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Uncaria gambier Roxb flavonoid extraction, antibacterial activity assessment against Escherichia coli, and flavonoid bio-nanoparticle preparation

Suparno Suparno[1*], Ariswan Ariswan[2], Heru Kuswanto[3], Eka Sentia Ayu Lestari[4], Deby Grace[5]

[1,2,3,4,5]*Physics Education Department, Faculty of Mathematics and Science, Universitas Negeri Yogyakarta, Indonesia.*

*Corresponding author: Suparno Suparno (*Email: suparno_mipa@uny.ac.id*)

Abstract

Escherichia coli (E. coli) was reported to cause 1.7 billion diarrhea cases per year in Indonesia, with a population of 275 million projected for 2024. This study reports the successful use of natural flavonoids from the medicinal plant Uncaria gambier Roxb (UGR) against E. coli. The flavonoid extraction was performed using the decoction method on small pieces of very hard UGR stem to replicate the traditional method, which has been proven effective for generations. The flavonoid content was determined using the aluminum chloride colorimetric assay and visible spectrophotometry. The antibacterial assessment was carried out using the Kirby-Bauer method. Furthermore, bio-nanoparticles were created using filtration, centrifugation, and subsequent characterization using the dynamic light scattering technique. The results showed that the flavonoid content in UGR was (31.55 ± 0.29) mg QE/g. By using the prediction curve and employing the same concentration unit, namely %, the effectiveness of 5% chloramphenicol was found to be the same as that of 0.0225% flavonoids. E. coli showed resistance to 5% chloramphenicol after 39 hours, which continued to decrease slowly, as indicated by the reduction in clear zone diameter. However, it did not show resistance against the flavonoid at all concentrations up to the last measurement at 75 hours. These findings suggest that UGR can be a promising source of natural flavonoids that have been proven to be efficacious in combating E. coli.

Keywords: Bacterial resistance, Chloramphenicol, Decoction, Diarrhea, Escherichia coli, Flavonoid, Kirby-Bauer method.

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1. Introduction

The number of reported cases of childhood diarrhea in Indonesia reaches 1.7 billion per year, and most of them are caused by *Escherichia coli* (*E. coli*). The death rate reaches approximately 525,000 children under 2 years of age [1]. *E. coli* has been observed to develop resistance to many antibiotics [2-5]. This resistance appears to develop over time. Chirila and colleagues, in their research involving 175 pathogenic isolates of *E. coli* during 1980-2016, found that 87% of isolates were resistant to tetracyclines and sulfonamides [6]. Marin et al., in his investigation of 403 *E. coli* isolates taken from healthy people in France during 1980-2010, showed that the increase in resistance among 152 isolates belonging to the sequence type was largely due to an increase in gene frequency, while the increase in virulence was due to an increase in gene frequency and clonal expansion [7]. Their findings showed that fighting against *E. coli* is extremely challenging.

E. coli is a cylindrical-shaped bacterium commonly found in the human gastrointestinal tract. These bacteria have spread

worldwide and have developed numerous strains. Some of these strains are pathogenic [8, 9] and can cause serious illnesses such as hemorrhagic colitis, which is severe watery diarrhea accompanied by sudden stomach cramps and possible vomiting. E. coli forms biofilms to trap food, defend against immune cells, protect against antibacterial agents, and spread virulence [10]. These Gram-negative bacteria are protected by double-layered membranes, with a cell wall located between these membranes as shown in Figure 1. Penetrating the double protection of *E. coli* is extremely challenging. Therefore, there is a pressing need for more extensive research to develop stronger antibacterial agents to fight *E. coli* [11]. This is the reason why we chose *E. coli* for this research, apart from the fact that it causes billions of infections and kills thousands of children per year. One effective approach to combat multi-resistant bacteria is to explore antibacterial agents based on traditional medicine, which have shown efficacy over decades.

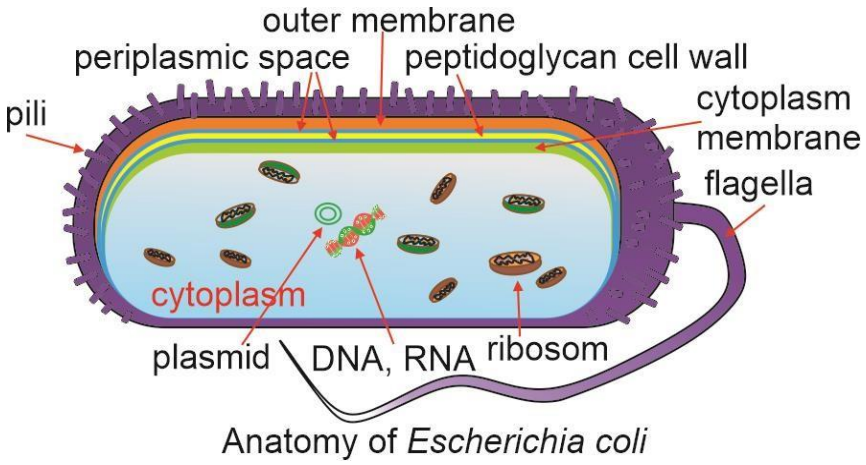


Figure 1.
Anatomy of Gram-negative *Escherichia coli*: the bacterial envelope consists of a cytoplasmic membrane (green), a cell wall (yellow) between two periplasmic spaces (blue), and an outer membrane (orange). Cytoplasm (sky blue) with many organelles, pili, and flagella (purple).

