Makara Journal of Science

Volume 25
Issue 4 *December*

Article 7

12-30-2021

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Cover Page Footnote

We acknowledge Indonesia Endowment Fund for Education/LPDP, Ministry of Finance, Republic of Indonesia for financial support, and partly supported by RISPRO-PRN-LPDP-PAJALE research grant. Thankfully into members of Research Center SMONAGENES, Brawijaya University for the discussions and laboratory facilities.

Makara Journal of Science, 25/4 (2021), 247-257
doi: 10.7454/mss.v25i4.1266

Antimicrobial Activity of Ferulic Acid in Indonesian Purple Rice through Toll-like Receptor Signaling

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Received August 4, 2021 | Accepted December 4, 2021

Abstract

Purple rice is a potential source of ferulic acid, which has antimicrobial properties. However, the inhibitory mechanism of ferulic acid on the growth of bacteria, particularly *Salmonella* and *Listeria*, has not been elucidated. This study aimed to determine the bioactivity of ferulic acid from purple rice as an antimicrobial agent against *Salmonella typhimurium* and *Listeria monocytogenes* using *in vitro* and *in silico* analyses. The antimicrobial activity of a purple rice ferulic acid extract was tested using the agar well diffusion method. Its effect on bacterial cells was observed using scanning electron microscopy. Ferulic acid was confirmed to have antimicrobial properties using *in silico* software to attenuate the binding of bacterial virulence factors (lipoproteins, lipopolysaccharides, and flagellins) to Toll-like receptors (TLRs) and to prevent interactions with peptidoglycans. The purple rice ferulic acid extract inhibited bacterial growth. The inhibitory effects included induction of a biofilm and shrinkage of *S. Typhimurium*, as well as osmotic lysis of *L. monocytogenes*. This activity was supported by the ability of ferulic acid to inhibit the binding of bacterial virulence factors with TLRs and block bacterial peptidoglycans. These findings indicate that the purple rice ferulic acid extract acts as antimicrobial, both directly to bacterial cells and indirectly through TLRs. We conclude that ferulic acid from Indonesian purple rice has a biological function as an antimicrobial agent.

Keywords: antimicrobial activity, ferulic acid, purple rice, Toll-like receptors

Introduction

Rice (*Oryza sativa* L.) is the main food crop for countries in Asia, including Indonesia [1, 2]. In general, people prefer to consume white rice, but the active compound content is less than in pigmented rice. Pigmented rice, such as brown, purple, dark purple, black, red, and mixed colored, can be used as an alternative to diversify healthy foods that contain various active compounds needed by the body [3, 4].

Purple rice is one of the pigmented rice varieties in Indonesia, which is produced from a cross between black rice and white rice, the local varieties of East Java Indonesia. The potential function of the purple rice from this cross has not been explored. However, purple rice in Thailand is rich in protein, fat, and fiber [5]. Varieties of rice resulting from crosses between black and white rice from Thailand also contain ferulic acid (FA), isoferulic acid, p-coumaric acid, and vanillic acid [6]. Another study reported that rice is a primary source of FA [7].

FA is one of the primary phenolic acids in rice, which is found in the outer layers of the seed, such as the pericarp and aleurone [1]. FA has antioxidant, anti-inflammatory [8], and antimicrobial effects against Gram-negative and -positive bacteria and yeast [9]. However, the mechanism underlying the role of FA in inhibiting bacterial growth remains unclear.

The pathogenic bacteria *Salmonella Typhimurium* and *Listeria monocytogenes* are the most common foodborne pathogens causing bacterial gastrointestinal disease [10]. *S. Typhimurium* belongs to nontyphoidal *Salmonella*, the

most prevalent cause of gastroenteritis, and has been linked to invasive systemic disease and high fatality rates in immunocompromised patients [11]. *L. monocytogenes* causes listeriosis, a systemic infection manifesting as bacteremia, which is generally complicated by meningo-encephalitis in immunocompromised people and the elderly, as well as fetal-placental infection in pregnant women [12]. *S. Typhimurium* and *L. monocytogenes* usually enter the digestive tract through contaminated food. *S. Typhimurium* and *L. monocytogenes* are considered major deadly human pathogens that can survive phagocytosis [13, 14]. The rate of antibiotic resistance in *S. Typhimurium* and *L. monocytogenes* has been increasing, which has complicated clinical therapy and increased morbidity and death [11, 15].

