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Antibacterial Ingredients That Cause Diarrhea Can Be Made from Black, Green, and White Tea Extract (*Camellia sinensis* L.)

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ABSTRACT

One cause of diarrhea is a bacterial infection. Tea (*Camellia sinensis*) is a steeping drink that has the efficacy of treating diarrhea. This looks at goals to decide the effect of formulations of three tea extracts that act as antibacterial causes of diarrhea and decide the most appropriate maceration temperature. Extraction was carried out with water solvents at temperatures 70°C, 100°C, and 121°C. The disc method conducted an antibacterial test against *E. coli* and *S. aureus*. The most optimal temperature for producing the highest inhibitory zone is the temperature of 100°C. Tea extract temperatures of 100°C are used for antibacterial testing. BSLT test showed the LC50 value of 1800 µg/mL. Tests on E. coli bacteria showed the highest results in a single white tea extract with a diameter of 9.750 mm, and tests on S. aureus showed the highest results in the three tea extract formula with a diameter of 9.65 mm. The inhibitory area evaluation of the system changed into finished using the simplex-centroid design method. It showed a non-huge linear model in the antibacterial test in opposition to *E. coli* and *S. aureus*.

1. Introduction

Diarrhea is an endemic ailment in Indonesia and is a capacity excellent occasion (KLB) followed by death. The number of diarrhea sufferers in 2016 was 3,176,079 patients, and there was an increase in 2017 to 4.274.790 diarrhea sufferers in health facilities (Indonesia, 2017). Dehydration due to diarrhea is one of the causes of death in diarrhea sufferers (Yushananta & Usman, 2018). Causes of diarrhea include infection, malabsorption, allergies, poisoning, and immune deficiency. One of the causes often found in diarrhea caused by a bacterial infection. Bacteria that often cause diarrhea are *Staphylococcus aureus, Vibrio cholera, Escherichia coli, Shigella flexneri*, and *Campylobacter jejuni* (Omwenga et al., 2011). Bacteria that enter the body with food and drink will reach the epithelial cells of the small intestine and cause infection so that the epithelial cells will be damaged. Fluids and food that are not absorbed will be pushed out, and diarrhea will occur. Generally, diarrhea can be treated by giving ORS, zinc, and antibiotics (N. Utami & Luthfiana, 2016). Antibiotics can inhibit the growth of bacteria or kill bacterial cells. Using synthetic antibiotics is expensive and impacts the environment because the residues are difficult to decompose (Utami, 2012).

Tea (Camellia sinensis) is a type of plant popular as a beverage. Based on the refining process, tea is divided into white, green, oolong, and black tea. The catechin content of the Assamica variety has benefits (Anjarsari, 2016). Bioactives in tea are reported to have benefits such as anticancer, antioxidant, antidiabetic, treating diarrhea, and lowering blood pressure. The chemical content of tea leaves is divided into four main groups: phenolic, non-phenolic, aromatic, and enzyme. Phenolic substances consist of flavonols and catechins (polyphenols). Non-phenolic substances include proteins, amino acids, chlorophyll, other dyes, organic acids, vitamins, resins, and minerals. The enzymes contained in tea are invertase, amylase, -glucosidase, oxymethylase, protease, peroxidase, polyphenol oxidase, pectase, and chlorophyllase (Towaha, 2013). The aroma of tea is classified into four groups, namely the carboxylate, phenolic, carbonyl, and free carbonyl fractions (Anggraini, 2017). Catechins, the main bioactive of tea, can bind to the peptidoglycan layer of bacterial cell walls. In addition, cell wall permeability is disturbed, which can cause molecules to detach from the cellular membrane (Tsou et al., 2017). Siregar (2017) reported that green and white tea have higher catechin content. However, the three types of tea are very different and tend to be expensive compared to black tea (Fuzi et al., 2017). Therefore, the formulations of the three types need to be tested to get the correct formulation and perspective for commercialization.

This study aims to determine the antibacterial activity of three types of tea extract and determine the effect of temperature on tea steeping to achieve the best antibacterial effect.

2. Methods

2.1. Materials

The materials used were black, green, and white tea samples, cultures of *Escherichia coli*, *Staphylococcus aureus*, chloramphenicol, *Artemia salina*, distilled water, seawater, NB (Nutrient Broth), NA (Nutrient Agar), and disc paper (a diameter of 6 mm).

2.1.1. Procedure Sampling

Black, green, and white tea were sampled from Ciwidey Tea Garden in Bandung. Samples were taken as dry tea leaves processed into black, green, and white tea. The sample is then crushed with a mixer. The sample is then filtered through 80 mesh.