The Dayak people native to the island of Kalimantan, Indonesia, have used *Uncaria gambier Roxb* (UGR), locally known as *Bajakah Kalalawit*, to effectively cure diarrhea. They boiled the chopped stems of UGR in water by half and drank a cup of the extract twice a day for a period of two to three days. This inspired us to investigate the flavonoid content of UGR extract because flavonoids have relatively strong antibacterial properties. Our previous investigations on a similar medicinal plant (*Bajakah Tampala*) showed relatively strong antifungal activity against Malassezia globosa and antibacterial activity against Bacillus subtilis [12, 13]. The effectiveness of flavonoids in fighting Gram-negative bacteria is depicted in Figure 2, which shows ten antibacterial mechanisms against *E. coli*. These ten mechanisms of action can work simultaneously, which makes flavonoids superior to existing antibiotics. A certain antibiotic generally only targets one organelle or one function of bacteria. Therefore, it is important to explore flavonoids extracted from UGR as antibacterial agents against E. coli to find antibiotic raw materials that are effective against multidrug-resistant bacteria.

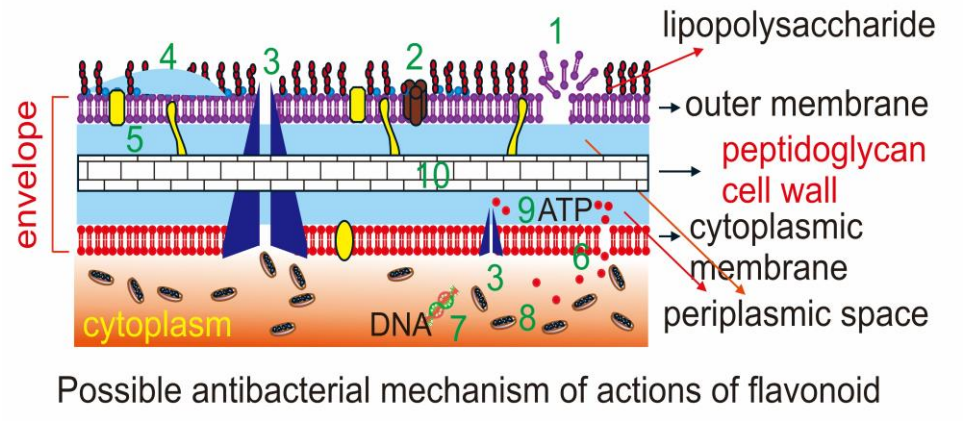


Figure 2.
Ten possible antibacterial mechanisms of flavonoid against *E. coli*: 1. membrane ruptures [14, 15], 2. porin inhibition [16, 17], 3. efflux pump inhibition [18, 19] 4. biofilm formation inhibition [20], 5. nucleic acid synthesis inhibition [21], 6. cytoplasmic membrane function disruption [22] , 7. DNA replication inhibition [23], 8. energy metabolism disruption [24], 9. electron transport inhibition and adenosine triphosphate (ATP) production disruption [25] 10. peptidoglycan synthesis inhibition [26]. Yellow cylinders in different shapes are protein. The red dots represent electron transports through cytoplasmic membrane via small pores or efflux pump.

In this paper, we present a comprehensive methodology for the extraction of flavonoids from UGR utilizing the decoction method. The total flavonoid content was determined through the aluminum chloride colorimetric assay [27] using a visible

spectrophotometer [28]. We compare the concentration of total flavonoids extracted from UGR with several other medicinal plants from Indonesia [29], India [30], and China [31]. Our study not only examines the efficacy of flavonoids as antibacterial agents against *E. coli* using the Kirby-Bauer disk diffusion method [32] but also compares their effectiveness with Chloramphenicol. *E. coli* developed strong resistance to Chloramphenicol starting at 39 hours. In contrast, *E. coli* did not show any resistance to flavonoids until the last observation at 75 hours. In addition, the preparation of flavonoid bio-nanoparticles was explained to open opportunities to produce physical and chemical antibiotics in a single product. All these findings promise valuable insights into the potential of flavonoids as natural antibacterial agents [33].

Flavonoids are polymers with the basic structure of flavan-3-ol monomers enriched with many aromatic and hydroxyl functional groups. The size of this polymer increases with the increase in flavonoid concentration. As the size of the flavonoid polymer increases, it becomes difficult for flavonoids to diffuse into the cytoplasm through outer membrane pores. This causes many flavonoid mechanisms of action to not be performed until the outer membrane is damaged. The tiny flavonoid bio-nanoparticles diffuse through porins, allowing many antibacterial mechanisms of action to be performed simultaneously, making them more efficient in killing bacteria. Therefore, we initiated the production of flavonoid bio-nanoparticles as additional work.

2. Materials and Methods

2.1. Sample Preparation

The main material, UGR, was purchased from Landak Regency, West Kalimantan Province, on the Indonesian island of Borneo. The decoction process involves boiling small pieces of the UGR stem in water to release their flavonoids and suspending the flavonoids in the water [34]. This method was selected to replicate the traditional medicine preparation of the Dayak people, which has been proven effective in fighting against E. coli, the bacteria that causes diarrhea. To begin, 50 grams of UGR were combined with 500 ml of water in a one-liter glass beaker and heated until boiling, reducing the water volume to 250 ml—a process that took approximately 52 minutes to halve the initial volume. The larger pieces of UGR were then removed, and the remaining solution was filtered using a coffee filter, resulting in a brownish UGR extract. Figure 3a shows small pieces of UGR stem that are ready for decoction, and Figure 3b shows the UGR extract containing 10 ppm, 20 ppm, and 30 ppm flavonoids.

