

METHANOL EXTRACT INHIBITION'S EFFECTIVENESS OF KAWISTA FRUIT VAR. DOMPU ON E-COLI THROUGH IN-VITRO

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ARTICLE INFO	ABSTRACT
<p>Published: April 25th, 2023</p> <p>Keywords: local plant, fruit pulp and seed, Kawista (<i>Limonia acidissima</i> L.) var. Domp, antibacterial, <i>Escherichia coli</i></p> <p>This work is licensed under CC BY-SA 4.0 </p>	<p><i>Diarrhea is an endemic disease that causes individuals to excrete feces with a liquid consistency that have frequency of three or more times a day. The disease is caused by gastrointestinal infection due to the introduction of disease agents that can be transmitted through food, water, eating utensils, hands and flies. One of the causative agents of diarrhea is Escherichia coli. The use of various types of diarrhea drugs is basically to prevent the proliferation of Escherichia coli bacteria in the gastrointestinal tract, such as the use of Kawista fruit pulp and seeds by local people as a cure for diarrhea. The purpose of this study was to test the inhibitory activity of methanol extract of Kawista fruit pulp and seeds (Limonia acidissima L.) Domp variant with 4 concentrations, 25%, 50%, 75% and 100% extract concentrations. Testing antibacterial activity using the agar well diffusion method, which is done by measuring the inhibition zone around the wells. The methanol extract of Kawista fruit pulp and seeds (Limonia acidissima L.) Domp variant was significantly able to inhibit the growth of Escherichia coli at extract concentrations of 25%, 50%, 75% and 100% with inhibition zone diameter respectively 14.5 mm, 20.5 mm, 25.25 mm and 26.75 mm. The 100% extract concentration is classified as the concentration with the highest effectiveness in inhibiting the growth of Escherichia coli bacteria. Based on the results obtained, it can be concluded that the methanol extract of Kawista fruit pulp and seeds (Limonia acidissima L.) Domp variant has potential antibacterial activity that can be used as a basic ingredient for making antibiotic drugs.</i></p>

INTRODUCTION

Diarrhea is an endemic disease that causes individuals to excrete feces with a liquid consistency with a frequency of three or more times a day or a condition where a person experiences bowel movements that are more frequent than usual. Diarrheal disease is included in the Extraordinary Occurrence (KLB) which is one of the largest diseases that occur in Indonesian society (Hastia & Ginting, 2019; Kemenkes, 2020). Based on a number of health reports recorded in the 2018 Riskesdas National Report, the percentage of diarrhea prevalence reached 6.8% (Kemenkes, 2018). Most cases of diarrhea experienced by toddlers at the age of under 5 years are caused because the level of endurance of the body in toddlers is still weak, so toddlers are more susceptible to the attack of bacteria that cause diarrhea (Fitriani et al., 2021; Qisti et al., 2021). Diarrhea is most common in areas that have low risk control factors. The most dominant main factors are low sanitation and clean water facilities, inadequate fecal disposal, and lack of hygiene behavior between humans (Iryanto et al., 2021; Simantupang et al., 2014).

Diarrhea is caused by gastrointestinal infections due to the entry of many parasites or viruses, bacteria that can be transmitted through food, water, eating utensils, hands and flies (Iryanto et al., 2021). The three main clinical syndromes of diarrhea include, acute liquid diarrhea lasting less than 2 weeks, bloody diarrhea, and persistent diarrhea lasting at least 2 weeks. Diarrhea causes electrolyte loss, dehydration, shock to cause death (WHO, 2017).

Escherichia coli is one of the most common bacterial agents of foodborne diarrheal bacteria and is known as pathogenic *Escherichia coli* (Wang et al., 2019) *Escherichia coli* is a gram-negative bacterium, a facultative anaerobic bacterium, and is a commensal bacterium often found in the large intestine of mammals (Alizade et al., 2019). Bacterial infection *E. Coli* can occur when a person consumes food contaminated with bacteria, for example, in undercooked meat. Some types of *E. coli*, including *Escherichia coli* strain O157:H7 (foodborne pathogen), can even produce toxins that can damage the walls of the small intestine and cause diarrhea mixed with blood (Achmad et al., 2020). Therefore, if you experience symptoms of diarrhea, immediate treatment is needed by taking anti-diarrheal drugs and consuming plenty of fluids to overcome the problem of dehydration.