2.1.2. Moisture Content ([AOAC], 2012)

The empty mass of the sealed porcelain cup was dried in an oven for 1 hour at 105 °C, and the cup was cooled in a desiccator for 30 minutes, after which it was weighed. In addition, no less than 3 g of simplicity from three types of tea leaves were placed in each cup, after which the total weight was measured. The porcelain cup containing Simplicia was placed back into an oven at 105°C for 3 hours. This weighing was repeated three times until a constant weight was obtained. The test was carried out in triple. Then, the water content is calculated using the following formula:

$$\text{Moisture content (\%)} = \frac{M2 - (M3 - M1)}{M2} \text{ x } 100\%$$

Information: M1: cup weight, M2: sample weight, and M3: weight of cup+dry sample

2.1.3. Extraction (Chapagai et al., 2017 Modification)

Extraction was carried out using the maceration technique. Tea leaf extract was made by weighing 10 g of each tea leaf. The solvent used in this maceration technique is 600 mL of distilled water. Extraction was carried out with three different temperature treatments, namely 70ºC, 100ºC, and 121ºC, or put in an autoclave for 15 min. It was then filtered with filter paper under vacuum and then concentrated with a rotary evaporator until the extract became dry. The resulting residue was then calculated as yield and used in the antibacterial assay.

2.1.4. Determination of Extract Yield ([AOAC], 2012)

The extract yield can be determined by calculating the ratio between the product's weight after extraction and the initial weight before extraction. The calculation of yield is as follows:

$$\text{Yield (\%)} = \frac{\text{dry weight}}{\text{simplicia weight}} \text{ x } 100\ \%$$

2.1.5. Preparation of Black, Green, and White Tea Extract Solutions (Modification of Shabri & Rohdiana, 2016)

Bottles as containers, measuring flasks, and pipettes are cleaned first. Then, a solution of black tea, green tea, and white tea extract was made with a concentration of 60000 ppm to test the optimization of the extraction temperature. The extract solution was then transferred to a vial.

2.1.6. Making NB (Nutrient Broth) and NA (Nutrient Agar) Media

A total of 0.26 g of NB and 2.8 g of NA were weighed. The medium was then dissolved in 10 ml of NB and 100 ml of NA in an Erlenmeyer flask. The Erlenmeyer was covered with cotton, aluminum foil, and plastic wrap. The mixture was then homogenized and heated on a hotplate. Then autoclaved for 15 minutes at 121ºC. After autoclaving, the NA medium was poured into a petri dish for the antibacterial test medium, while the NB medium was kept in an Erlenmeyer for bacterial rejuvenation. Rejuvenation and pouring of the media are carried out in laminar airflow. Then, the media is tightly closed to prevent contamination from the outside.

2.1.7. Bacterial Rejuvenation (Modification of Milanda et al., 2021)

A total of 1 ose of Escherichia coli and Staphylococcus aureus cultures were inoculated into different NB media. The bacteria were kept in a shaking incubator at 37 oC for 24 hours. After the test, the bacteria reached a turbidity of 0.5 McFarland. Bacterial turbidity was

measured with a spectrophotometer at 625 nm to obtain an absorbance of 0.08-0.10; if it was too concentrated, NB medium could be added. The absorbance is equivalent to the standard McFarland turbidity of 0.5 at a bacterial concentration of 108 CFU/mL (colony-forming unit).

2.1.8. Optimization of Temperature with Antibacterial Test (Modification of Maria & Angelina 2014)

The bacterial suspension equal to 0.5 McFarland was inoculated on the NA medium. 100 L of bacterial suspension was pipetted into each cup containing previously prepared NA media and then spread evenly. Each disk paper was immersed in a vessel containing black, green, and white tea at 70, 100, and 121 oC with a concentration of 60,000 ppm of tea, chloramphenicol, and sterile distilled water so that the plate paper was wet. The soaked plate paper was placed on the surface of the NA medium inoculated with bacteria. It was then incubated at 37 oC for 24 hours for bacterial growth. Then, the formed clear zone was observed to determine the inhibitory ability of the three tea extracts, and the average diameter of the inhibition zone was measured using the thickness. Experiments were performed with two types of bacteria. Cytotoxicity Test with Shrimp Lethality Test (BSLT) (Modification of Meyer et al., 1982) Shrimp eggs with Arthemia Salina extract are placed in a jar and seawater is added. The aerator is placed in the jar. Eggs were incubated for 48 h. Preparation of Test Stock Solution. The test solution was prepared by dissolving the extract in seawater. The test solution was prepared using several concentrations of 1000, 2000, 4000, 6000, 8000, and 10000 g/mL.