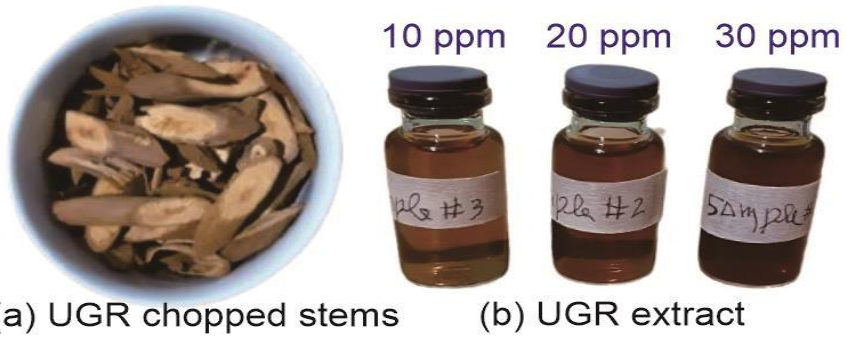


Figure 3.
(a) small pieces of UGR, (b) extract UGR containing (10-30) ppm flavonoid.

2.2. Flavonoid Concentration Determination

The $AlCl_3$ colorimetric assay was used to determine the concentration of flavonoids [35]. To prepare the sample, 0.5 mL of UGR extract was diluted in 4.5 mL of ethanol (dilution factor $D_F = 10$). Quercetin powder from Sigma Aldrich was used to prepare five different concentrations of quercetin samples: 6 ppm, 8 ppm, 10 ppm, 12 ppm, and 14 ppm. To determine the absorbances of the quercetin samples, 0.1 mL of 10% aluminum chloride ($AlCl_3$), 0.1 mL of 1 M potassium acetate (CH_3COOK), 1.5 mL of ethanol, and 2.8 mL of water were added to 0.5 mL of each quercetin sample. The same amount of aluminum chloride assay was added to the diluted flavonoid sample to determine its absorbance. These five quercetin samples were incubated for 30 minutes at 25°C [36]. The absorbance measurements were performed using a visible spectrophotometer by scanning between 400 and 800 nm, and the absorption peak was found at 435 nm. The results were used to draw a calibration curve, and the linear regression equation was found to be $Y = 0.0423X + 0.034$ with $R^2 = 0.9974$. This linear regression equation was then used to calculate the concentration of the diluted flavonoid. The total flavonoid concentration of the UGR extract was calculated using Equation 1:

$$C_T = \frac{C\,VD_F}{m} \qquad (1)$$

Where C_T represents total flavonoid concentration, C denotes the concentration of the diluted flavonoid sample, V stands for volume, D_F is the dilution factor, and *m* is the mass.

2.3. Flavonoid Bio-Nanoparticle Preparation and Size Measurement

Flavonoid bio-nanoparticles are different from the green synthesis of metal nanoparticles. We believe that green synthesis reduces the efficacy of metal nanoparticles since the coating causes metal nanoparticles to release their “magic bullet” metal ions. Flavonoid bio-nanoparticles are biopolymer flavonoids that are made as small as nanoparticles through a series of filtrations and centrifugations of pure flavonoid. To prepare flavonoid bio-nanoparticles, the flavonoid extract

undergoes a series of filtration processes using 0.22-micrometer Millipore filters on a syringe. Subsequently, the filtered extract is centrifuged at a speed of 4000 rpm three times for 10 minutes each to separate large particles from the supernatant [37]. The supernatant is then transferred from the centrifuge tube to a cuvette using a pipette. This must be done gently since the sediment is easy to break upon syringe suction. Particle size determination is conducted using the dynamic light scattering (DLS) technique [38], and the result is presented in Figure 4. More detailed results are presented as supplementary material (Figure S1).

2.4. Antibacterial Observation

Prior to the experiment, nutrient broth (NB), a liquid medium that is nutrient-rich and conducive to E. coli growth, and nutrient agar, which is a solid medium used to test antibacterial agents, had to be prepared [39]. The inhibition of flavonoids was observed in a petri dish containing nutrient agar rich with E. coli (Migula) Castellani and Chalmers ATCC 11229 strain AMC 198, where 5 impregnated disks were placed in one petri dish. Three of these disks were impregnated with flavonoids (10 ppm, 20 ppm, and 30 ppm), one disk was impregnated with water as a negative control, and another disk with 5% chloramphenicol as a positive control. The clear zone diameter representing antibacterial agent efficacy around the disks was measured 3 times at different positions [40]: horizontally, vertically, and diagonally. Measurements were taken every 3 hours over a period of 75 hours.

2.5. Statistical Analysis

The measurement of physical quantities was carried out three times each, and the presented data were the average of these three measurements, except for the DLS data, which were averaged from five measurements performed by the software. The clear zone diameter data of four groups of antibacterial agents were compared to one another by performing t-test analysis: two samples assuming unequal variances. The comparisons were made between 5% chloramphenicol and 30 ppm flavonoid, between 5% chloramphenicol and 20 ppm flavonoid, between 5% chloramphenicol and 10 ppm flavonoid, between 30 ppm flavonoid and 20 ppm flavonoid, between 30 ppm flavonoid and 10 ppm flavonoid, and between 20 ppm flavonoid and 10 ppm flavonoid. The null hypothesis for the comparison of 5% chloramphenicol and 30 ppm flavonoid, Ho: there is no significant difference in the clear zone diameter between 5% chloramphenicol and 30 ppm flavonoid. The alternative hypothesis (Ha): there is a significant difference in the clear zone diameter between the two groups. Both hypotheses hold for all six comparisons above, resulting in six two-tailed p-values from the t-test analysis presented in Table 2. More detailed statistical analysis is presented as supplementary material (Figure S2).