Kawista fruit (*Limonia acidissima* L.) has been known by the public and used for various purposes, including for the benefit of health. Kawista fruit grows naturally in Sri Lanka, India, Myanmar, and Indochina, then spreads to Malaysia and Indonesia. Kawista trees have also been introduced to America. In Indonesia, Kawista grows naturally in the northern coastal areas of Java island and most of Lombok and Sumbawa (West Nusa Tenggara) islands (Erliananda et al., 2022; Vijayakumar et al., 2013)

Kawista fruit contains carbohydrates, proteins, and various vitamins, such as vitamin B, vitamin C, thiamin and riboflavin (Vijayvargia & Vijayvergia, 2014). In addition, Kawista fruit is known to contain several other phytochemical compounds such as alkaloids, saponins, phenols, and flavonoids (S. Pandey et al., 2014). Kawista fruit itself has several benefits from those known as antitumor, anti-inflammatory, antipyretic, analgesic, antioxidant, anti-diabetic activity as well as anti-diarrheal (Islam et al., 2020; Singhania et al., 2020; Sonawane et al., 2016)

Other supporting studies such as research by Naidu et al. (2014) using hexane extract, chloroform and methanol *Limonia acidissima* L. against antibacterial activity, the results showed that methanol showed good antibacterial activity with high inhibitory zones in several bacteria, one of which was *Escherichia coli* bacteria. In research conducted by Thomas and Ponnammal (2005) showed that 0.1 mL methanol extract of fruit flesh and seeds of *Limonia acidissima* L. in *Escherichia coli* and *Staphylococcus aureus* bacteria carried out by disc diffusion method can provide inhibitory zones of 40 mm and 43 mm. Furthermore, the results of antimicrobial activity *in vitro* showed that methanol extract of fruit flesh and Kawista seeds provided a good inhibitory zone effect on *Escherichia coli* bacteria by 22 mm (Patil, 2012). In Jayashree and Londonkar's (2014) study, Kawista fruit flesh extract has great potential against the antimicrobial activity of several microorganisms tested, one of which is *Escherichia coli*. Among the extracts tested, methanol showed the highest antimicrobial activity.

Research Ponnuraj et al. (2015) also reports that a concentration of 500 mg/mL is the optimum dose against the inhibitory zone. Based on several research results related to the inhibitory power of Kawista fruit extract against *Escherichia coli* bacteria caused by the higher the concentration of Kawista fruit extract, the greater the zone or obstacle to the growth of *Escherichia coli* bacteria. Antibacterial test results show methanol extract of Kawista fruit can inhibit the growth of *Escherichia coli* bacteria.

Based on the above background, it is necessary to conduct testing to determine the effectiveness of the use of Kawista fruit as an alternative to diarrhea treatment in addition to using chemical drugs. This is done as an effort to develop plants that have medicinal properties and an effort to find antibacterial sources derived from natural ingredients. The discovery of antibacterial sources derived from natural materials can help overcome the problem of bacterial resistance, especially pathogenic bacteria (Oroh et al., 2015)

This study aimed to determine whether methanol extract of fruit flesh and Kawista seeds (*Limonia acidissima* L.) var. Dompu concentrations of 25%, 50%, 75% and 100% can inhibit the growth of *Escherichia coli* bacteria and methanol extracts of fruit flesh and seeds of Kawista (*Limonia acidissima* L.) var. Dompu concentration has the best growth inhibition ability of *Escherichia coli* bacteria. This study is expected to provide a comprehensive picture of the effects of fruit flesh and seeds of Kawista on the inhibitory power of *Escherichia coli* bacteria to update the data.

METHOD

This type of research is true experimental research with the *in-vitro* method, which is a type of research used to look for the influence of certain treatments on others and researchers can modify research subjects. This study used a simple experimental design (Post-test Only Control Group Design). This design was used with the aim of determining the relationship between the inhibitory power of Kawista fruit extract and the growth of *Escherichia coli* bacteria, with the treatment group model can be seen in Table 1.

Table 1. Design of Treatment Grouping Method Based on Methanol Extract Concentration of Fruit Flesh and Kawista Seeds (*Limonia acidissima* L.)

No	Group	Treatment
1.	Group 1 (K1)	The group of <i>Escherichia coli</i> bacteria given Methanol extract of Kawista fruit flesh and seeds with a concentration of 25%.
2.	Group 2 (K2)	The group of <i>Escherichia coli</i> bacteria given Methanol extract of Kawista fruit flesh and seeds with a concentration of 50%.
3.	Group 3 (K3)	The group of <i>Escherichia coli</i> bacteria given Methanol extract of Kawista fruit flesh and seeds with a concentration of 75%
4.	Group 4 (K4)	The group of <i>Escherichia coli</i> bacteria given Methanol extract of Kawista fruit flesh and seeds with a concentration of 100%.

5.	Group 5 (K5)	The group of <i>Escherichia coli</i> bacteria given <i>chloramphenicol</i> as a positive control.
6.	Group 6 (K6)	The group of <i>Escherichia coli</i> bacteria given <i>Aquadest</i> as a negative control.