2.1.9. BSLT Test

The bioactivity test was performed on a plate with 48-hour shrimp Artemia Salina Leach larvae. The test plate is calibrated with 5 ml of seawater and marked with a marker. 1 ml of seawater was placed on the test plate. Ten 24-hour shrimp larvae were placed on the test plate. 0.5 ml of the test solution was added to the plate and calibrated to 5 ml with seawater so that the concentration of the test solution was 100, 200, 400, 600, 800, and 1000 g/ml. Larvae were incubated for 24 h, and the number of dead larvae was counted. The LC50 value was determined by Probit analysis.

2.1.10. Black Tea, Green Tea, and White Tea Extract Formulation Test

Preparation of Test Stock Solution (Ouedrhiri et al., 2016). First, a solution of 1800 ppm black, green, and white tea was made. This concentration is determined from the LC50 value resulting from the BSLT test. Furthermore, different tea solution mixtures were made using the simplex-centroid design method (Table 1).

2.1.11. Antibacterial Test (Modification of (Maria & Angelina, 2014)

The bacterial suspension equal to 0.5 McFarland was inoculated on NA media using a scatter plate. 100 L of bacterial suspension was pipetted into each cup containing previously prepared NA media and then spread evenly. Each disc paper was immersed in a vessel containing black, green, and white tea extracts at 100 oC. The soaked disk paper was placed on the surface of the NA medium inoculated with bacteria. It was then incubated at 37C for 24 hours for bacterial growth. The clear zone formed was then observed to determine the inhibitory power of the three tea-type preparations, and the average diameter of the inhibition zone was measured using the thickness. The tests were carried out with two bacteria types, *Escherichia coli* and *Staphylococcus aureus*.

Tabel 1 Simplex-centroid design tea solution mix

Number Eksperimental Test	Black Tea (ppm)	Green Tea (ppm)	White Tea (ppm)
1	0	0	1800
2	0	1800	0
3	1800	0	0
4	0	900	900
5	900	0	900
6	900	900	0
7	600	600	600

2.2. Modeling Analysis

The modeling analysis was carried out after the response from the formula test to the antibacterial test. For this purpose, the data of the measurement results of the inhibition zone are entered into the program Design Expert 10.0® (Tests). The input data results for each response to all formulas are then analyzed using Design Expert 10.0®. At this point, the program Design Expert 10.0® provides a polynomial model that fits the results of the measurements of corresponding areas of the inhibition zone.

3. Results and Discussion

3.1. Results

The water content of simplicia of black, green, and black tea was measured gravimetrically. The average water content (Figure 1) produced by black tea is 4.41%, green tea is 6.686%, and white tea is 6.334%. These results indicate that 100 g of simplicia black, green, and white tea contained 4,411, 6,686, and 6,335 g of physically bound water that can be removed by heating at a temperature of about 105oC.

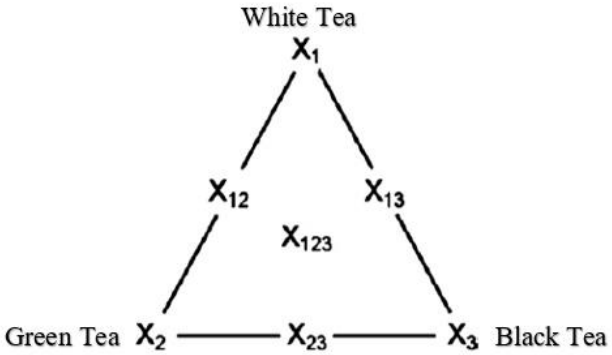

Figure 1. Mixture design for the formulation of three types of tea extract

The extract yields are shown in the graph in Figure 2. The yields of the most extensive tea extracts at 70 C were black tea, white tea, and green tea extracts, which were 26.522%, 18.385%, and 14.502%. The yield of tea extract at a temperature of 100 C from the largest is black tea leaf extract, green tea, and white tea, which is 24.026%, 16.18%, and 11.531%. As for the extract at a temperature of 121 C, the most considerable extracts were black tea leaf extract, green tea, and white tea, with yield values of 32.674%, 32.016%, and 23.155%.