3. Results

This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn.

3.1. The Result of Total Flavonoid Concentration Calculation

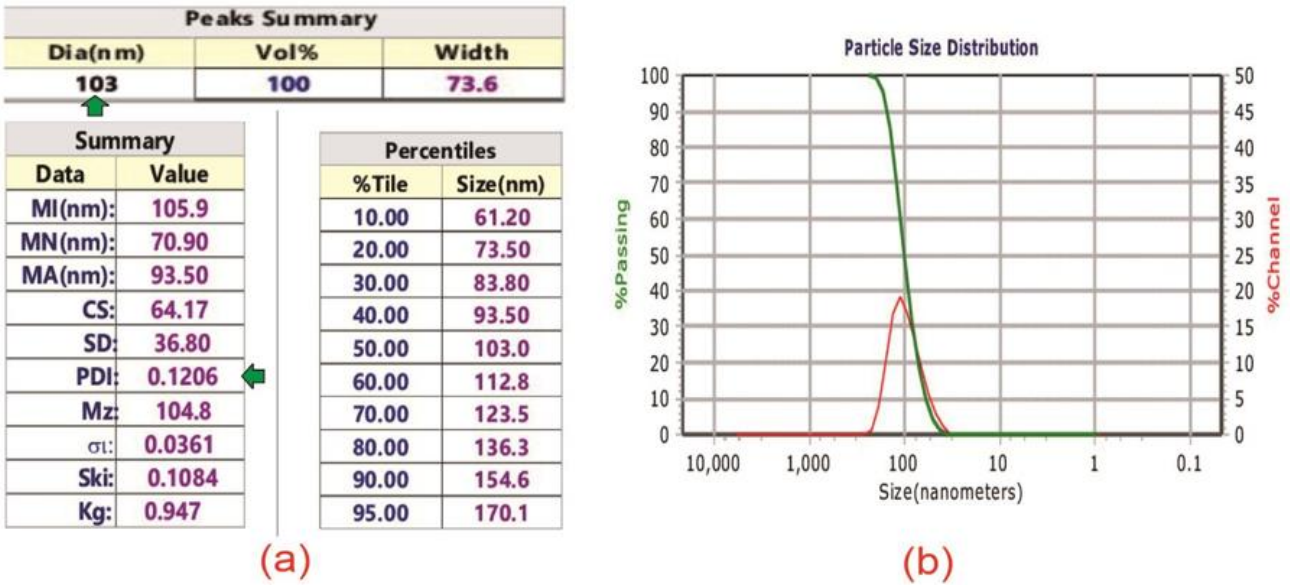
The absorbance measurements of the diluted flavonoid sample using a visible spectrophotometer were conducted three times, and the results are presented in Column 2 of Table 1. The calculations of the diluted flavonoid concentrations using the linear regression equation based on these absorbances are presented in Table 1, Column 3. These three calculated diluted flavonoid concentrations were then utilized to compute the total flavonoid content using Equation 1. The resulting total flavonoid contents are provided in Column 7 of Table 1. The average and standard deviation of these total flavonoid contents are presented in the last column of Table 1. The total content of flavonoid of UGR extract was (31.55 ± 0.29) mg QE/g.

Table 1.
Total flavonoid concentration of *Uncaria gambier Roxb.*

Name	Abs.	Conc. (µg QE/ml)	Mass (g)	Vol. (ml)	D_F (x)	Conc. (mg QE/g)	Avg. Conc. (mg QE/g)
UGR	0.393	8.486998	0.0135	5	10	31.43332	31.55 ±0.29
	0.399	8.628842	0.0135	5	10	31.95867	
	0.391	8.439716	0.0135	5	10	31.25821	

3.2. Flavonoid Bio-Nanoparticle Characteristics

The results of flavonoid bio-nanoparticle size measurement are presented in Figure 4. Figure 4a on the left displays polydispersity index (PDI) of 0.1206 (horizontal green arrow) and average flavonoid bio-nanoparticle size of 103 nm (vertical green arrow). Figure 4b shows particle size distribution (depicted by red curve) showing a normal Gaussian distribution graph.



Peaks Summary		
Dia(nm)	Vol%	Width
103	100	73.6

Summary	
Data	Value
MI(nm):	105.9
MN(nm):	70.90
MA(nm):	93.50
CS:	64.17
SD:	36.80
PDI:	0.1206
Mz:	104.8
σι:	0.0361
Ski:	0.1084
Kg:	0.947

Percentiles	
%Tile	Size(nm)
10.00	61.20
20.00	73.50
30.00	83.80
40.00	93.50
50.00	103.0
60.00	112.8
70.00	123.5
80.00	136.3
90.00	154.6
95.00	170.1

Figure 4.
(a) Polydispersity index and average size, and (b) size distribution curve.

3.3. Flavonoid Antibacterial Efficacy

The results of clear zone diameter measurements are presented in Figure 5. This figure shows the clear zone diameter of three flavonoid samples (10 ppm, 20 ppm, and 30 ppm) and chloramphenicol 5%. The average clear zone diameters of flavonoids at 10 ppm, 20 ppm, and 30 ppm were (6.69 ± 0.19) mm, (7.02 ± 0.23) mm, and (7.54 ± 0.42) mm, respectively. These results confirm that flavonoids extracted from UGR exhibit antibacterial activity. In contrast, disk D did not display a clear zone, indicating that water did not inhibit the growth of E. coli [41]. On the other hand, disk E showed a relatively large clear zone diameter with an average of (15.79 ± 1.91) mm, indicating that chloramphenicol has strong antibacterial efficacy against these bacteria [42].