S. Typhimurium is a facultative anaerobic Gram-negative rod-shaped bacteria with a flagellum [16], whereas *L. monocytogenes* is a Gram-positive flagellated bacteria [17]. Gram-negative bacteria contain lipopolysaccharides (LPS), which play a role in activating the innate immune system via Toll-like receptor 4 (TLR4) interactions. In Gram-positive bacteria, particular lipoproteins (LPP) interact with TLR2 [18]. Flagellin, an immunodominant protein in the flagellum, also acts as a virulence factor [19] that activates TLR5 [20]. Both bacteria contain peptidoglycans in their cell wall, which are recognized by the peptidoglycan recognition protein (PGRP) [18, 20].

In this study, FA antimicrobial activity will be determined in these two bacteria. The inhibitory mechanism of *S. Typhimurium* and *L. monocytogenes* in response to a purple rice ferulic acid extract (PRFAE) will be observed through microscopic analysis. Scanning electron microscopy (SEM) has been used to examine the effect of antimicrobial agents on microorganism cell morphology [22]. Antimicrobial activity will be confirmed by the agar well diffusion method. The mechanism will be described by observing the interaction among FA, bacterial virulence factors, and TLRs *in silico* to predict the potential of FA for inhibiting bacterial infection in digestive tract cells. The potential of FA to inhibit the binding of peptidoglycans to PGRP will also be determined. Inhibiting *S. Typhimurium* and *L. monocytogenes* with the PRFAE is expected to prevent gastrointestinal infection, reduce the rate of mortality from foodborne infection, and protect gastrointestinal epithelial cells from inflammation. Therefore, our study focuses on investigating the antimicrobial activity of the PRFAE in *S. Typhimurium* and *L. monocytogenes* through *in vitro* and *in silico* studies.

Materials and Methods

The PRFAE was prepared according to a previous study [23] with modifications. Fifty grams of purple rice powder was mixed in 300 mL of 0.5 M sodium hydroxide and held at a constant speed on a water bath shaker for 4 hours at 60 °C. The mixture was added to 96% ethanol (three times the initial volume) and neutralized with 37 N hydrochloric acid. The final mixture was filtered through Whatman filter paper 41 using a vacuum on a Buchner funnel, and excess ethanol was removed with a rotary evaporator for about 30 min to obtain the concentrated extract. A brown extract indicated the presence of FA. The FA from purple rice was identified based on the same Rf value to standard FA using thin-layer chromatography. The PRFAE concentrations used for the antimicrobial assay were 250, 500, and 750 mg/mL.

Pure *S. Typhimurium* and *L. monocytogenes* cultures were purchased from the Laboratory of Microbiology, Medical Faculty, Brawijaya University (Malang, Indonesia). Each bacterium was cultured in Luria Bertani (LB) broth medium (Bioworld) at 37°C overnight. The optical density of the bacterial cultures was 10^8 cells/mL, as measured using a UV-vis spectrophotometer (SmartSpec Plus™, Bio Rad Laboratories Inc., Hercules, CA, USA) at 600 nm [24].

The antimicrobial activity assay was carried out by the agar well diffusion method, as described previously [25] with modifications. The bacterial cultures were transferred to Mueller Hinton Agar medium on poured plates. The wells were prepared with an 8 mm borer and filled with 100 µL of the PRFAE. The plates were incubated at 37 °C for 18 hours.

The effect of the PRFAE on the bacterial cells was examined by SEM cellular analysis as modified from a previous study [26]. A total of 15 mL of LB medium was inoculated with 150 µL of bacterial culture and 150 µL of the PRFAE and incubated at 37 °C for 18 hours. The cultures were centrifuged at 2,000 × g, 4 °C for 10 min. The pellets were washed twice with 0.9% sodium chloride and resuspended in 3% glutaraldehyde for 30 min. The fixed samples were washed three times in phosphate-buffered saline for 15 min, dehydrated through an ethanol series (30, 50, 70, 80, 90, and 96%), and dried. The slides were coated with platinum and observed by SEM (Hitachi TM3000; Tokyo, Japan).

The FA and peptidoglycan structures for the *in silico* study were downloaded from NCBI PubChem (CID 445858, https://pubchem.ncbi.nlm.nih.gov/compound/ Ferulic-acid; and CID 9816401, https://pubchem.ncbi.nlm.nih.gov/compound/9816401). The protein structures were downloaded from RSCB, as follows: LPP (2RA2, https://www.rcsb.org/structure/2ra2), LPS (3VQ2, https://www.rcsb.org/structure/3vq2), flagellin (1IO1, https://www.rcsb.org/structure/1IO1), TLR2 (6NIG, https://www.rcsb.org/structure/6NIG), TLR4 (2Z63, https://www.rcsb.org/structure/2Z63), TLR5 (3J0A, https://www.rcsb.org/structure/3J0A), and PGRP (1YCK, https://www.rcsb.org/structrue/1YCK). The FA structure was converted to a pdb format using PyRx software,

while the protein structures were prepared using Discovery Studio 2020 software to remove water molecules and ligands bound to the proteins. Interactions among FA, LPP, LPS, Flagellin, and the TLRs were docked using the blind docking Hex 8.0.0 Cuda with Shape+Electro+DARS and a root mean square deviation value ≤ 2 Åas docking parameter and visualized using Discovery Studio 2020. PyMol determined the binding sites and the two-dimensional structures were visualized with LigPlot (modified from [27]).