This study also used a Complete Randomized Design (RAL) with 3 treatment groups consisting of 1 experimental group in the form of Kawista fruit flesh extract (*Limonia acidissima* L.) with concentrations of 25% (treatment 1/K1), 50% (treatment 2/K2), 75% (treatment 3/K3) and 100% (treatment 4/K4) and 2 control groups consisting of positive control groups (*chloramphenicol*) and negative controls using aquades.

The classification of fruit maturity can be seen in 7 maturity indices, namely from dark green or unripe fruit to yellow orange or fully ripe. Index 1: young or immature and dark green, not suitable for picking. Table of Contents 2: mature starting level, dark green with a slight yellowness between the eyes at the base, can be picked and is suitable for export. Index 3: mature, overall green eyes with 1-2 eyes at the base yellow, suitable for export. Index 4: the fruit begins to ripen, 25% of the eyes at the base of the fruit turn yellowish-orange. Index 5: almost 50% of eyes become yellowish-orange. Index 6: more than 75% of eyes are yellowish-orange. Index 7: cook ranum, eyes are orange yellow (Lustini, 2019). This study used Kawista fruit flesh with the level of fruit maturity at index 1, which is immature in the form of young fruit with a green or yellow shell and white or cream flesh and has a plain, bitter or sour taste (wry).

This research will be conducted at the Research Laboratory of the Faculty of Medicine of Al-Azhar Islamic University Mataram. The time of this study is carried out starting in February 2023 and is estimated to be completed in February 2023. The samples in this study were *Escherichia coli* bacteria.

The tools used in this study are petridish, ose, bunsen, test tube (Pyrex, Japan), analytical scale/balance, erlenmeyer, measuring flask, beaker glass, caliper/digital caliper with 0.5 mm accuracy, micropipette, glass spatula, blender, electric stove, laminar air flow cabinet, autoclave, incubator, vacuum rotary evaporator, measuring cups, filter paper and bunsen. The ingredients used in this study were: Kawista fruit flesh extract (*Limonia acidissima* L.), Methanol Pa (E.Merk), aquades, MHA media (Mueller Hinton Agar), 70% alcohol, rubber bands, plastic, aluminum foil, and *Escherichia coli* test bacteria. Then the research procedures carried out were sterilization of tools, making McFarland solution turbidity standards, preparation of fruit flesh and Kawista seeds, making extracts of fruit flesh and Kawista seeds, making MHA media (Mueller Hinton Agar), making concentrations of fruit meat and seed extract solutions, making test bacteria, inhibitory power tests, and finally data analysis. The diameter of the obstacle zone of each treatment is then calculated the total diameter of the obstacle zone and the average diameter of the obstacle zone of each treatment.

RESULT AND DISCUSSION

Manufacture of Methanol Extract of Kawista Plant (*Limonia acidissima* L.) Var. Dompou

Young Kawista fruit that has been separated between the skin and flesh is then sliced and blended so that fruit powder and Kawista seeds with a final weight of 373.27 grams are obtained. The extraction method used is maceration method using 800 mL methanol solvent. The resulting fiber was then concentrated using a rotary evaporator at 40° C and obtained a thick extract of 23.5 grams. From the viscous extract that has been obtained, the percent yield of methanol extract is obtained. The obtained viscous extract is then diluted with sterile aquades. The dilution aims to produce some concentration that will be used to inhibit the growth of *Escherichia coli*. The weight of fruit flesh powder and Kawista seeds, the weight of viscous extract and the percent of methanol extract ration are presented in table 2.

Table 2. Weight of Powder and Viscous Extract of Fruit Flesh and Kawista Seeds and Percent Yield of Methanol Extract

Type	Weight (grams)	Randemen (%)
Kawista Powder	373.27	-
Methanol Extract	23.5	6.296

Test of the Effectiveness of Methanol Extract of Kawista Plant (*Limonia acidissima* L.) Var. Dompou Against *Escherichia coli* Bacteria

Antibacterial activity test of methanol extract of fruit flesh and Kawista seeds against the growth of *Escherichia coli* bacteria was carried out using the sumuran diffusion test method. The antibacterial activity test is carried out using the sumuran diffusion method, because the diffusion method is one of the methods that is often used for the hole/*sumuran* method. This method can be used to see the resulting clear area around the wellhole created.

The clear area around the well is called the inhibition zone. The use of this method is based on research by Prayoga (2013) and Haryati et al. (2017) which states that the well method is better and wider in the inhibition zone than the disk method. This is because the well method occurs osmolarity process from extract concentrations higher than the disk method. The welling method of each hole is filled with extract concentration so that osmolarity occurs more thoroughly and more homogeneously and the concentration of the extract produced is higher and stronger to inhibit bacterial growth.