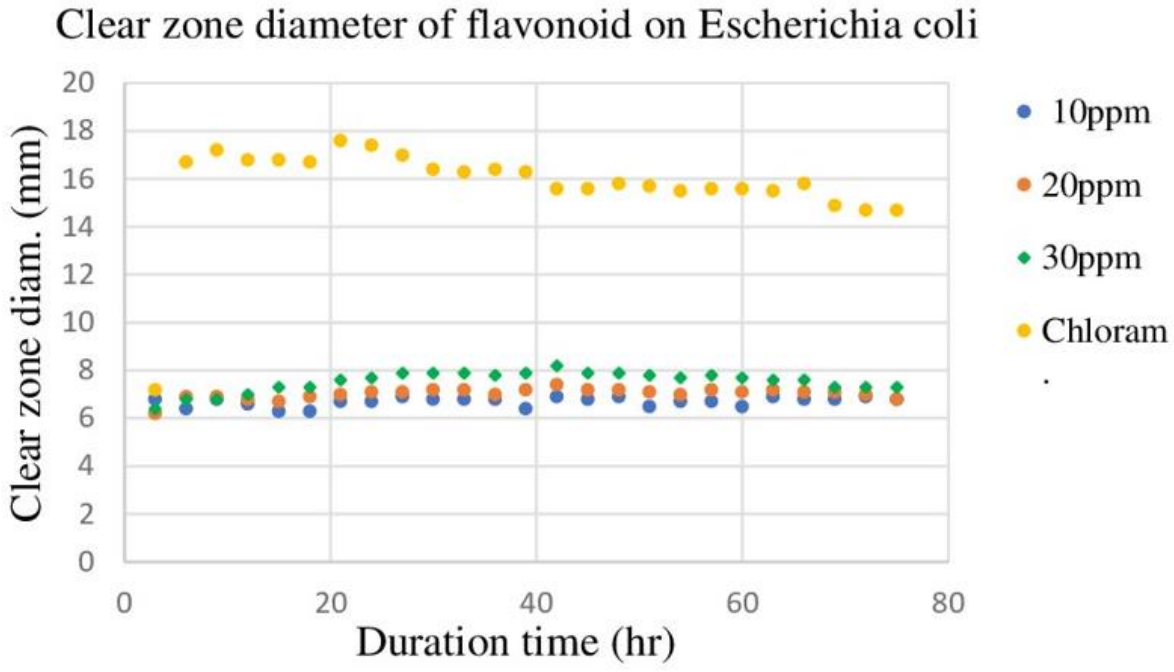


Figure 5.
Clear zone diameter of flavonoids and chloramphenicol on *Escherichia coli.*

Comparison of the diameter of the clear zone between chloramphenicol 5% and flavonoids with different concentrations was analyzed statistically using t-test analysis. The two-tailed p-values of these comparisons are presented in Table 2. The first, second, and third rows in Table 2 are the respective comparisons between 5% chloramphenicol and 30 ppm, 20 ppm, and 10 ppm flavonoids. The fourth and fifth rows in Table 2 are the respective comparisons between flavonoids 30 ppm and flavonoids 20 ppm and 10 ppm. The last row of Table 2 is a comparison between 20 ppm and 10 ppm flavonoids. All p-values are listed in the last column of Table 2.

Table 2.
P two-tail values of comparison chloramphenicol and flavonoids.

Chloramphenicol	Flavonoid			P two-tail value
5%	-	-	30 ppm	1.50×10^{-17}
5%	-	20 ppm	-	5.77×10^{-18}
5%	10 ppm	-	-	6.87×10^{-18}
-	-	20 ppm	30 ppm	7.45×10^{-6}
-	10 ppm	-	30 ppm	3.17×10^{-10}
-	10 ppm	20 ppm	-	2.75×10^{-6}

3.4. Escherichia Coli Resistance Development

The observation on E. coli resistance development is also presented in Figure 4. This figure illustrates that after 39 hours in contact with E. coli, the clear zone diameter of chloramphenicol consistently decreases until the last measurement at 75 hours. In contrast, E. coli did not show any resistance developments at all flavonoid concentrations up to the last measurement at 75 hours.

4. Discussion

The total content of flavonoids extracted from UGR, which was (31.55 ± 0.29) mg QE/g, is notably high. This flavonoid content surpasses that of 30 other Indonesian medicinal plants [29], 21 Indian medicinal plants [30], and 93 Chinese medicinal plants [31]. Most of these produced less than 20 mg QE/g, with the largest flavonoid content found in *Cosmos caudatus* at (29.93 ± 0.01) mg QE/g [29]. These comparisons underscore the superiority of UGR's flavonoid content, solidifying its status as a prominent source of flavonoids for future antibiotics.

Flavonoid bio-nanoparticles have been successfully made with a size of 103 nm, which is still 3% above the upper limit. Therefore, more effort should be made to reduce the current size. The PDI was 0.1206, and the size distribution was a tight bell-like graph indicating the homogeneity of the solution [43]. The small size of the particles enables them to diffuse into the cytoplasm to produce reactive oxygen species (ROS) and to inhibit many internal cell organelles through outer membrane pores [44, 45]. Such products are expected to increase efficacy against E. coli, as they combat E. coli with double physical and chemical "magic bullets" simultaneously [46-49]. In vitro antibacterial assessment is required in the near future to provide evidence of the efficacy of flavonoid bio-nanoparticles.