Results and Discussion

The strength of the antimicrobial activity is shown in Table 1. The PRFAE at concentrations of 250, 500, and 750 mg/mL inhibited *S. Typhimurium* and *L. Monocytogenes*; thus, confirming its antimicrobial activity (Table 1). However, the diameters of the inhibitory zones of the 250 and 500 mg/mL concentrations for *S. Typhimurium*, and the 500 and 750 mg/mL concentrations for *L. monocytogenes* were not significantly different.

The responses of the bacterial cells treated with 0, 250, 500, and 750 mg/mL of the PRFAE were observed by SEM. The SEM micrographs of *S. Typhimurium* and *L. monocytogenes* are shown in Figure 1. The *S. Typhimurium* cells treated with 250–750 mg/mL PRFAE shrank to 0.6–1.6 µm long, while the untreated controls (0 mg/mL) were intact and 2–3.5 µm long. This result indicates that *S. Typhimurium* was unable to grow well with the PRFAE.

Although the cells that shrank were almost the same size in each treatment, different concentrations of the PRFAE had different effects. The cells shrunk with a flat surface in the 250 mg/mL treatment. However, the 500 mg/mL treatment induced the formation of a biofilm, as indicated by the attached and clustered cells. The 750 mg/mL concentration resulted in small, shrunken cells, with uneven surfaces that were partially lysed, as indicated by the amount of cell debris in the field.

The *L. monocytogenes* treated with the PRFAE appeared damaged, compared to normal cells that appeared intact with a flat surface (Figure 1). Furthermore, increasing the PRFAE concentration resulted in more cell damage. The pores at the end of the cells and the cell surface became uneven at 750 mg/mL

The interactions among FA, the TLRs, and the bacterial virulence factors are illustrated in Figures 2, 3, and 4. Figure 2 shows that LPP was bound to TLR2 in some residues. The binding sites changed when the TLR2-LPP complex interacted with FA, and TLR2 interacted with FA first before LPP. However, when LPP interacted with FA before TLR2, they were the same residues as the TLR2-LPP complex binding site (Ser2, Gly3, and Asp19). These findings show that FA inhibited the binding of TLR2 and LPP while interacting with the TLR2-LPP complex, and when TLR2 interacted with FA first. However, when LPP interacted with FA first, FA failed to inhibit TLR2 and LPP binding, indicating that FA did not interact with LPP at the LPP-TLR2 binding site; thus, LPP could still bind to TLR2. The lowest binding energy occurred when the TLR2-LPP complex formed, while the highest occurred when the TLR2-FA complex formed.

Table 1. Inhibitory Zones of the Purple Rice Ferulic Acid Extract

Concentration (mg/mL)	Inhibition zone diameter (mm)	
	S. typhimurium	*L. monocytogenes*
0	0.00[a]	0.00[a]
250	12.13[b]	11.13[b]
500	11.63[b]	12.25[c]
750	15.75[c]	12.38[c]

* Different letters in the same column indicate a significant difference (Tukey's HSD; $P < 0.05$).

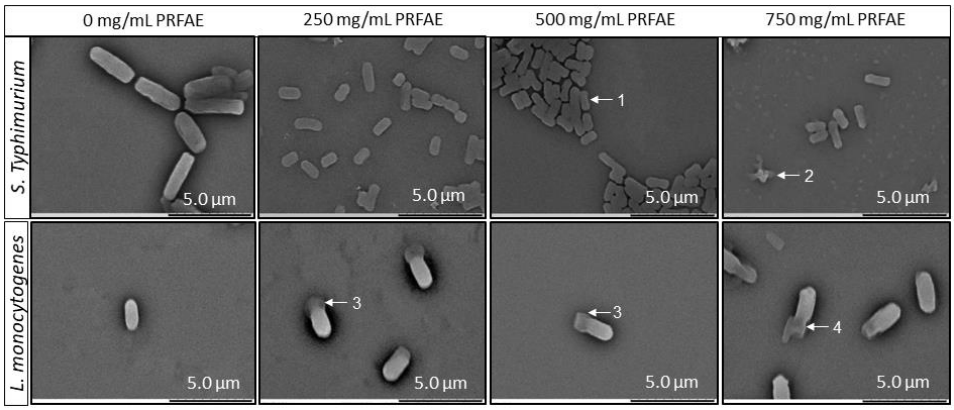

Figure 1. SEM Micrograph of *S. Typhimurium* and *L. monocytogenes*. PRFAE: Purple Rice Ferulic Acid Extract. White Arrow Shows Biofilm Formation (1) and Cell Debris (2) in *S. typhimurium* and Pore Formation (3) and Osmotic Lysis (4) in *L. monocytogenes* (12.000× Magnification)

