 26°C. The monocyte and salivary leukocyte layers were added to HBSS (1:1) and pipetted. Monocytes and salivary leukocytes were filtered on each coverslip (@ 100 µL), placed in a microplate and incubated for 15 minutes. 1 cc of RPMI medium, 20 µL of stain and 5 µL of fungizone were added to each well and incubated for 20 minutes, washed twice to eradicate any contamination and, finally, treated according to the group.[15]

The monocyte group consisted of six groups: a control group; a monocyte isolates untreated *S. mutans* group; a monocyte isolate induced by *S. mutans group;* a Gurami thorn group: @ 100 µL of monocyte isolates were added to 200 µL of Gurami thorns and 100 µL of *S. mutans;* a Gurami scales group: @ 100 µL of monocyte isolates were added to 200 µL of Gurami scales and 100 µL of *S. mutans.*

The salivary leukocytes group consisted of six groups: a control group: untreated salivary leukocytes isolates; *S. mutans* group: salivary leukocytes isolates induced by *S. mutans*; Gurami thorns group: @ 100 µL of salivary leukocytes isolates were added to 200 µL thorns of Gurami and 100 µL of *S. mutans*; Gurami scale group: @ 100 µL of salivary leukocytes isolates were added to 200 µL of scales Gurami and 100 µL of *S. mutans*; Kuniran thorn group: @ 100 µL of salivary leukocytes isolates were added to 200 µL of Kuniran thorns and 100 µL of *S. mutans*; Kuniran scales group: @ 100 µL of salivary leukocytes isolates were added to 200 µL of Kuniran thorns and 100 µL of *S. mutans*. All groups were painted with Tripan Blue to facilitate analysis of cell viability. Treatment ensued with *S. mutans* (@ 100 µL and thorns/scales @ 200 µL.

Cell viability was analyzed by counting the number of living cells (white) under the inverted microscope at 200 times magnification from five fields of view. The data obtained was subsequently analyzed by means of ANOVA and LSD Tests.

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RESULTS

The results of the viability of salivary monocyte and leukocyte cells exposed by thorns and scales of Kuniran and Gurami fish after being induced by *S. mutans* were as follows. The ANOVA analysis results were p=0.000 (p<0.05), while the LSD test produced a significant difference between the control with Gurami thorns and scales and the control with Kuniran thorns and scales. However, there were no significant differences between Gurami thorns and Gurami scales p=0.86 (p>0.05), Gurami thorns with Kuniran scales p=0.14 (p> 0.05), Gurami thorns with Kuniran scales p=0.147 (p>0.05), Gurami scales with Kuniran scales p=0.765 (p>0.05).

The ANOVA analysis results were p=0.000 (p<0.05), while the LSD Test indicated a significant difference between the control group with Gurami thorns and scales and the control group with Kuniran thorns and scales. However, there were no significant differences between Gurami thorns with Gurami scales p=0.86 (p>0.05), Gurami thorns with Kuniran thorns p=0.14 (p>0.05), Gurami thorns with Kuniran scales p=0.147 (p>0.05) and Gurami scales with Kuniran scales p=0.765 (p> 0.05). These results illustrated that Kuniran thorns and scales possess the same ability to increase the viability of salivary leukocytes, whereas Gurami scales increased the viability of salivary leukocytes compared to their scales. It could be said that the thorns and scales of Kuniran and Gurami fish usually increased both the viability of monocytes and salivary leukocytes (Tables 1-6 and Figures 1-4).

Table 1. Normality test of monocyte cells viability One-sample Kolmogorov-Smirnov test

		Treatment	Viability of monocyte cells
N		24	24
Normal Parameters[a,b]	Mean	3.5000	92.4167
	Std. Deviation	1.74456	59.96007
Most Extreme Differences	Absolute	.138	.401
	Positive	.138	.235
	Negative	-.138	-.401
Kolmogorov-Smirnov Z		.678	1.966
Asymp. Sig. (2-tailed)		.748	.001

a. Test distribution is normal; b. Calculated from data.

Table 2. Test of homogeneity of monocyte cells viability

Levene Statistic	df1	df2	Sig.
.467	5	18	.796

Table 3. One-way ANOVA of monocyte cell viability

	Sum of squares	df	Mean square	F	Sig.
Between groups	82665.333	5	16533.067	12146.743	.000
Within groups	24.500	18	1.361		
Total	82689.833	23			

Table 4. Normality test of salivary leucocyte viability One-sample Kolmogorov-Smirnov test

		Treatment	Viability of monocyte cells
N		24	24
Normal Parameters[a,,b]	Mean	3.5000	44.8333
	Std. Deviation	1.74456	27.64160
Most Extreme Differences	Absolute	.138	.375
	Positive	.138	.222
	Negative	-.138	-.375
Kolmogorov-Smirnov Z		.678	1.837
Asymp. Sig. (2-tailed)		.748	.002

a. Test distribution is Normal; b. Calculated from data.