This study used methanol extracts of fruit flesh and Kawista seeds that were inserted into the well hole on MHA media (Muller Hinton Agar) which had been homogenized with bacteria and then incubated until an inhibitory zone was seen in the area around the disc. Kawista fruit extract inserted into the well hole consisted of methanol extract of fruit flesh and Kawista seeds 25%, 50%, 75% and 100% accompanied by positive control (*chloramphenicol*) and negative control (aqudest). Test of antibacterial activity of methanol extract of fruit flesh and Kawista seeds is presented in table 3.

Table 3. Antibacterial Activity Test of Viscous Extract of Fruit Flesh and Kawista Seeds against *Escherichia coli*

Treatment Group	Resistance Zone Area (mm)				Number of Diameters	Avg. (mm)	Meaning
	I	II	III	IV			
(K1) 25%	15	15	13	15	58	14,5	Able to inhibit the growth of <i>Escherichia coli</i> bacteria
(K2) 50%	21	20	20	21	82	20,5	Able to inhibit the growth of <i>Escherichia coli</i> bacteria
(K3) 75%	25	26	25	25	101	25,25	Able to inhibit the growth of <i>Escherichia coli</i> bacteria
(K4) 100%	27	26	27	27	107	26,75	Able to inhibit the growth of <i>Escherichia coli</i> bacteria
Control (+)	32	33	32	32	129	32,25	Able to inhibit the growth of <i>Escherichia coli</i> bacteria
Control (-)	0	0	0	0	0	0	Unable to inhibit the growth of <i>Escherichia coli</i> bacteria

Graphs of the diameter of the inhibition of antibacterial activity of methanol extracts of fruit flesh and Kawista seeds of various concentrations are presented in Figure 1.

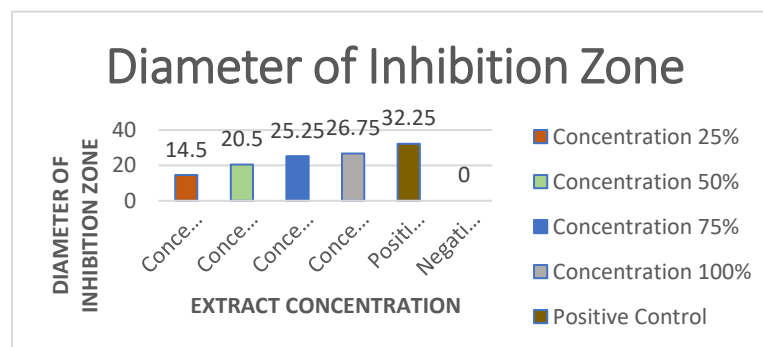


Figure 1. Diameter of Inhibition Zone Chart of Methanol Extract of Fruit Flesh and Kawista Seeds of Various Concentrations

Data Analysis

Data on the growth of inhibitory power of *Escherichia coli* bacteria were analyzed using a normality test using statistical analysis in the form of a One Way Anova test and then continued with the post Hoc Test Tukey test, but before the statistical test was carried out, a normality test must be carried out first to ensure the data is normally distributed ($P > 0.05$). The normality test used uses the Shapiro Wilk normality test. The purpose of his Shapiro Wilk test was to identify whether a random variable follows a normal distribution of data with a number of less than 50. If the data is not normally distributed, the data analysis process uses the Kruskal-wallis test and the

Mann-whitney Advanced test. The Normality Test using the Shapiro Wilk Test is presented in table 4.

Table 4. P-Value on Shapiro Wilk Normality Test

Treatment Group	P Value	Information
(K1) 25%	0,001	Not normally distributed
(K2) 50%	0,024	Not normally distributed
(K3) 75%	0,001	Not normally distributed
(K4) 100%	0,001	Not normally distributed
Control (+)	0,001	Not normally distributed

Based on Table 4. It can be seen that the Shapiro-Wilk test shows that all data have significance values below 0.05 ($p < 0.05$) so it is concluded that the data are not normally distributed. This indicates that the data used is non-parametric data so it is continued with the Kruskal-wallis test. The Kruskal-wallis test is presented in table 5.