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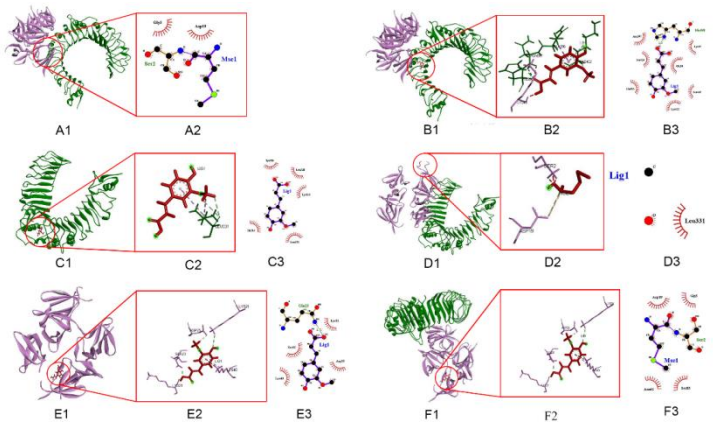

II

Receptor	Ligand	Interactions	Bond type	Total Energy (kJ/mol)
TLR2	LPP	Ser2	Hydrogen	-553.4
		Gly3, Asp19	Hydrophobic	
TLR2-LPP	FA	His398	Hydrogen	-220.09
		Lys49, Glu50, Ser445, Lys422, Gln396, Ser424, Asn397	Hydrophobic	
TLR2	FA	Tyr326, Lue328, Tyr332, Leu331, Ile314	Hydrophobic	-197.43
TLR2-FA	LPP	Leu331	Hydrophobic	-511.99
LPP	FA	Gln23	Hydrogen	-204.77
		Lys21, Asp25, Lys40, Ser32	Hydrophobic	
TLR2	LPP-FA	Asp19, Gly3, Ser85, Asn61	Hydrophobic	-548.67
		Ser2	Hydrogen	

Figure 2. The Interaction among FA, TLR2, and LPP. I. The Conformations of the FA, TLR2, and LPP Interactions, (A1) the TLR2-LPP Complex, (A2) 2D Structure; (B1) the TLR2-LPP Complex Interacted with FA, (B2) 3D and (B3) 2D Structures; (C1) the TLR2-FA Complex, (C2) 3D and (C3) 2D Structures; (D1) the TLR2-FA Complex Interacted with LPP, (D2) 3D and (D3) 2D Structures; (E1) the LPP-FA Complex, (E2) 3D and (E3) 2D Structures; (F1) the LPP-FA Complex Interacted with TLR2, (F2) 3D and (F3) 2D Structures. Red Represents FA, Green is TLR2, and Purple is LPP. II. Summary Table of Interactions, Bond Types, and Total Energy of the Docking Results

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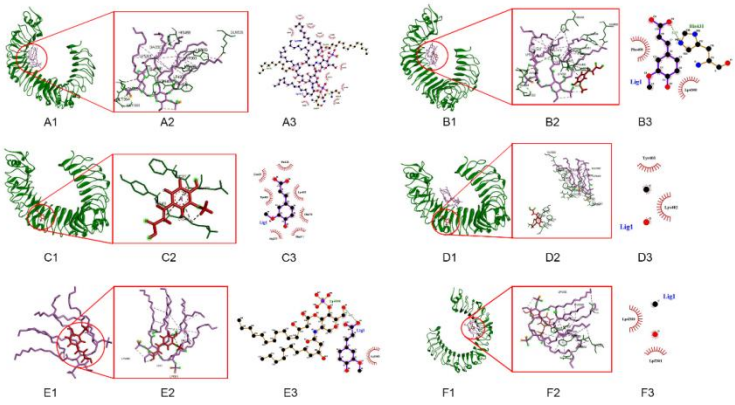

II

Receptor	Ligand	Interactions	Bond Type	Total Energy (kJ/mol)
TLR4	LPS	His456, Gln505, His431, Phe408, Arg382, Ser360, Lys362, Asn383, Gly364, Gly384	Hydrophobic	-442.37
TLR4-LPS	FA	Phe408, Lp4300	Hydrophobic	-186.62
		His431	Hydrogen	
TLR4	FA	His426, Lys402, Glu376, Phe377, Arg355, Tyr403, Glu425	Hydrophobic	-188.25
TLR4-FA	LPS	Tyr403, Lys402	Hydrophobic	-437.90
LPS	FA	Lp4300	Hydrogen	-187.38
		Lp5301	Hydrophobic	
TLR4	LPS-FA	Lp4300, Lp5301	Hydrophobic	-436.98

