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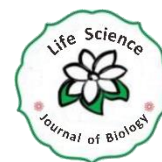

Non-Ribosomal Inhibition of The Antibacterial Compound from Ethanol Extract of *Gnetii gnemon*

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Abstract

Melinjo (Gnetii gnemon) is a plant species originating from Peninsular Malaysia and Indonesia, known that contain several bioactive compounds that can be utilized as antibacterial agents. The aim of this study is to analyze the profile of the melinjo leaf bioactive compound which has an antimicrobial function by inhibiting the growth of S. aureus and activity in ribosomal expression. The profile analysis of the bioactive compound of melinjo leaf was carried out by screening phytochemical study, HPLC, and analysis of antibacterial activity was carried out using turbidimetry method against Bacillus cereus (Bc), Escherichia coli (Ec), and Staphylococcus aureus (Sa). The ethanol extract of melinjo leaves contains tannins, saponins, alkaloids, flavonoids, and triterpenoids, with the highest levels being flavonoids 67,117ppm. Analysis of bacterial growth using OD600 showed relatively lower antibacterial activity compared to the control. The identification of RNA expression showed that the antibacterial compound of melinjo leaf extract did not have inhibition activity through the ribosome pathway it was thought to have an antibacterial mechanism through non-ribosomal inhibition of bacteria. Our findings in this study are that the ethanol extract of Melinjo leaves has antibacterial activity through a non-ribosomal inhibitory mechanism.

Abstrak

Melinjo (*Gnetii gnemonii*) merupakan tanaman yang berasal dari Semenanjung Malaysia dan Indonesia, diketahui mengandung beberapa senyawa bioaktif yang dapat dimanfaatkan sebagai antibakteri. Penelitian ini bertujuan untuk menganalisis profil senyawa bioaktif daun melinjo yang memiliki fungsi antimikroba dengan menghambat pertumbuhan *S. aureus* dan aktivitas ekspresi ribosomal. Analisis profil senyawa bioaktif daun melinjo dilakukan dengan studi skrining fitokimia, HPLC, dan analisis aktivitas antibakteri dilakukan dengan metode turbidimetri terhadap *Bacillus cereus* (*Bc*), *Escherichia coli* (*Ec*), dan *Staphylococcus aureus* (*Sa*). Ekstrak etanol daun melinjo mengandung tanin, saponin, alkaloid, flavonoid, triterpenoid, dengan kadar flavonoid tertinggi yaitu 67,117ppm. Analisis pertumbuhan bakteri menggunakan OD_{600} menunjukkan aktivitas antibakteri ekstrak daun melinjo relatif lebih rendah dibandingkan kontrol. Identifikasi ekspresi RNA menunjukkan bahwa senyawa antibakteri ekstrak daun melinjo tidak memiliki aktivitas penghambatan melalui jalur ribosom diduga memiliki mekanisme antibakteri melalui penghambatan bakteri non ribosom. Temuan kami dalam penelitian ini ekstrak etanol daun Melinjo memiliki aktivitas antibakteri melalui mekanisme non-ribosomal inhibisi.

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INTRODUCTION

Melinjo is one of the *Gnetaceae* family plants originating from the tropics, belongs to the Gnetaceae family, and is widely cultivated in Southeast Asia (Kato *et al.*, 2011). The community generally consumes young leaves as vegetables for daily food and seeds as ingredients for processing melinjo chips. Melinjo leaves contain active compounds such as alkaloids, saponins, steroids, and tannins (Muadifah *et al.*, 2019) The active compound of either melinjo seed and melinjo peel extract can inhibit the growth of *Bacillus cereus Staphillococcus aureus Enterobacter aerogenes, Pseudomonas aeruginosa*, but did not show, and antifungal activity because it could not inhibit *Aspergillus flavus* growth (Parhusip & Sitanggang, 2011). Melinjo generally has a flavonoid content ranging from 0.85 to 3.14 mg quercetin equivalent (QE) g^{-1} sample. The highest free radical scavenging activity was found in roots, 37.27 mg of vitamin C equivalent antioxidant capacity (VCEAC) g^{-1} sample, the antioxidant activity of flavonoids in melinjo was equivalent to the antioxidant activity of vitamin C (Kato *et al.*, 2011).