Figure 5 shows that flavonoids (10 ppm, 20 ppm, and 30 ppm) and chloramphenicol exhibit antibacterial activity. Chloramphenicol had the highest efficacy among the four antibacterial agents, as indicated by the average clear zone diameter (15.79 ± 1.91) mm. However, both are measured in different concentration units, namely chloramphenicol in % and flavonoids in ppm. Statistical analysis showed that the efficacy of flavonoids increases with the increase in concentration (Table 2 shows all p-values < 0.05). This allows us to draw a prediction curve to estimate the concentration of flavonoids that can produce the same clear zone diameter as that produced by 5% chloramphenicol. The linear regression equation for this prediction curve is obtained as $Y = 0.0425X + 6.2367$ with $R^2 = 0.9868$. In this equation, Y is equal to 15.79 mm and X is the calculated flavonoid concentration. By using this equation, a clear zone diameter of (15.79 ± 1.91) mm can be produced by a flavonoid concentration of 224.8 ppm, which is equivalent to 0.0225%. This flavonoid concentration is still much lower than that of chloramphenicol and can be achieved practically. The prediction curve is presented as supplementary material Figure S3.

The high efficacy of flavonoids can be understood because flavonoids can inhibit and disrupt ten different parts of E. coli simultaneously, as shown in Figure 2. In fact, chloramphenicol targets only one, namely ribosomes [50, 51]. Chloramphenicol inhibits ribosomal assembly [52]. All commercial antibiotics are designed to target specific bacterial sites. Ciprofloxacin, for example, targets DNA topoisomerase and gyrase to prevent DNA replication [53]. There are two significant implications of these findings. First, these findings demonstrate the superiority of flavonoids over the commercial antibiotic chloramphenicol, making flavonoids the primary choice for future antibiotic raw materials. Second, these findings demonstrate baseline concentrations of flavonoids when used for antibacterial assessment. Most studies were conducted using much lower concentrations, so the flavonoids appeared inferior compared to the positive control.

Finally, E. coli showed relatively weak resistance to chloramphenicol starting at 39 hours and increasing slowly until the end of observation at 75 hours, as shown by a consistent decrease in clear zone diameter. E. coli, which is resistant to chloramphenicol, was discovered by other researchers [51]. In contrast, E. coli did not show the development of resistance to flavonoids at all concentrations. The fact that the flavonoid concentrations used in this research were still very low shows the superpower of flavonoids in preventing E. coli resistance, and this is in line with the results of many other researchers [50-53]. These findings highlight the interesting potential of flavonoids as antibacterial agents and will be the main choice as raw materials for future antibacterial drugs to combat multidrug-resistant bacteria. However, further research is needed to reveal the spectrum of bacteria that can be killed by flavonoids and at which concentrations they can be killed.

5. Conclusions

UGR has been proven to be rich in natural flavonoids, making it the primary source of flavonoids as raw materials for future antibiotics. With a flavonoid content of (31.55 ± 0.29) mg QE/g, it is superior to many medicinal plants from Indonesia, India, and China. Therefore, this important medicinal plant must be protected from illegal trade.

At first glance, the efficacy of flavonoids was lower than that of 5% chloramphenicol. However, they are measured in different concentration units. Using the prediction curve and employing the same concentration unit, namely %, the efficacy

of 5% chloramphenicol was found to be the same as that of 0.0225% flavonoids. This underscores the superiority of flavonoids over chloramphenicol. On the other hand, E. coli showed weak development of resistance to chloramphenicol, as indicated by a consistent decrease in clear zone diameter from 39 hours to the final measurement at 75 hours. In contrast, E. coli did not show any resistance to flavonoids at all concentrations until the last measurement. The results of this study demonstrate that flavonoids not only produce much higher efficacy than chloramphenicol but also exhibit strong power in preventing the development of E. coli resistance. These findings highlight the promising potential of flavonoids as future antibiotic raw materials to combat bacterial resistance. However, more research needs to be conducted to reveal the efficacy of flavonoids against many multi-drug resistant bacteria.

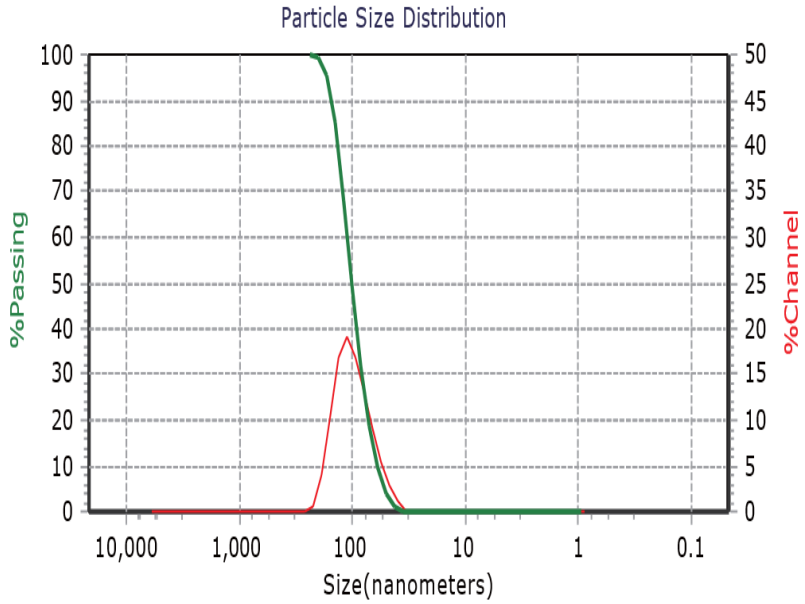
In addition, the success of preparing bio-nanoparticles opens opportunities to produce physical and chemical antibacterial agents in a single product to combat multi-resistant bacteria using these "double magic bullets." However, more research should be conducted to produce much smaller sizes of bio-nanoparticles to serve this purpose, and antibacterial assessments of flavonoid bio-nanoparticles should also be carried out in the future.