Table 5. Kruskal-Wallis Test Results

Variable	Treatment	N	Mean Rank	P Value	Information
Diameter of the Inhibition Zone	Methanol extract of fruit flesh and Kawista seeds 25%	4	6,50	0,000	There are differences between treatments
	Methanol extract of fruit flesh and Kawista seeds 50%	4	10,50		
	Methanol extract of fruit flesh and Kawista seeds 75%	4	14,63		
	Methanol extract of fruit flesh and Kawista seeds 100%	4	18,38		
	Positive Control (<i>Chloramphenicol</i>)	4	22,50		
	Negative Control (Aquades)	4	2,50		

The data obtained using Kruskal-wallis showed an overall result with a value of 0.000, the value is smaller than 0.05, so it can be concluded that there is a significant influence on the administration of methanol extracts of fruit flesh and Kawista seeds on the activity of *Escherichia coli* bacteria. The greatest inhibition was found in the positive control treatment based on the mean rank obtained which was 22.50, followed by methanol extract treatment of fruit flesh and Kawista seeds 100% (18.38), treatment 75%, treatment 50%, treatment 25% and negative control.

Furthermore, to determine the differences between sample groups, the analysis was continued using the Mann-Whitney follow-up test. Further Mann-Whitney tests are presented in Table 6.

Table 6. Mann-Whitney Advanced Test

Sample 1-Sample 2	Sig value.	Information
25% Extract and 50% Extract	0,017	There are differences
25% Extract and 75% Extract	0,015	There are differences
25% Extract and 100% Extract	0,015	There are differences
25% Extract and Positive Control	0,015	There are differences
25% Extract and Negative Control	0,011	There are differences
Extract 50% - Extract 75%	0,017	There are differences
50% Extract - 100% Extract	0,017	There are differences
Extract 50% - Positive control	0,017	There are differences
Extract 50% - Negative control	0,013	There are differences
Extract 75% - Extract 100%	0,022	There are differences
Extract 75% - Positive control	0,015	There are differences
Extract 75% - Negative Control	0,011	There are differences
100% extract - Positive control	0,015	There are differences
100% Extract - Negative Control	0,011	There are differences
Positive control - Negative control	0,011	There are differences

Based on table 6, it can be seen that all treatment groups experienced significant differences from one another ($p < 0.05$). It can be concluded that the antibacterial activity of each treatment group of methanol extract of fruit flesh and Kawista seeds experienced differences that can be seen from the diameter of the inhibitory zone formed.

Extraction of Kawista Plant (*Limonia acidissima* L.) Var. Dompu

Making methanol extract of fruit flesh and Kawista seeds is carried out using the maceration method, where young Kawista fruit is separated between the skin and flesh, then the fruit is thinly sliced and wet sorting is carried out to separate the fruit flesh from impurities or foreign materials, then washed using running water until clean. This washing aims to reduce dirt and microorganisms in plants (Prasetyo & Inorih, 2013) then drained and dried by aerating until free of residual water.

The dried flesh and seeds of the Kawista are then blended. Extraction is carried out using the maceration method. Maceration is one of the extraction methods carried out through soaking the material powder in an extractor solution (Putra et al., 2014). The use of maceration method in this study is to extract the active substance in the flesh of the Kawista fruit which is easily soluble in the extracting liquid, does not expand in the extractor, and does not contain benzoin (Rahmawati et al., 2016). The principle of the maceration method is based on the distribution of solutes or the distribution of solubilable components to the ratio of two types of solvents that do not mix with each other. This study used methanol solvent, where methanol was chosen as a solvent because methanol is a solvent that can be used to extract flavonoids. In addition, methanol is also a polar solvent so that it can be used to extract flavonoid glycoside group compounds (Agustina, 2017).

Flavonoids are one of the most abundant groups of secondary metabolite compounds found in plant tissues. Flavonoids belong to the class of phenolic compounds with the chemical structure C6-C3-C6. Flavonoid compounds are synthesized by plants as a defense system and in their response to infection by microorganisms. Flavonoids work as antibacterial with several mechanisms of action, including inhibiting nucleic acid synthesis, inhibiting cytoplasmic membrane function and inhibiting energy metabolism of bacteria. Due to its antibacterial properties, this study tried to extract flavonoid compounds and various other antibacterial compounds in Kawista fruit to be tested against *Escherichia coli* bacteria (Ajie, 2015).

The flesh and seeds of Kawista macerated with methanol solvent until completely submerged for 1 day (1x24 hours). This 1-day soaking aims to maximize the process of taking chemical compounds contained in fruit meat samples (Kamal & Prayitno, 2020). After soaking, the maserat is separated until filtrate and residue are obtained. The residue is then macerated again with methanol with 3 repetitions. The total methanol volume used was 800 mL. The final fiber formed is then evaporated using a rotary evaporator with a temperature of 40° C. The viscous extract is then calculated as a percentage of its yield.