For Indonesia local food products, melinjo seeds are commonly used as raw material for masing ‘emping’ (traditional cracker in Indonesia) and as supplementary soup material (Parhusip & Sitanggang, 2011). The results of our previous study showed that the methanol extract of melinjo leaves contained alkaloids, flavonoids, triterpenoids, tannins, and saponins (Muadifah *et al.*, 2019). It was reported that, stilbenoids from melinjo showed moderate antimicrobial activity via a diphenyl-picrilhydrazil-hydrate (DPPH) radical scavenging effect, including lipase and α-amylase inhibition activity (Kato *et al.*, 2011). Moreover, ethanol extract of *G. gnemon* fruit potentiated production of Th cytokines such as I L-2 and IFN-γ in PP cells irrespective of Con-A stimulation, whereas, no effect on the production of the Th2 cytokines IL-4 and IL-5 was observed (Barua *et al.*, 2015). Ethanol extract of *G. gnemon* leaves has antibacterial activity against *S. aureus* bacteria with a minimum inhibitory concentration at a concentration of 640 mg/ml (Dayoh *et al.*, 2021).

The activity of melinjo as an antimicrobial is not yet understood in-depth, so an in-depth study of the mechanism of the reaction of antibacterial compounds with receptor targets in the form of ribosomal (Dersch *et al.*, 2017), non-ribosomal or protein synthesis in housekeeping enzymes (Li *et al.*, 2018). Its ability as a natural antimicrobial through melinjo protein can also be used as a natural food. Gg-AMP peptides isolated from melinjo seeds have antibacterial activity against types of Gram-positive and Gram-negative bacteria (Kato *et al.*, 2011). Analysis of the antibacterial activity of melinjo leaves, especially in bacteria that cause infection needs to be known in-depth and developed in the form of extracts, fractions or pharmaceutical preparations. The aims of this research is to analyze the profile of the bioactive compound of melinjo leaf extract through quantitative using HPLC, its activity as a natural antibacterial and its effect on mRNA expression of R50S Ribosomal Gene. The activity of melinjo as an antimicrobial has not been understood in-depth, so an in-depth study of the reaction mechanism of antibacterial compounds with receptor targets, either ribosomal (Dersch *et al.*, 2017), non-ribosomal (Li *et al.*, 2018) or protein synthesis is needed as house-keeping enzymes (Ma *et al.*, 2016). Antibiotics have various inhibitory mechanisms, such as tetracyclines inhibiting translation by preventing aa-tRNA binding to the A site (Jenner *et al.,* 2013). At the same time, the smaller molecule natamycin stabilizes tRNA binding in

the A site, inhibiting translocation and stimulating miscoding (Polikanov *et al.,* 2014). Hence, Erythromycin is a macrolide antibiotic that inhibits mRNA translation and 50S ribosomal subunit assembly in bacterial cells. An essential mechanism of erythromycin resistance is the methylation of 23S rRNA by erm methyl transferase enzymes. A 50S ribosomal subunit formation model suggests that the precursor particle accumulating in Erythromycin-treated cells is the target for methyl transferase activity. Hybridization experiments identified the presence of 23S rRNA in the 50S precursor particle (Pokkunuri & Champney, 2007).

METHODS

Materials used were leaf extracts of melinjo, H_2O, Et-OH 70% (Sigma Aldrich), glacial acetic acid, HCl (p), Mg, $FeCl_3$ 1%, H_2SO_4 (p), $CHCl_3$, Met-OH, Dragendroff Reagents, Mayer reagents, nutrient agar (NA), nutrient broth (NB), clindamycin (Merck), aluminum foil, bacterial strain of *S. aureus* (Sa), *B. subtitulis* (Bc), *E. coli* (Ec), Glycerin, propylenglycol, methylparaben, phenolphthalein, liquid paraffin, KOH (Sigma Aldrich), Ribosomal Primer sets (ILT1, ILT2, ILT3, ILT,4, ILT5, and ILT6), RNA extraction KIT, PCR Reaction KIT, and agarose gel (1%). The tools used in this study were blenders (Toshiba), horn spoons, maserators, analytical scales (Mettler Toledo AL204®), stainless spoons, ovens (Memmert UP400), potentiate crucifixes, filter paper, tweezers, laminar air flow (LAF) cabinet, Autoclave (HL 36Ae), Spectrofotometry UV-Vis, qPCR, electrophoresis, Ose Needles, Micropipette (Rainin E1019705K), pH meter (Hanna), Bunsen burners, and general glass tools (Pyrex).
This primer serves to read and amplify the target gene.