Figure 3. The Interaction among FA, TLR4, and LPS. I. The Conformations of the FA, TLR4, and LPS Interactions, (A1) TLR4-LPS Complex, (A2) 3D and (A3) 2D Structures; (B1) the TL4-LPS Complex Interacted with FA, (B2) 3D and (B3) 2D Structures; (C1) the TLR4-FA Complex, (C2) 3D and (C3) 2D Structures; (D1) the TLR4-FA Complex Interacted with LPS, (D2) 3D and (D3) 2D Structures; (E1) the LPS-FA Complex, (E2) 3D and (E3) 2D Structures; (F1) the LPS-FA Complex Interacted with TLR4, (F2) 3D and (F3) 2D Structures. Red Represents FA, Green is TLR4, and Purple is LPS. II. Summary Table of Interactions, Bond Types, and Total Energy of the Docking Results

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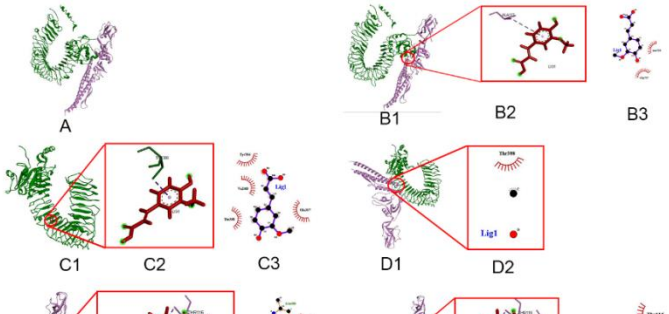

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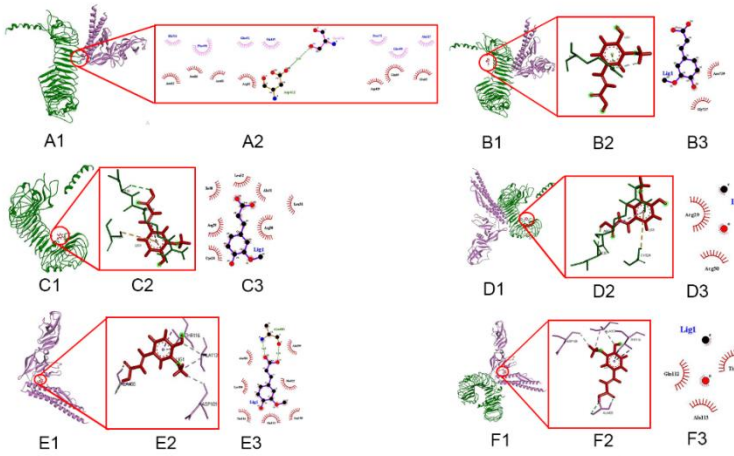

III

Receptor	Ligand	Interactions	Bond Type	Total Energy (kJ/mol)
TLR5 (A)	Flagellin	-	-	-347.89
TLR5 (B)	Flagellin	His524, Phe498, Gln451, His449, Ser474, Pro473, Glu499, Ala527	Interfaces 2 bonds	-299.42
		Asp412	Hydrogen	
		Asn82, Asn86, Asn85, Arg95, Asp419, Gln89, Glu93	Hydrophobic	
TLR5 (A)-Flagelin	FA	Asn739, Gly737	Hydrophobic	-199.10
TLR5 (B)-Flagelin	FA	Asn739, Gly737	Hydrophobic	-138.13
TLR5 (A)	FA	Tyr364, Val340, Thr388, Gln387	Hydrophobic	-189.68
TLR5 (B)	FA	Leu52, Ala31, Leu51, Arg50, Cys24, Arg29, Ile30	Hydrophobic	-179.65
TLR5 (A)-FA	Flagellin	Thr388	Hydrophobic	-276.50
TLR5 (B)-FA	Flagellin	Arg29, Arg50	Hydrophobic	-296.98
Flagelin	FA	Ala400	Hydrogen	-196.20
		Ala399, Gln112, Asp109, Ala113, Thr116, Lys384, Ala381	Hydrophobic	
TLR5 (A)	Flagellin-FA	Thr116, Ala113, Gln112	Hydrophobic	-267.15
TLR5 (B)	Flagellin-FA	Thr116, Ala113, Gln112	Hydrophobic	-296.38