The yield presentation is a comparison of the final weight with the initial weight multiplied by 100%, the yield is also related to the amount of bioactive content contained in the simplisia (Dewatisari et al., 2018). The extraction results of 373.27 g of dried fruit flesh and Kawista seed powder using 800 mL of solvent produced a thick extract of fruit flesh and Kawista seeds as much as 23.5 grams, then calculated the percentage of extract yield and obtained as much as 6.296%, while the percentage of extract yield obtained by previous research, namely research conducted by Kusuma et al. (2022), a yield of 24.8% was obtained from the powder of Kawista fruit flesh weighing 273 g. The difference in yield percentage results obtained with research conducted by Kusuma et al. This is because in this study the sample used is estimated to still contain a lot of fat so that it cannot be drawn by methanol and the difference in the weight of the extracted powder. Particle size also affects the percentage of yield produced, where research by Maulida and Guntarti (2015) states that reducing the particle size of simplisia can increase the amount of active substance and the amount of extract yield. In addition, according to Wijaya et al. (2019), the yield of an extract is strongly influenced by several factors, including: material size, extraction time, extraction temperature, type of material, type of solvent, and the ratio of the amount of solvent to the material.

Research conducted by Nachimuthu et al. (2014) showed yield results obtained from chloroform extract 2.20%, petroleum ether extract 2.25%, water extract 8.42% and ethyl acetate extract 2.97%. This shows that the yield of methanol extract obtained is more when compared to chloroform solvents, petroleum ether solvents and ethyl acetate, but the yield of methanol extract produced is less than the yield of water extract. This difference can occur due to the difference in polarity of the solvent. Methanol has a polarity of 34, while water has a polarity of 74 (I. M. Kusuma et al., 2022). The more polar the solvent of the chemical components in the plant sample will be perfectly extracted, resulting in more extracts (I. M. Kusuma et al., 2022).

The obtained viscous extract is then diluted with sterile aquades. The dilution aims to produce some concentration that will be used to inhibit the growth of *Escherichia coli*. In dilution, aquades are more often used to dilute a substance with a high concentration because many biochemical reactions can take place only in solutions of aquades. In addition, the addition of aquades causes the concentration to change and the volume is enlarged but the number of moles of solute remains, so it does not change the bioactive substances in the extract (Harjanti, 2004; Konay et al., 2019).

Antibacterial Activity Test of Kawista Fruit (*Limonia acidissima* L.) Var. Dompu

Antibacterial activity test against *Escherichia coli* bacteria in this study was carried out with a well diffusion approach (agar diffusion) on MHA Agar Media (Muller Hinton Agar). This study uses *Escherichia coli* bacteria as objects because these bacteria are normal flora that are often found in the human intestine, but if the population in the gastrointestinal tract is excessive it can cause primary infections such as diarrhea (Sumampouw, 2018). The purpose of this study is to prove the bioactive content in Kawista fruit whether it is able to treat diarrheal diseases or other gastrointestinal infections caused by *Escherichia coli* bacteria, so that these bacteria are used as test targets.

The well diffusion method is the most frequently used method. The use of this method is based on research by Prayoga (2013) and Haryati et al. (2017) which states that the well method is better and wider in the inhibition zone than the disk method. This is because the well method occurs osmolarity process from extract concentrations higher than the disk method. The welling method of each hole is filled with extract concentration so that osmolarity occurs more thoroughly and more homogeneously and the concentration of the extract produced is higher and stronger to inhibit bacterial growth. The medium used for growth and antibacterial activity testing is the Mueller Hinton Agar media. Mueller Hinton Agar media is the best media for test sensibility examination (by slurble diffusion method) on non-fastidious bacteria (both aerobic and facultative anaerobes) including *Escherichia coli* bacteria (Indrayati & Amelia, 2019).

Testing antibacterial activity in Kawista fruit flesh using methanol extracts of fruit flesh and Kawista seeds with various concentrations, namely 25%, 50%, 75% and 100%. The concentration was taken based on research conducted by Kusuma and Jastian (2021) and Kusuma et al. (2022) the two studies used methanol extracts of Kawista fruit peels with concentrations of 25%, 50% and 75% but used different bacteria, namely *Propionibacterium acnes* and *Staphylococcus aureus* bacteria, respectively. The results of Kusuma and Jastian's (2021) research show that methanol extract of Kawista fruit peel at concentrations of 25%, 50% and 75% has anti-bacterial activity on *P.acnes* with inhibitory diameter values sequentially, namely 10.80 mm, 12.40 mm and 13.31 mm, in the strong category. And the results of research by Kusuma et al. (2022) showed that methanol extract of Kawista fruit peel has antibacterial activity against *S. aureus* at concentrations of 25%, 50% and 75% with inhibitory diameters of 15.49 mm, 17.37 mm, and 22.49 mm respectively. Based on these results, Kawista fruit meat extract was carried out using various concentrations to test antibacterial activity against *Escherichia coli*.