Table 1. Primer Set for complementary Ribosome 50S

Primer	Ret. Time	Area
ILT1	TTC AAC AAT TTA ATG TTT AAG	*5' PCR Primer (+20bp) complementary to nucleotide +20 to 120 of 50S Ribosomal Gene of rplK S. aureus*
ILT2	TTG TAG TAC CCT AAG ACA TTT	*3' PCR Primer (+120) complementary to nucleotide +20 to 120 of 50S Ribosomal Gene of rplK S. aureus*
ILT3	GGA TAC AGT TCG ACG TCC AAC	*5' PCR Primer (+20) complementary to nucleotide +20 to 120 of 50S Ribosomal Gene of rplK E. coli*
ILT4	TTG TAG TAC CTT AAG ACG TTT	*3' Primer (+120) complementary to nucleotide +20 to 120 of 50S Ribosomal Gene of rplK E. coli*
ILT5	TTC AAC ATT TTT ATG TCG ATG	*5' Primer (+20) complementary to nucleotide +20 to 120 of 50S Ribosomal Gene of rplK B. cereus*
ILT6	TTA TAA TAC TAC CAC ACA TTC	*3' Primer (+120) complementary to nucleotide +20 to 120 of 50S Ribosomal Gene of rplK B. cereus*

(*www.NCBI.com)*

Preparation and Extraction

Gnetum gnemon leaves were taken from Tulungagung area, East Java from community gardens, cleaned, simplified, dried, and mashed using a skid machine to be filtered using Whatman paper to obtain simplicia. The simplicia were then extracted by maceration method using 80% methanol for 4 days. The extract is thickened by vaporizing the solvent to produce the extract (Muadifah *et al.*, 2019).

Qualytative and Quantitative Analysis of Secondary Metabolite

Phytochemical tests were carried out to determine the content of secondary metabolites in ethanolic extracts of melinjo leaves such as tannins, flavonoids, alkaloids, polyphenols, and steroids using phytochemical screening reagents (Rizal *et al.*, 2018), which following previous studies, which included: tannin and polyphenol screening using $FeCl_3$ solution, flavonoid screening using Shinoda reagent, alkaloid test using Dragendorff reagent, while steroid test using anhydrous CH_3COOH and H_2SO_4. A positive test is indicated by the formation of a specific color from each compound. Quantitative analysis was carried out using HPLC, at the UIN Malang Chemical Laboratory. Samples (concentrated extract) of melinjo leaves are injected into the HPLC machine with the analysis conditions: column: C18; V. injection = 2 μL; movable = acetonitrile: potassium dihydrogen phosphate in water pH 3 (10:90); flow rate = 1 mL / minute; λ = 210 nm (Tarigan *et al.*, 2020).

Antibacterial Activity

The antibacterial activity of the extract using the Turbidity method. As much as 25 ml of Nutrient Agar (NA) media was mixed with 25 L of the appropriate test bacterial suspension, dengan menggunakan konsentrasi 80% ethanol extract of melinjo leaves following our previous studied (Muadifah *et al.*, 2019) The antibacterial activity of extracts was tested to determine the ability of a bioactive compound to inhibit bacterial growth. Two types of bacteria were used *B. subtilis* (*Bs*), *E. coli* (*Ec*), and *S. aureus* (*Sa*) which were cultured in NB media then calculated OD(600nm) then perform in UV-Vis Spectrophotometry (Balouiri *et al.*, 2016). Bacteria were cultured at 37^0C for 3 hour and OD measurements were carried out at 1, 2, 3 hours incubation. The positive control used chloramphenicol, while the negative control used DMSO. We used different extract concentrations 10%, 15%, and 25% (Chalke *et al.*, 2016).

Bacterial RNA Isolation

Harvest bacterial cultured after 5hr in eppendoft, then extract bacterial RNA using RNA extraction kit. The Total RNA Mini Kit Bacterial Cultured was designed for total RNA purification from fresh cultured cells. Detergents and chaotropic salt are used to lyse cells and inactivate RNase while RNA is bound by the glass fiber matrix of the RNA spin column. Once any contaminants have been removed, using the Wash Buffer (containing ethanol), the purified total RNA is eluted by RNase-free Water and ready for use in various downstream applications (Shu *et al.*, 2014; Wu *et al.*, 2013).