Table 5. Test of salivary leucocyte homogeneity

Levene Statistic	df1	df2	Sig.
.612	5	18	.692

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Table 6. One-way ANOVA of salivary leucocytes viability

	Sum of squares	df	Mean square	F	Sig.
Between groups	17551.833	5	3510.367	2938.912	.000
Within groups	21.500	18	1.194		
Total	17573.333	23			

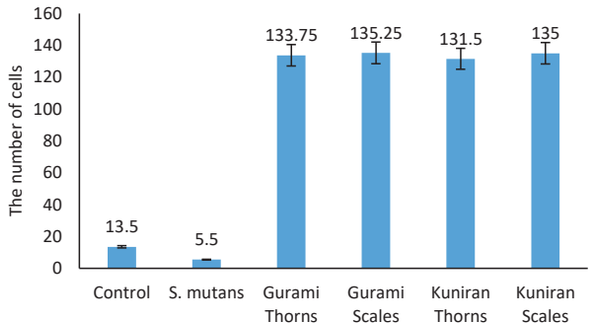

Figure 1. Diagram of monocytes viability

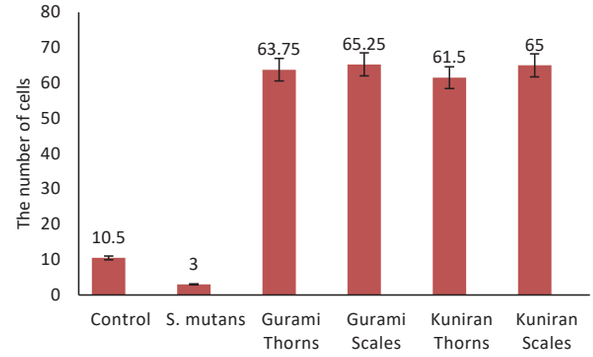

Figure 3. Diagram of salivary leukocytes viability

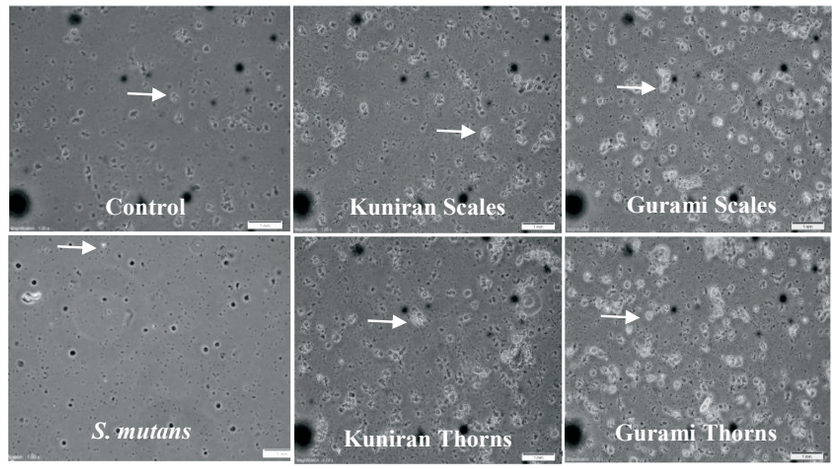

Figure 2. Microscopic description of monocytes viability of Kuniran and Gurami fish scales and thorns (arrow). Observations conducted with an inverted microscope at 200x magnification.

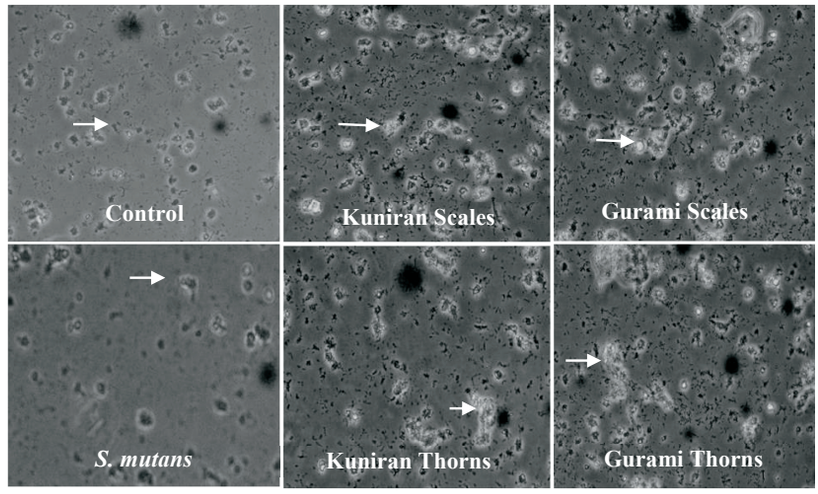

Figure 4. Microscopic description of salivary leucocyte viability of Kuniran and Gurami fish scales, thorns (arrow). Observations using a inverted microscope at 200x magnification.