The results obtained using methanol extract samples of fruit flesh and Kawista seeds were all extract treatment groups 25%, 50%, 75% and 100% formed inhibition zones against *Escherichia coli* bacteria with an average diameter of 14.5 mm, 20.5 mm, 25.25 mm, 26.75 mm, respectively. Based on these data, it shows that the higher the concentration, the higher the inhibitory zone produced. This is because the higher the concentration of the extract tested, the more antibacterial active substances contained in it, so that the effectiveness in inhibiting bacterial growth will be better and the average diameter of the resulting inhibitory zone will differ for each concentration (Mawan et al., 2018). In addition, between 75% and 100% extract concentrations have a low average diameter increase, which can be caused by various factors such as uneven distribution of bacteria in the media, less pipetting volume or errors in the measurement process.

An antibacterial substance has weak inhibitory activity if an inhibitory zone is formed with a diameter of 5 mm or less, is declared medium if it has an inhibitory zone of 5-10 mm, is declared strong if it has an inhibitory zone of 10-20 mm, and is declared very strong if it has an inhibitory zone of 20 mm or more, so that it can be known that the antibacterial inhibition category of methanol extract of fruit flesh and Kawista seeds is on average classified as a very strong category. This result is in accordance with the research of Veryanti and Kusuma (2021) which states that methanol extract of Kawista fruit peel is able to inhibit *Escherichia coli* bacteria by forming the highest inhibitory zone of 22.71% at a concentration of 80%. This proves that the extract of Kawista fruit flesh has better activity than the skin of the fruit.

In addition, the concentration of extracts tested falls into the sensitive, intermediate, and resistant categories. The concentration of 25%, 50%, 75%, 100%, positive control is included in the sensitive category and negative control does not appear to form an inhibitory zone at all. According to Yuniarty and Hasjim (2017), the classification of Inhibitory Power Diameter (DDH) is categorized based on the large diameter of the inhibitory zone formed by traditional medicines, it is said to be sensitive when the diameter of the resistance zone is >12 mm, intermediate when the diameter of the resistance zone is 4-12 mm and resistant when the diameter of the resistance zone is < 4 mm. Based on this, all extract treatments are included in the sensitive category.

Based on the results of the normality test data, data on methanol extracts of fruit flesh and seeds of Kawista (*Limonia acidissima* L.) were not normally distributed ($p>0.05$). Therefore, a non-parametric test was carried out, namely the *Kruskal-wallis* test to determine the differences between treatment groups.

Based on the results of the *Kruskal-wallis* test, there was a significant difference in each treatment group with a value of ($p = 0.000$) where the value was smaller than 0.05 so that it can be concluded that methanol extract of fruit flesh and Kawista seeds can inhibit the growth of *Escherichia coli* bacteria. This is because methanol extract of Kawista fruit flesh and seeds (*Limonia acidissima* L.) has antibacterial activity. The greatest inhibition was found in the positive control treatment based on the *mean rank* obtained which was 22.50, followed by methanol extract treatment of fruit flesh and Kawista seeds 100% (18.38), treatment 75%, treatment 50%, treatment 25% and negative control. This is supported by research conducted by Pandey et al. (2014) stating

that methanol extracts of fruit flesh and Kawista seeds have the highest antibacterial activity on bacteria *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Bacillus subtilis*.

After the Kruskal-wallis test, the Mann-whitney test was carried out. Based on the test results showed that all treatment groups experienced significant differences from one another ($p < 0.05$). It can be concluded that the antibacterial activity of each treatment group of methanol extract of fruit flesh and Kawista seeds experienced differences that can be seen from the diameter of the inhibitory zone formed.

The negative control group with the positive control group had a difference in inhibition power, where the negative control did not produce an inhibitory zone at all. This happened because the negative control group did not have antibacterial activity. The negative control used is aquadest. According to Muljono et al. (2016), the use of aquades as a negative control is absolute to rule out the possibility of antibacterial effects. This is in accordance with the results in this study, where all wells given aquades do not have an inhibition zone. The positive control used is to use *Chloramphenicol*. The choice of *Chloramphenicol* as a positive control is based on the fact that *Escherichia coli* bacteria have experienced resistance to several antibiotics besides that this antibiotic is bacteriostatic with a broad spectrum that is active against gram-negative and gram-positive bacteria, able to inhibit the attachment of amino acids from bacteria so as to inhibit the growth of *Escherichia coli* (Goetie et al., 2022). Bacteria are said to be resistant when they produce an inhibitory zone diameter of <20 mm (Utomo et al., 2018). In this study the diameter produced by *chloramphenicol* against *Escherichia coli* bacteria was 32.25 mm so that it was categorized as very strong, which category was the same as the inhibitory power produced by methanol extract of Kawista fruit concentrations of 50%, 75% and 100% (Very strong).