Figure S1.
Particle size analysis.
Bio-nanoparticle size measurement using dynamic light scattering (DLS).

Microtrac Total Solutions in Particle Characterization	- Particle Size Analysis -	C:\Microtrac\FLEX 11.1.0.6\Databases\Grace.MDB

-Tabular Data –

Size(nm)	%Chan	%Pass	Size(nm)	%Chan	%Pass
6540	0.00	100.00	15.19	0.00	0.00
5500	0.00	100.00	12.77	0.00	0.00
4620	0.00	100.00	10.74	0.00	0.00
3890	0.00	100.00	9.03	0.00	0.00
3270	0.00	100.00	7.60	0.00	0.00
2750	0.00	100.00	6.39	0.00	0.00
2312	0.00	100.00	5.37	0.00	0.00
1944	0.00	100.00	4.52	0.00	0.00
1635	0.00	100.00	3.80	0.00	0.00
1375	0.00	100.00	3.19	0.00	0.00
1156	0.00	100.00	2.690	0.00	0.00
972.0	0.00	100.00	2.260	0.00	0.00
818.0	0.00	100.00	1.900	0.00	0.00
687.0	0.00	100.00	1.600	0.00	0.00
578.0	0.00	100.00	1.340	0.00	0.00
486.0	0.00	100.00	1.130	0.00	0.00
409.0	0.00	100.00	0.950	0.00	0.00
344.0	0.00	100.00			
289.0	0.00	100.00			
243.0	0.60	100.00			
204.4	3.92	99.40			
171.9	10.27	95.48			
144.5	16.95	85.21			
121.5	19.15	68.26			
102.2	17.00	49.11			
85.90	13.24	32.11			
72.30	9.19	18.87			
60.80	5.50	9.68			
51.10	2.80	4.18			



43.00	1.24	1.38			
36.10	0.14	0.14			
30.40	0.00	0.00			
25.55	0.00	0.00			
21.48	0.00	0.00			
18.06	0.00	0.00			

- Measurement Info -	
Title	
bajakah eka	
Identifiers	
bajakah eka	
bajakah eka	
Database Record	45
Run Number	Avg of 5
Date	12/5/2023
Time	2:01 PM
Acquired Date	12/5/2023
Acquired Time	2:11 PM
Serial Number	W3936
Calculated Data	
Above Residual	0
Below Residual	0
Loading Index	7.07E-1
Conc. Index : cc/ml	0.655 : 5.48E-5
RMS Residual	0.076%
Cell Temp (C)	25.32

-SOP Info-	
BAJAKAH EKA	
Timing	
Setzero Time	60 (sec)
Run Time	30 (sec)
Number of Runs	5
Multi-Run Delay	0 (min)
Delay First Meas.	Disabled
Analysis	
bajakah eka	
Refractive Index	1.59
Transparency	Transp
Shape	Spherical
WATER	
Refractive Index	1.33
Low Temperature	20.0
Low Temp. Visc.	1.002
High Temperature	30.0
High Temp. Visc.	0.797
Options:	
Analysis Type	Distribution
Filter: Resolution	Std: Norm
Sensitivity	Standard

Viscosity(cp)	0.8840
Reflected Pwr (uW)	2.40
User Defined Calculations	
Name	**Value**
Recalculation Status	
DB-Meas:: Original :	

Algorithm	2.0
Perspective	
Progression	Standard
Distribution	Intensity
Upper Edge(nm)	6540
Lower Edge(nm)	0.8
Residuals	Disabled

Summary	
Data	**Value**
MI(nm):	105.9
MN(nm):	70.90
MA(nm):	93.50
CS:	64.17
SD:	36.80
PDI:	0.1206
Mz:	104.8
si:	0.0361
Ski:	0.1084
Kg:	0.947

Percentiles	
%Tile	Size(nm)
10.00	61.20
20.00	73.50
30.00	83.80
40.00	93.50
50.00	103.0
60.00	112.8
70.00	123.5
80.00	136.3
90.00	154.6
95.00	170.1

Peaks Summary		
Dia(nm)	Vol%	Width
103	100	73.6

FLEX

1.1.0.6

Figure S2.
Statistical Analysis.
A t-Test analysis: Comparison between Chloramphenicol and Flavonoid (10 ppm, 20 ppm, 30 ppm)