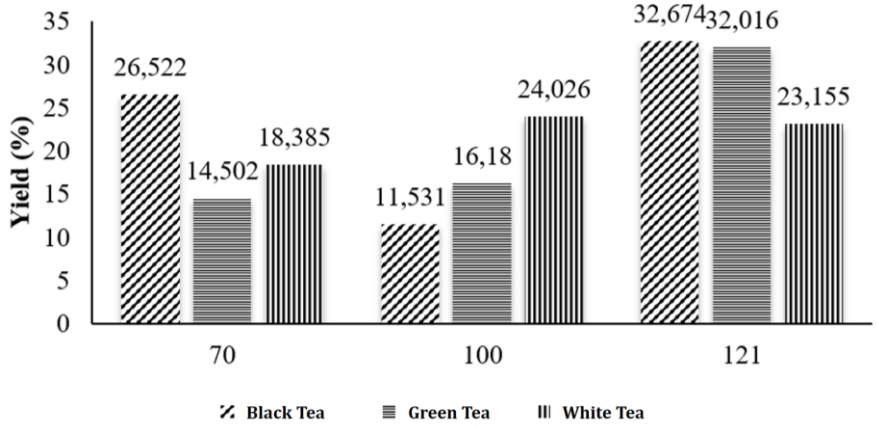

Figure 2. Yield of black tea leaf extract, green tea, and white tea

Figure 3 shows the results of zones of inhibition against *E. coli* produced by three different types of tea extracts at different maceration temperatures. Figure 4 shows the inhibition zone produced against *S. aureus* with different maceration temperatures. The highest inhibition zone was produced by the maceration temperature of 100°C from the extracts of the three types of tea. Tests on E. coli showed an inhibition zone of 17.03 mm, higher than that of S. aureus of 11 mm.

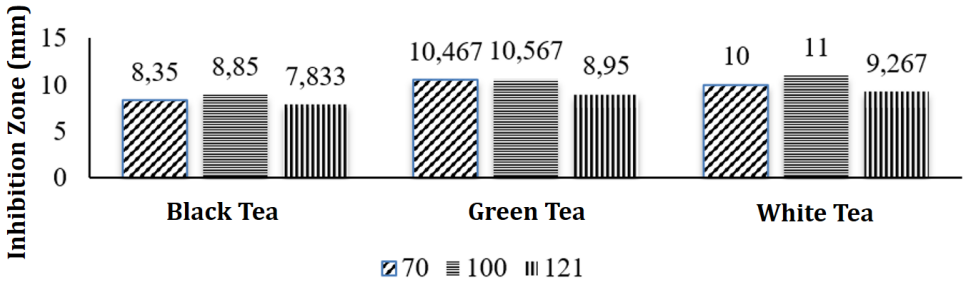

Figure 3. Results of zones of inhibition against E. coli produced by extracts of three different teas at different steeping temperatures

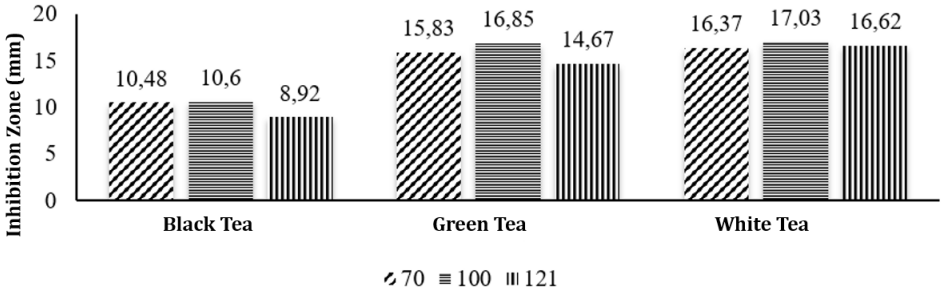

Figure 4. The inhibition zone produced against S. aureus with different maceration temperatures.

The cytotoxicity test of the tea extract was performed using the BSLT (Brine Shrimp Lethality Test) method. The experiment was conducted by observing the mortality of Artemia Salina shrimp larvae when they were given a black, white, and green tea solution. The tea samples used were macerated at a temperature of 100^0C. The result of larval mortality was measured by the value of Lethality Concentration 50 (LC_{50}) in g/mL. The LC_{50} value indicates a concentration that can kill as much as 50% of the shrimp larvae population. This

concentration value is obtained by calculating probit analysis. The LC50 values produced by black, green, and white tea were 1762.69 g/mL, 1463.24 g/mL, and 1243.38 g/mL (Table 2).