Figure 4. The Interactions among FA, TLR5, and Flagellin. I. The Conformations of the FA, TLR5 (Domain A), and Flagellin Interactions, (A) 3D Structure of the TLR5-flagellin Complex, (B1) the TL5-flagellin Complex Interacted with FA, (B2) 3D, and (B3) 2D Structures; (C1) the TLR5-FA Complex, (C2) 3D and (C3) 2D Structures; (D1) the TLR5-FA Complex Interacted with Flagellin, (D2) 2D Structure; (E1) the Flagellin-FA Complex, (E2) 3D and (E3) 2D Structures; (F1) The Flagellin-FA Complex Interacted with TLR5, (F2) 3D and (F3) 2D Structures. II. The Conformations of the FA, TLR5 (Domain B), and the Flagellin Interactions, (A1) the TLR5-Flagellin Complex, (A2) 2D Structure; (B1) the TL5-flagellin Complex Interacted with FA, (B2) 3D, and (B3) 2D Structures; (C1) the TLR5-FA Complex, (C2) 3D and (C3) 2D Structures; (D1) the TLR5-FA Complex Interacted with Flagellin, (D2) 2D and (D3) 3D Structures; (E1) the Flagellin-FA Complex, (E2) 3D and (E3) 2D Structures; (F1) the Flagellin-FA Complex Interacted with TLR5, (F2) 3D and (F3) 2D Structures. Red Color Represents FA, Green Color is TLR5, and Purple is Flagellin. III. Summary Table of the Interactions, Bond Types, and Total Energy of the docking Results

LPS bound to TLR4 in some residues (Figure 3). When the TLR4-LPS complex interacted with FA, there were two identical residues in the binding sites (Phe408 and His431). However, when TLR4 interacted with FA before LPS and LPS interacted with FA before TLR4, the binding sites were different from those of the TLR4-LPS complex. Thus, FA reduced binding of the complex if TLR4 interacted with LPS first. However, if FA interacted with TLR4 or with LPS first, FA inhibited the binding between TLR4 and LPS. The lowest binding energy resulted from the TLR4-LPS complex, while the highest occurred with the TLR4-LPS-FA complex.

TLR5 domains A and B were used to test which could interact with flagellin and FA. Figure 4 shows that flagellin interacted with TLR5 (B), but not with TLR5 (A). The binding sites changed when the TLR5-flagellin complex interacted with FA, and when TLR5 interacted with FA before flagellin, and flagellin interacted with FA before TLR5. These results show that FA inhibited binding between TLR5 and flagellin, when TLR5 interacted with FA first and when flagellin interacted with FA first. The lowest binding energy resulted from the TLR5-flagellin complex, while the highest occurred in the TLR5-flagellin-FA complex.

Peptidoglycans and PGRP were added to confirm the interactions between FA and TLR signaling. Figure 5 shows that FA is bound to the peptidoglycans with hydrogen bonds (Figure 5A). The interaction between the peptidoglycan-FA complex and PGRP (5B) showed that FA blocked the interaction between PGRP and peptidoglycans; thus, only FA bound to PGRP. These results indicate that FA inhibited the recognition of peptidoglycans by PGRP.

FA has antimicrobial activity against various types of bacteria. Previous studies have shown that FA inhibits *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Listeria monocytogenes* [28], *S. epidermidis* [29], *Citrobacter freundii*, *Enterobacter aerogenes*, *E. cloacae* ssp. *Cloacae*, *Klebsiella oxytoca*, *Proteus hauseri*, *P. mirabilis*, and *Salmonella enterica* ssp. *Enterica* [30]. However, there is still a lack of information about the antimicrobial mechanism.

FA was extracted from local purple rice grown in East Java Indonesia and evaluated for its antimicrobial activity in *in vitro* and *in silico* analyses. The *in vitro* study was conducted to observe the inhibition of *S. Typhimurium* and *L. monocytogenes* cells by the PRFAE using SEM. The inhibition of bacterial virulence factors was analyzed *in silico* using the TLRs. The concentrations of the PRFAE used were 250, 500, and 750 mg/mL, which were based on the minimum inhibitory concentration of a FA standard, which ranges from 100 to 1,250 µg/mL [28]. The FA content obtained from the extract using a previous method [23] was 1.90–2.91 mg/g. We obtained a brown extract, indicating the presence of FA. This was supported by previous studies using a specific extraction process to obtain FA, which was indicated by the brown color of the extract [22, 31]. Small amounts of other phenolic acids were also present. The phenolic acids that have been identified in rice include ferulic, coumaric, sinapic, gallic, protocatechuic, p-hydroxybenzoic, vanillic, syringic, caffeic, chlorogenic, cinnamic, and ellagic acids, and the most abundant phenolic acid found in the endosperm, bran, and whole-grain is FA [32].