Bacterial 50S Ribosome Genetic Expression

First Complementary DNA Synthesis (cDNA). The first cDNA thread was synthesized using the Super ScriptTM First-Strand cDNA Synthesis kit. The total reaction volume used was 20 μL. Into a sterile micro tube 2 μg RNA, 1 μL dNTPs 10 mM and 1 μL oligo (dT) 12-18 10 pmol / μL and sterile MFW (molecular free water) up to 10 μL are then incubated at 65°C for 5 minutes. After that, the mixture was immediately put into ice and added with 2μL of 10x RT buffer, 4μL of 25mM MgCl2, 2μL of DTM 0.1M and 1μL of RNaseOUTTM Recombinant RNase Inhibitor. After that the mixture was incubated at 42°C for 2 minutes, and the reaction mixture was added with Super ScriptTM II reverse transcptase, then incubated for 50 minutes. After the reaction is complete, the enzyme inactivation is carried out by incubating the mixture at 70°C for 15 minutes. RNaseH was added to the reaction mixture to remove

RNA, followed by incubation at 37ºC for 20 minutes and finally the temperature was conditioned at 10ºC. Amplification of Target Gene Fragments with Specific Primers. The PCR reaction was carried out with a total volume of 30 µl consisting of 600ng first strand cDNA (first strand cDNA) as molds, primary pairs of ILT1 and ILT2 each with a concentration of 10 mM as much as 1 µl; 3 µl 10x PCR buffer; 0,6 µl dNTPs 10 mM; 0,3 µl Taq DNA polymerase 5 units/ µl and NFW (nuclease free water) until it reaches a volume of 30 µl. The PCR program consists of pre-denaturation at 94ºC for 30 seconds followed by a specific cycle consisting of denaturation at 94ºC for 30 seconds, annealing temperature corresponding to 45 seconds, and elongation at 72ºC for 1 minute 30 seconds. PCR results were checked by electrophoresis on 1.2% agarose gel. EF primers are used as controls. Each primer has a different temperature and number of cycles. Quantification of RT-PCR Results. Electrophoresis results from PCR were analyzed for the expression quantity of each gene using UnScan gel IT software (Chien *et al.*, 2015; Wu *et al.*, 2013).

RESULT AND DISCUSSION

Qualitative and Quantitative Analysis of Secondary Metabolites of *G. gnemon* Leaves

Gnetum gnemon leaves extract has secondary metabolites, tannins, saponins, alkaloids, flavonoids, and triterpenoids (Muadifah *et al.*, 2019) but further analysis is needed to determine the levels of each of these compounds. HPLC was running with specific conditions: column: C18; V. injection = 2 µL; movable = acetonitrile: potassium dihydrogen phosphate in water pH 3 (10:90); flow rate = 1 mL / minute; λ = 210 nm (Tarigan *et al.*, 2020). The results of the analysis can be seen in Figure 1 and Table 1. Previous study was reported that several bioactive compounds are found in *G. gnetum* seeds such as saponins, tannins, and flavonoids (Kato *et al.*, 2011).

Table 2. The profile of secondary metabolites of *G. gnemon* leaves extract

Peaks	Ret. Time	Area	Conc (ppm)	Compound	Screening
1	1.514	354015	-	-	-
2	2.758	676338	1.057	Tannins	+
3	3.865	990785	37.391	Saponins	+
4	6.132	629173	-	-	-
5	6.998	598629	4.074	Alkaloids	+
6	7.249	851363	67.117	Flavonoids	+
7	9.074	1963805	-	-	-
8	10.492	767138	30.435	Triterpenoids	+
9	12.021	268512	-	-	-
10	13.111	751386	-	-	-