The positive control group with concentration groups of 25%, 50%, concentration of 75% and concentration of 100% had different diameters of inhibitory zones formed. This is due to the different mechanisms of antibacterial activity in inhibiting bacteria between the positive control group with a concentration group of 25%, concentration of 50%, concentration of 75% and concentration of 100%. According to Taufiq et al. (2015), positive control of chloramphenicol has the mechanism of disrupting cell wall synthesis, inhibiting protein synthesis, impairing nucleic acid synthesis, inhibiting metabolic pathways, and the β -lactam agent *chloramphenicol* inhibits bacterial cell wall synthesis by interfering with enzymes needed for peptidoglycan coating synthesis. While the mechanism of the extract group does not attack bacteria singly because it contains different antibacterial active compounds in each extract group.

The ability of fruit flesh and Kawista seeds in inhibiting the growth of *Escherichia coli* bacteria due to the content of bioactive substances such as flavonoids, alkaloids, glycosides, saponins, tannins, coumarins and tyramine derivatives which are antioxidants and antibacterials. This is in accordance with the research of Kusuma et al. (2020) which shows that Kawista fruit contains alkaloids, flavonoids, saponins, and tannins. Research by Rini and Supriatno (2017) also shows that Kawista fruit extract contains various active compounds such as alkaloids, saponins, tannins, triterpenoids, and polyphenols.

The mechanism of action of flavonoids works as antibacterial by having 3 activities, namely inhibiting nucleic acid synthesis, inhibiting cell membrane function, and inhibiting energy metabolism (Rijayanti, 2014). Saponins have antibacterial activity with a mechanism of lowering surface tension resulting in increased permeability or damage to bacterial cells and resulting in intracellular compounds coming out (Ngajow et al., 2013). Alkaloids have the ability as antibacterial and inhibitory mechanisms by disrupting the constituent components of peptidoglycan in bacterial cells so that the cell wall layer is not formed intact and causes cell death (Amalia et al., 2017). Saponins act as antibacterial with a mechanism that damages cell wall permeability so that it can cause cell death (Astriani et al., 2021). While secondary metabolites of tannins have antibacterial activity by inhibiting the enzymes reserve transcriptase and DNA topoisomerase so that the formation of bacterial cells cannot be formed (Ngajow et al., 2013).

Research by Rini and Supriatno (2017) also proves that Kawista fruit extract has antibacterial activity against the growth of *Escherichia coli* bacteria, where the higher the concentration of Kawista fruit extract, the greater the antibacterial activity against the growth of *Escherichia coli* bacteria. This is in accordance with this study, namely the higher the concentration of methanol extract, fruit flesh and Kawista seeds, the higher the inhibitory zone produced.

Research conducted by Supriatno and Rini (2018) also shows that the content of antibacterial active compounds in Kawista fruit has the ability to inhibit the growth of *Escherichia coli* bacteria. These bacteria are one of the classes of Gram negative bacteria. The cell wall of Gram negative bacteria consists of one or more thin peptidoglycan layers and a membrane on the outside of the peptidoglycan layer. Its cell wall contains only a small layer of peptidoglycan and does not contain teichoic acid, therefore the cell wall of gram-negative bacteria is more susceptible to physical shocks, such as the administration of antibiotics or other antibacterial materials (Mpila et al., 2012).

CONCLUSION

Methanol extract of Kawista fruit flesh and seeds (*Limonia acidissima* L.) var. Dompu concentrations of 25%, 50%, 75% and 100% have a significant effect in inhibiting the growth of *Escherichia coli* bacteria with an average diameter of 14.5 mm, 20.5 mm, 25.25 mm and 26.75 mm, respectively. Methanol extract of Kawista fruit flesh and seeds (*Limonia acidissima* L.) var. Dompu concentration of 100% is classified as the concentration with the highest effectiveness in inhibiting the growth of *Escherichia coli* bacteria with an average diameter of 26.75 mm.

The limitations in this study are not fractionating extracts, not screening phytochemicals and not identifying the structure of compounds in each isolate because of limited research time and requiring separate methods to determine specific types of compounds. Therefore, researchers suggest that phytochemical screening be carried out to determine the active substance content of Kawista fruit flesh, fractionation is carried out to obtain pure extracts from Kawista fruit flesh, and further research is carried out to identify active compounds contained in methanol isolates that have antibacterial activity.

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