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DISCUSSION

As caries-related bacteria penetrate deeply into dentin, coming into close proximity to the pulp, inflammatory cells (such as lymphocytes, macrophages and neutrophils) infiltrate the bacterium-invaded area resulting in the development of pulpitis. Many types of cytokines and adhesion molecules are responsible for the initiation and progression of pulpitis.[16] Bacteria (*S. mutans*) then spread systemically through the blood vessels in the oral cavity to other parts of the body. At this point, the role of immunocompetent cells such as monocytes, macrophages, neutrophils, lymphocytes is crucial in preventing the spread of *S. mutans* from the oral cavity to other parts of the body's systems. Immunocompetent dental pulp cells have an important function in maintaining the structural integrity of connective tissue. These cells include odontoblasts which produce pro-inflammatory cytokines and express adhesion molecules in response to pathogens such as pathogen-associated molecular patterns (PAMP) which are structures expressed by microorganisms. Generally, the initial recognition of microbial pathogens is mediated by pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and the nucleotide-binding oligomerization domain-like receptors (NLRs).[17–19]After recognition by receptors, the cells will initiate proinflammatory cytokines and oxidant-producing phagocytic processes, both of which cause damage to cells and tissues. Therefore, a material is required that can increase cell resistance to damage caused by bacterial infections, especially *S. mutans*.

The research reported here has proved that the thorns and scales of Kuniran and Gurami fish can inhibit the growth of *S. mutans* by increasing the viability of monocytes and salivary leukocytes. Kuniran and Gurami fish thorns and scales increased the viability of monocytes and salivary leukocytes due to their content, including: amino acids, flavonoids and unsaturated fatty acids (omega 3, omega 6). Amino acids play a role in the vitamin B process (especially that of B5 and B6), produce leucine and isoleucine in protein synthesis, form antibodies, activate various types of hormones, energy providers, ketone and glucose makers. Arginine can strengthen the immune system, while methionine nourishes blood vessels, reduces inflammation and treats allergies. Glycine can be employed for wound healing and arginine for various metabolic urea synthesis, lymphocyte proliferation and wound healing. Glutamine is one of the three amino acids present in glutathione which are antioxidant compounds used as ingredients in leukocytes metabolism. Glutathione is one of the antioxidants with a role in protecting cells from damage caused by reactive oxygen. On the other hand, the main components of cell membranes are phospholipids, glycolipids and cholesterol. These components contain polyunsaturated fatty acids which are highly susceptible to oxidation that causes free radicals.[20,21]

Antioxidants can prevent cell damage caused by donating hydrogen electrons to free radicals. Antioxidants can give hydrogen atoms to lipid radicals (R •, ROO •) and transform them into more stable forms. In addition, antioxidants can also slow the rate of auto-oxidation resulting in the presence of amino acids, thought to positively affect body cell metabolism, thereby enhancing cell resistance to infection. The flavonoid content is thought to work through its inhibiting of the production of Nitric Oxide (NO) through the mechanism of the cytokine-induced NO synthase (iNOS) enzyme. It may also inhibit arginine transport through the mechanism of Cationic Amino Acid Transporter-2 mRNA (CAT-2 mRNA). It is said that flavonoids are potential cancer-reducing compounds which can inhibit oxidation reactions induced by enzymes or non-enzymes and act as a good source of hydroxyl and superoxide radicals that protect membrane lipids from reactions that can damage cells (monocytes and salivary leukocytes).[22]

Unsaturated fatty acids constitute the main component of phospholipids which act as a constituent of cell membranes, DNA and proteins. DNA represents a cell's genetic device, while proteins play an important role as enzymes, receptors, antibodies, matrix formers and cytoskeleton. Research has demonstrated that omega-3 polyunsaturated fatty acid fish oil reduces stress-induced oxidative DNA in vascular endothelial cells.[23] Therefore, all bioactive components of Kuniran and Gurami fish thorns and scales increase cell viability. The conclusion of this research is that the thorns and scales of Kuniran (*U. moluccensis*) and Gurami (*O. goramy*) fish can increase the viability cells of salivary leukocytes and monocytes against *S. mutans*.

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