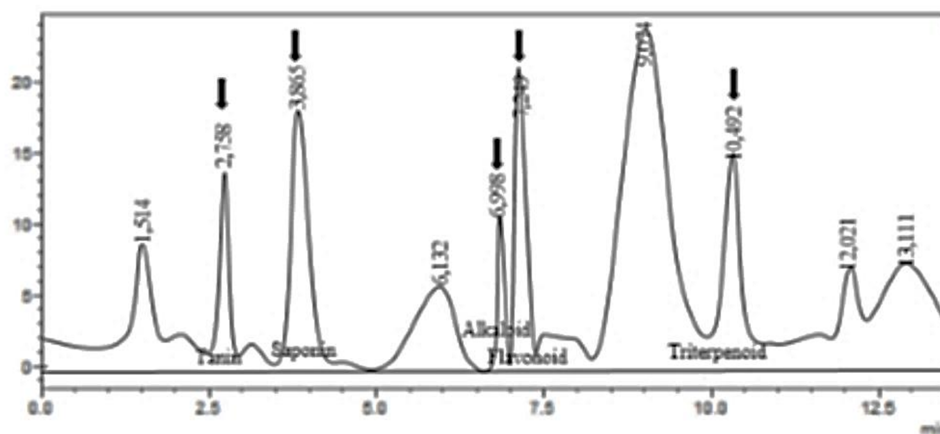
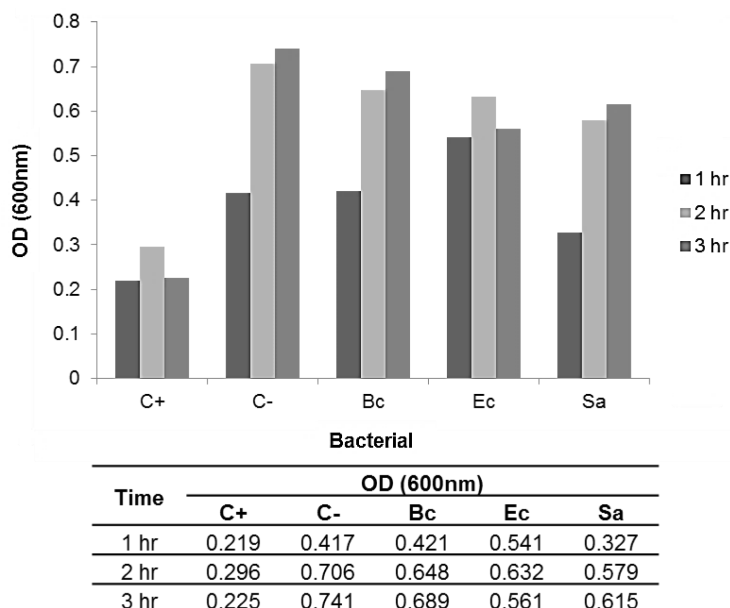


Figure 1. HPLC Chromatogram of Melinjo Leaf Extract

Antibacterial Activity

To identify the antibacterial activity of melinjo leaf extract against bacterial growth, we cultured three *Bs, Ec*, and *Sa* bacteria by adding 80% melinjo leaf extract, and measured Optical Density (OD600) at 1-3 hours of culture (Chasanah *et al.*, 2015). OD results (Figure 2) show that antibacterial activity tends to reduce OD values in *Bs, Ec*, and *Sa* bacteria. *G. gnetum* leaves antibacterial effectiveness in inhibiting the growth of these three bacteria is relatively very small. Our result shown that the larger the absorbance in *Bc*, the smaller the *Ec* and *Sa*. Unfortunately, *Ec* is belongs to gram-negative bacteria with different cell walls, while *Bs* and *Sa* are gram-positive. However, the absorbance of *Ec* at the 3rd hour was getting smaller, from 0.632 to 0.56, this showed that the longer the incubation time of the extract against bacterial inoculation, the more bacteria died. Antibacterial activity can be seen from the OD value in all bacteria which is relatively smaller than the positive control. The best antibacterial activity of melinjo extract against *Sa* with an OD value of 0.615 at 3 days incubation. Our previous study using the disc-diffusion method showed that 80% methanol extract of melinjo leaves can inhibit *S. aureus* bacterial growth with the largest inhibition zone of 13.08 mm (Muadifah *et al.*, 2019). In this research, we conduct antibacterial activity based on turbidity resulting from light scattering by bacteria. This method quantifies bacterial growth by measuring the optical density at 600 nm (OD600), which is mainly used as a quick and affordable method to monitor the growth of bacteria during their culture in liquid media but has also been applied for testing antibacterial properties of nanostructures (Haase *et al.*, 2017).



Time	OD (600nm)				
	C+	C-	Bc	Ec	Sa
1 hr	0.219	0.417	0.421	0.541	0.327
2 hr	0.296	0.706	0.648	0.632	0.579
3 hr	0.225	0.741	0.689	0.561	0.615

Figure 2. Antibacterial activity Melinjo Leaves Extract agains *Bc*, *Ec*, and *Sa*

RNA Expression

In this research we analyzed gene expression using PCR, a primer is needed to remove specific 50S Ribosomes from three bacteria, ILT1-ILT2 (*S. aureus*), ILT3-ILT4 (*E. coli*), and ILT5-ILT6 (*B. cereus*). Basically, there are many of methods that can be used to analyze the antibacterial activity of a drug compound. Antimicrobial activity assay can be developed to found of a drug compounds, epidemiology and predict outcomes of therapy. Pathogenic bacteria and microorganisms can infect human body tissues by destroying and damaging cell function (Nash *et al.*, 2015). Antibacterial has a way to kill various bacteria, ranging from disrupting cell membranes that play a role in producing energy in the form of ATP (Wang *et al.*, 2017), disrupting cell walls, inhibiting the synthesis and metabolism of biological compounds both proteins, or enzymes that play a role in survival cells, and inhibits nucleic acid synthesis through replication blockages (Donadio *et al.*, 2021; Lobritz *et al.*, 2015). Generally referred to as ribosomal and nonribosomal antibacterial activity inhibition (Kapoor *et al.*, 2017; MacGowan *et al.*, 2016).