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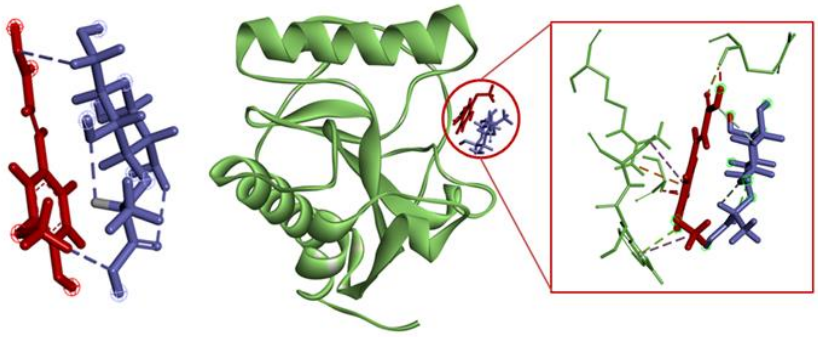

A B

II

Receptor	Ligand	Interactions	Bond Type	Total Energy (kJ/mol)
Peptidoglycan	FA	Lig1, Lig2	Hydrogen	-135.6
PGRP	Peptidoglyca-FA	Gln27, Arg88 Trp69, Cys70 Ala95	Hydrogen Hydrophobic Unvavorable bump	-270.9

Figure 5. Interaction among FA, Peptidoglycans, and PGRP. I. The Conformations of FA, Peptidoglycans, and PGRP Interaction, (A) the Peptidoglycan-FA Complex, (B) the Peptidoglycan-FA Complex Interacted with PGRP. Red Represents FA, Purple is Peptidoglycans, and Green is PGRP. II. Summary Table of Interactions, Bond Types, and Total Energy of the Docking Results

The PRFAE had *in vitro* and *in silico* antimicrobial activities and produced inhibition zones around the bacteria growing on the medium. The antimicrobial activity of the PRFAE against *S. Typhimurium* and *L. monocytogenes* was classified as weak (10–15 mm) based on the diameter of the inhibition zones [33]. Nevertheless, the bacterial cells exposed to the PRFAE revealed several effects, including biofilm formation, shrinkage, the presence of cell debris in S. *Typhimurium*, uneven surfaces, and the formation of pores that caused osmotic lysis in *L. monocytogenes*. These effects could have been caused by direct contact of individual bacterial cells with the PRFAE during preparation for the SEM observations. Biofilms are a type of bacterial defense [34]. Cell shrinkage indicates that the bacteria failed to grow to their maximum size due to antimicrobial effects. The cell debris indicated lysed cells, while the presence of uneven surfaces and pores indicated damage to the bacterial cells [35].

FA also inhibited and attenuated the binding of bacterial virulence factors with TLRs. We have proposed a mechanism for the antimicrobial activity of purple rice FA based on the results shown in Figure 6. FA enters the cell through the TLRs. FA inhibits TLR recognition of the bacterial virulence factors which induces several effects leading to bacterial cell death. Bioactive compounds affect bacterial virulence by competing with the bacteria for binding to cell receptors [13]. Based on this study, administering FA prevents infection by binding to the TLRs; thus, the bacterial virulence factors, which are native ligands of the TLRs, are not recognized. In contrast, when bacteria have infected cells and their virulence factors have been recognized by TLRs, FA attenuated the binding which reduced TLR activation. Failed binding of ligands to the TLRs prevents excessive TLR activation, thus avoiding continuous production of pro-inflammatory cytokines and chemokines, and the development of inflammatory-related diseases [36]. Bacterial virulence factors are a new alternative target for antimicrobial therapy to deactivate bacteria in the host [37] and alleviate host tissue damage [38].

FA also inhibited the binding of peptidoglycans to the PGRP. Peptidoglycans are cell wall components that maintain cell shape, integrity, and viability [39]. The PGRP recognizes the peptidoglycans of the bacterial cell wall [40]. The activation of PGRP is mediated by TLR signaling [41]. This finding also confirms the potential of FA as a bacterial anti-virulence factor. Based on the effects of FA on bacterial cells and its ability to bind with virulence factors, the PRFAE may act on the cell wall and cytoplasmic membrane of the bacteria, leading to loss of membrane integrity and release of cytoplasmic contents. Disrupting membrane pores causes loss of the membrane potential, the rapid release of intracellular components, and cell death [42].