Time (hr)	10ppm	20ppm	30ppm	Chloram.
3	6.8	6.2	6.4	7.2
6	6.4	6.9	6.8	16.7
9	6.8	6.9	6.8	17.2
12	6.6	6.8	7	16.8
15	6.3	6.7	7.3	16.8
18	6.3	6.9	7.3	16.7
21	6.7	7	7.6	17.6
24	6.7	7.1	7.7	17.4
27	6.9	7.1	7.9	17
30	6.8	7.2	7.9	16.4
33	6.8	7.2	7.9	16.3
36	6.8	7	7.8	16.4
39	6.4	7.2	7.9	16.3
42	6.9	7.4	8.2	15.6
45	6.8	7.2	7.9	15.6
48	6.9	7.2	7.9	15.8
51	6.5	7.1	7.8	15.7
54	6.7	7	7.7	15.5
57	6.7	7.2	7.8	15.6
60	6.5	7.1	7.7	15.6
63	6.9	7.2	7.6	15.5
66	6.8	7.1	7.6	15.8
69	6.8	7.1	7.3	14.9
72	6.9	7	7.3	14.7
75	6.8	6.8	7.3	14.7
Average diam.	6.7	7.024	7.536	15.752
Standard Dev.	0.187617	0.230269	0.423207	1.913347

Ho= There is no significant different between chloramphenicol and Flavonoid
Ha= There is significant different between chloramphenicol and Flavonoid
Ho is rejected when P<0.05
Ha is accepted when P>0.05

Comparing Chloramphenicol and Flavonoid 30 ppm
t-Test: Two-Sample Assuming Unequal Variances

Chloram.	***30ppm***
Mean 15.752	7.536
Variance 3.813433	0.186567
Observations 25	25

Hypothesized	0	P< 0.05, Ho is rejected
df	26	
t Stat	20.54	
P(T<=t) one-ta	6.77E-18	
t Critical one-t	1.705618	
P(T<=t) two-ta	1.35E-17	
t Critical two-t	2.055529	

Conclusion
Chloramphenicol efficacy was significantly higher than Flavonoid 30 ppm

Comparing Chloramphenicol and Flavonoid 20 ppm
t-Test: Two-Sample Assuming Unequal Variances

	Chloram.	***20ppm***
Mean	15.752	7.024
Variance	3.813433	0.055233
Observations	25	25
Hypothesized	0	
df	25	
t Stat	22.18728	
P(T<=t) one-ta	2.89E-18	
t Critical one-t	1.708141	
P(T<=t) two-ta	5.77E-18	P< 0.05, Ho is rejected
t Critical two-t	2.059539	

Conclusion
Chloramphenicol efficacy was significantly higher than Flavonoid 20 ppm

Comparing Chloramphenicol and Flavonoid 10 ppm
t-Test: Two-Sample Assuming Unequal Variances

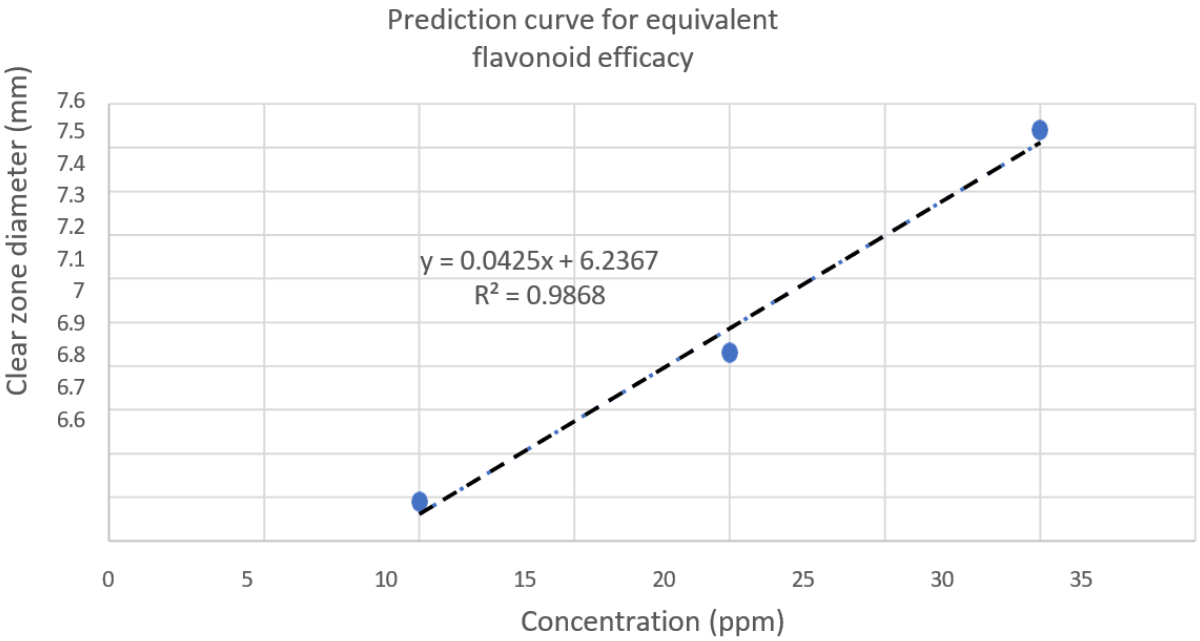
	Chloram.	*10ppm*
Mean	15.752	6.7
Variance	3.813433	0.036667
Observations	25	25
Hypothesized	0	
df	24	
t Stat	23.06633	
P(T<=t) one-ta	3.44E-18	
t Critical one-t	1.710882	
P(T<=t) two-ta	6.87E-18	P< 0.05, Ho is rejected
t Critical two-t	2.063899	

Conclusion
Chloramphenicol efficacy was significantly higher than Flavonoid 10 ppm

Figure S3.
Prediction curve equivalent efficacy

Prediction curve for equivalent flavonoid efficacy	
Flav. Conc. (ppm)	C. Z. diam.(mm)
10	6.69

20	7.03
30	7.54
224.78	15.79



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