We succeeded in extracting the RNA of bacteria *Bs*, *Ec*, and *Sa* that incubated using methanol extract of Melinjo leaves, concentrations of 1.71; 3.8, 1.73 (x40ng/µl) RNA, and control 0.13 (x40ng/µl) using clindamycin against *Sa*. Furthermore, the reverse transcript was reversed to produce complementary DNA (cDNA), and the resulting cDNA expression was relatively the same. In this study an initial analysis was carried out whether the antibacterial compounds from melinjo leaves have antibacterial activity through the mechanism of ribosomal and nonribosomal activity. To identify this mechanism, complete isolation of RNA from bacteria that has been cultured, then synthesized cDNa as a gene library. The resulting cDNA was used as a template for the bacterial Ribosome 50S genetic expression test with primers ILT1, ILT2, ILT3, ILT4, ILT5, and ILT6 that had been previously designed. PCR results (Figure 4) were compartable with *Bs, Ec***,** and *Sa* bacteria. The results of Ribosome 50S RNA expression (Figure 2) from bacteria showed that all PCR gene R50S results were well-expressed, relatively

no genetic expression was affected by the effect of melinjo leaf extract antibacterial. This shows that the possibility of antibacterial compounds of melinjo leaf extract has an inhibition mechanism that does not pass through the ribosomal (non-ribosomal) inhibition pathway (Figure 3).

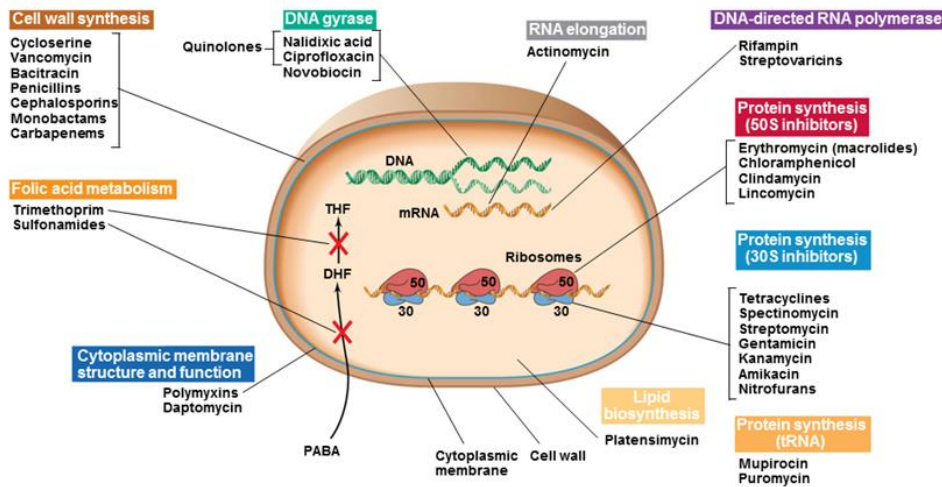
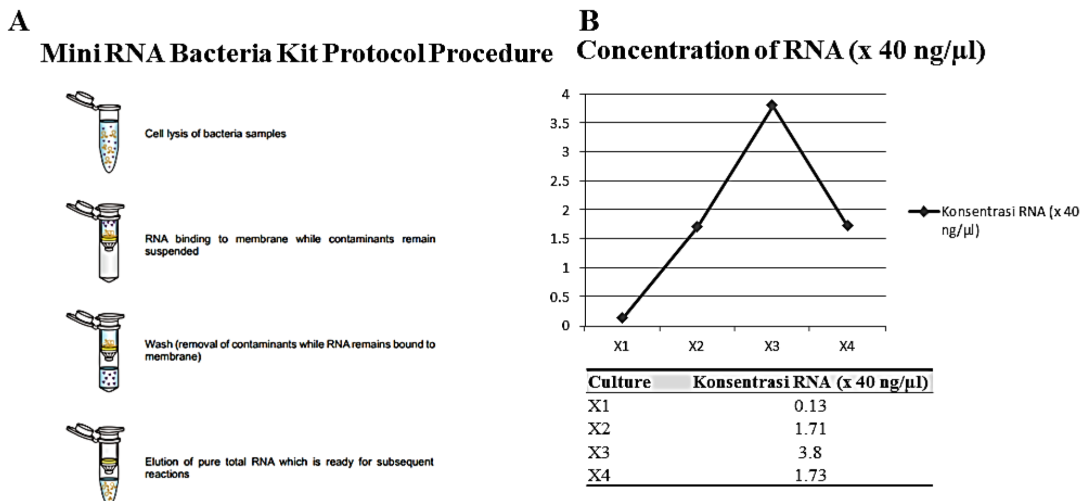