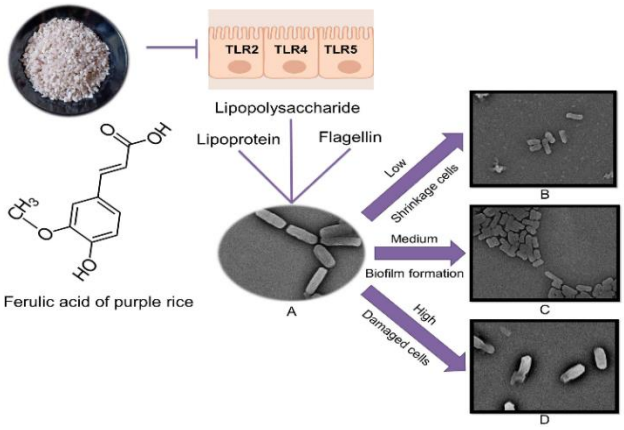

Figure 6. The Inhibition of Bacteria by Purple Rice Ferulic Acid through the Toll-like Receptors. (A) Normal, (B) Shrunk, (C) Biofilm Formation, and (D) Damaged Bacterial Cells at Low, Medium, and High Concentrations of the PRFAE

The PRFAE revealed different actions when inhibiting the growth of *S. Typhimurium* and *L. monocytogenes*, which may have been influenced by differences in the cell wall structure of the two bacteria. *L. monocytogenes*, a Gram-positive bacterium, was damaged and lysed, while the size of *S. Typhimurium*, a Gram-negative bacterium, was stunted but it maintained cell shape at low PRFAE concentrations. This result suggests that the PRFAE was more potent against *L. monocytogenes* than S. *Typhimurium*. The cell membranes of Gram-positive bacteria are weaker than those of Gram-negative bacteria due to the structure of the single peptidoglycan layer [43]. Gram-negative bacteria tend to be more resistant to antimicrobial agents because of the LPS outer membrane, a unique lipid layer in Gram-negative bacteria [44].

The result of broken and disrupted membranes is similar to previous SEM studies that reported destroyed *L. monocytogenes* cells treated with an olive oil polyphenol extract [45] or apple phloretin [46]. However, this result differs from other studies reporting [34] damaged cells and changes in the size of *L. monocytogenes* treated with lactic acid. Pitted, deformed, and broken *Salmonella* cells treated with an *Acacia nilotica* extract have also been reported [47]. Another study [48] reported that lactic acid shrinks *Salmonella* cells. However, the factors affecting cell size were not determined [49].

Another important finding was that the PRFAE induced biofilm formation by *S. Typhimurium*. Biofilms are characterized by adherent and aggregated cells with a clumped structure [26]. Biofilms are microbial communities that attach to and produce a matrix that wraps around the cell [50]. Biofilms are related to bacterial virulence factors, one of which is LPS [51], a component of the outer membrane of Gram-negative bacteria that plays a role in the interaction between the

bacterium and the environment [52]. *S. Typhimurium* is a Gram-negative bacterium [53]; thus, it can form biofilms. The stages of biofilm formation include initial attachment, irreversible attachment, microcolony development by proliferation, biofilm formation, and dispersal [54]. Biofilms form in response to cellular stress [55]. In addition, bacteria coordinate their gene expression, and some use a quorum-sensing system to form biofilms [56]. The biofilm formation by *S. Typhimurium* demonstrates that exposure to the PRFAE was stressful.

Several studies have shown that low concentrations of antibiotics induce the formation of biofilms in various bacterial species [57]. Previous studies have suggested that a sub-minimal inhibitory concentration of antibiotics can induce biofilms to form. The concentration is influenced by the type of antibiotic and the bacterial species [55]. Surprisingly, *S. Typhimurium* formed a biofilm after exposure to 500 mg/mL of the PRFAE. The extract provided an inhibitory effect at 250 mg/mL, but the cells were protected by a biofilm at 500 mg/mL. The highest concentration of 750 mg/mL was required to lyse the bacterial cells. Determining the proper concentration of antimicrobial agent is essential to prevent biofilm formation, thus avoiding antimicrobial resistance.

The findings of this study show that the PRFAE had antimicrobial properties, and thus has important implications for developing purple rice as an antimicrobial agent. The antimicrobial properties of the PRFAE also support an antiaging function through TLR signaling by avoiding recognition of the TLR activator, which prevents inflammation and helps maintain the epithelial cells of the gastrointestinal tract.

Conclusion

The PRFAE had antimicrobial activity against *S. Typhimurium* and *L. monocytogenes*. The TLRs inhibition induced biofilm formation, shrinkage, and pores to form in the bacterial cells that caused cell death. The results of this study indicate that the purple rice FA is a promising antimicrobial agent to prevent infection by pathogenic bacteria.

Acknowledgements

We acknowledge the Indonesia Endowment Fund for Education/LPDP, Ministry of Finance, the Republic of Indonesia for financial support, and partly supported by RISPRO-PRN-LPDP-PAJALE research grant. We thank Dewi Ratih TS and members of the Research Center SMONAGENES, Brawijaya University for the discussions and laboratory facilities.

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