Figure 3. Mechanism of antibacterial agent activity in cellular organism (MacGowan *et al.*, 2016)

Our understanding of how antibiotics induce bacterial cell death is much more essential to know drug-terget interaction. Antibiotics classified into bactericidal drugs (induced cell death) or merely inhibit cell growth. Currently, most of antibiotics possess bactericidal antimicrobials by inhibit cell wall, DNA, RNA, or protein synthesis (Kohanski *et al.*, 2010). Ribosomes become one of the targets of drug interactions. The bacterial 70S ribosome is composed of two ribonucleoprotein subunits, the 30S and 50S subunits either 30S or 50S ribosomes (Kapoor *et al.*, 2017) . Some drugs that interact with 30S Ribosomes are aminoglycosides, tetracyclines, while macrolides, streptogramins, and phenicols against 50S ribosomes (Katz & Ashley, 2005; Kohanski *et al.*, 2010; Patel *et al.*, 2001). Moreover, some antibiotics such as erythromycin, clindamycin, lincomycin, chloramphenicol, linezolid etc. have been shown to be among the 50S ribosome inhibitors (MacGowan *et al.*, 2016).



Culture	Konsentrasi RNA (x 40 ng/µl)
X1	0.13
X2	1.71
X3	3.8
X4	1.73

Figure 4. Gene Expression, (A) RNA Bacterial Kit Protocol Procedure, (B) RNA concentration was measured using UV-Vis Spectrofotometry at 280nm

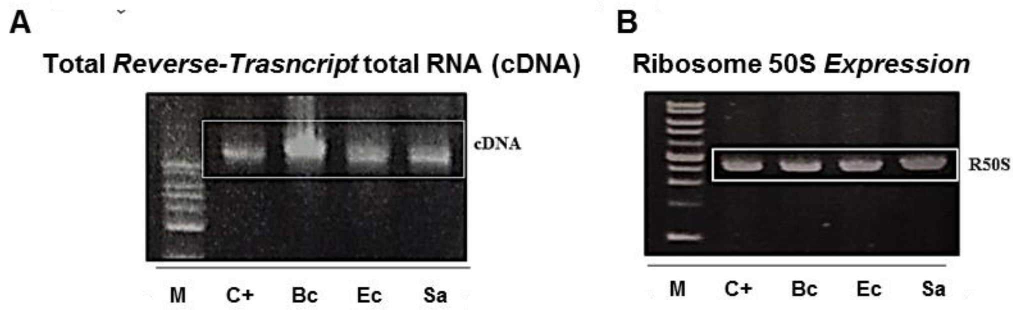
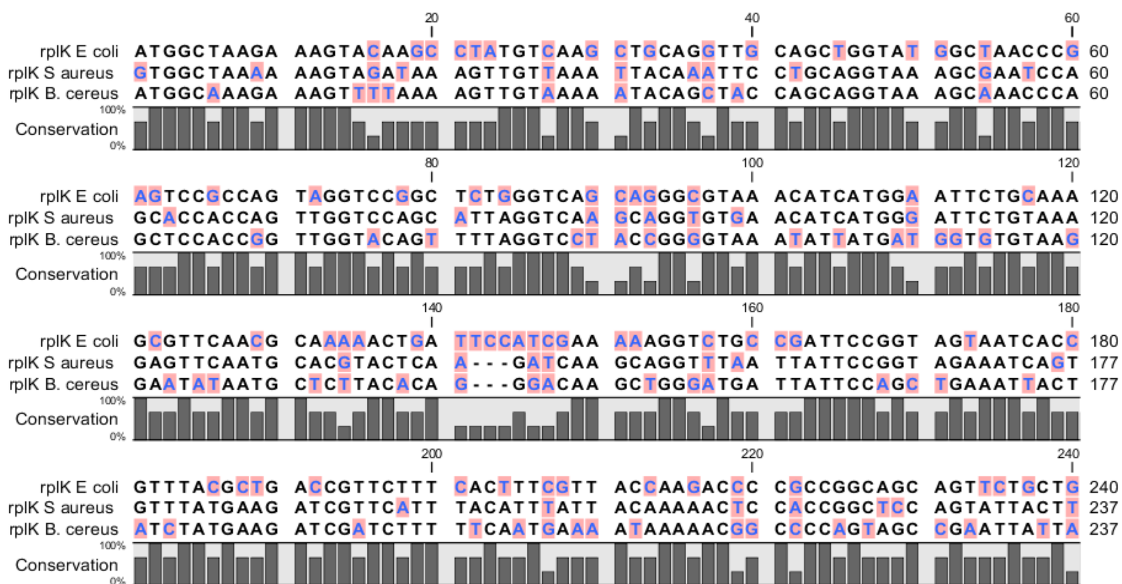


Figure 5. Gene Expression; Synthesis of cDNA using reverse transcript (RT)-PCR (A), Gene expression of 50S Ribosomes (B)

The bacteria used in this study were *Bc, Ec,* and *Sa*. These three bacteria show the mechanism of interaction with antibacterial compounds which are almost the same melinjo leaves, have a relatively similar expression pattern. To analyze the similarity and kinship of the Ribosome 50S gene, sequence alignment is performed by comparing the three genes of the bacteria. B. cereus and S. aureus are gram-positive bacteria, whereas E. coli are gram-negative bacteria, possibly having relatively different sequences. Alignment results (Figure 4) show that the R50S gene from the three bacteria does not conserve; the conservation level is only around 60%. The result raises an interesting question: Why do these three bacteria have the same R50S interaction with the antibacterial extract of melinjo leaf? However, the similarity of R50S is very low. Our supposed all bacteria have a similar pathway in antibacterial inhibition mechanism, by non-ribosomal inhibition, supposed to be both of membrane or cell wall inhibition aligns (Dharuman *et al.*, 2021).



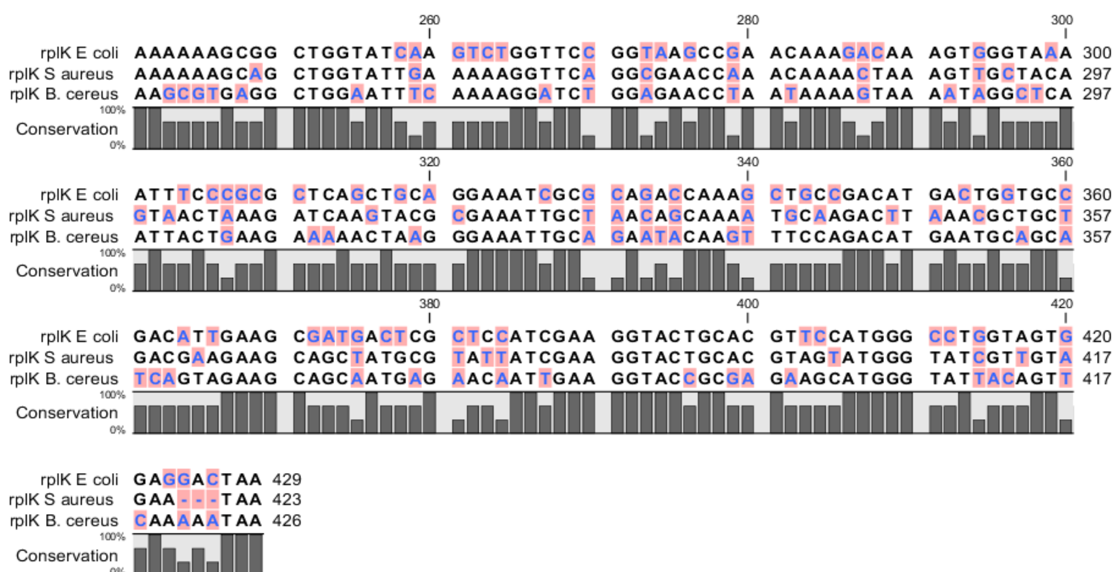


Figure 6. Sequence aligment of gene R50S of *B. cereus, E. coli*, and *S. aureus*

CONCLUSION

The methanol extract of melinjo leaves contains tannins, saponins, alkaloids, flavonoids, triterpenoids, with the highest levels being flavonoids 67,117 ppm. Analysis of bacterial growth using OD600 showed relatively lower antibacterial activity compared to control. The identification of Ribosomal RNA gene expression showed that the antibacterial compound of melinjo leaf extract did not have inhibition activity through the ribosome pathway it was thought to have an antibacterial mechanism through non-ribosomal inhibition of bacteria.

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