Table 2. LC_{50} value of tea extract

Sample	Value LC_{50} (µg/mL)
Black Tea	1762.69
Green Tea	1463.24
White Tea	1243.38

Zones of inhibition of extract formulations and individual extracts showed conflicting results. Figure 6 shows the results of the formulation of extract anti-S. aureus, positive control, and negative control. Zones of inhibition produced by each formulation and the control could significantly prevent the growth of E. coli bacteria. White tea samples (9.75 mm) produced the largest zone of inhibition at a significant pandlt level; 0.05. Duncan's analysis showed the homogeneity of white tea samples with white, green, and black tea formulas. This can be seen in the same letter in the graph, which shows no significant difference at the 5% level. Figure 7 shows the mathematical model in the ternary diagram for the response of the inhibitory zone of the extract formula to *E. coli*. It can be seen in Figure 7 that a single sample of white tea produced the highest inhibition zone. The blue to yellow color indicates the inhibition zone from lowest to highest.

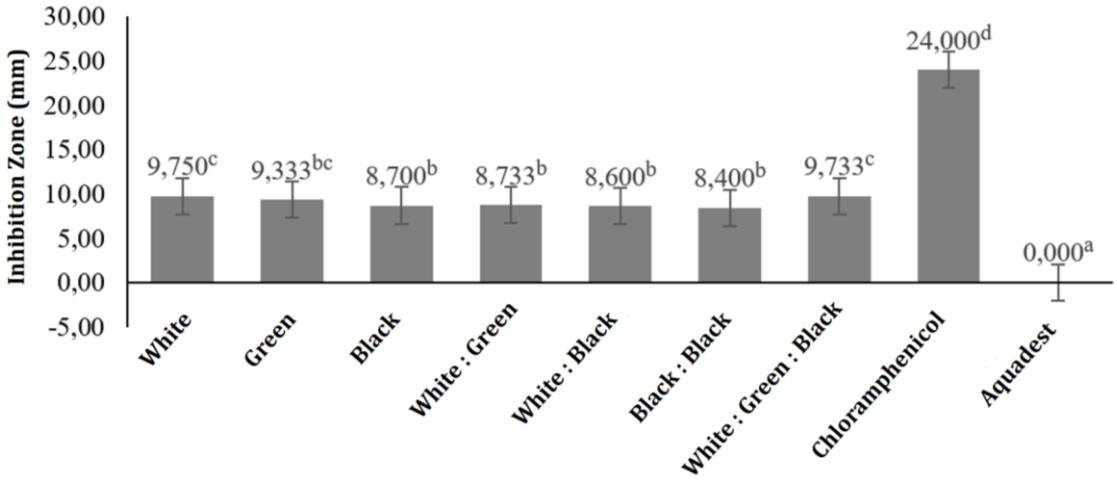

Figure 6. The results of the inhibition zone of the extract formula, positive control, and negative control against *S. aureus*.

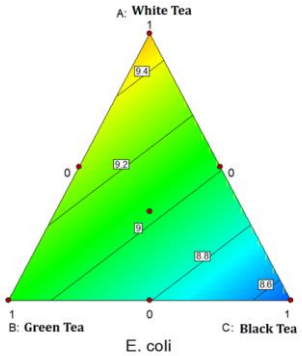

Figure 7. shows that a single sample of white tea produced the highest inhibition zone. The blue to yellow color indicates the inhibition zone from lowest to highest.

3.2. Discussion

Moisture content analysis can be done using drying, distillation, and Karl Fischer titration methods (Atma, 2018). The moisture content of black, green, and white tea was dried using oven drying. The principle of this method is to remove the sample's water content by heating it to 105 oC so that the physically activated air in the sample evaporates and a constant weight is obtained. (Harun & Rahmawati, 2022). Tea is a hygroscopic material that quickly absorbs air from the air during storage. Air absorption will increase tea's water content (Zayadi et al., 2016). Regulation No. 12 of 2014 of the Food and Drug Administration of Indonesia (BPOM) on Quality Requirements for Traditional Medicines states that the allowed water content of simplicia is less than 10% (BPOM, 2014). Figure 1 shows the air content in black, green, and white tea, 4.411%, 6.686%, and 6.335%, respectively. Based on Yenita A. (2018), the water content of green tea is 9.513%, and black tea is 7.697%, and based on the research of Widyasanti et al. (2018), the water content of white tea is 6.9%. The water content produced from each type of tea is different. This is due to the different processing processes for each type of tea. According to Kusumaningrum et al. (2013), those who were processed through the enzymatic oxidation process had lower air content than those who did not undergo enzymatic oxidation. Oxidation by this enzyme changes the tannin compound into its derivative compounds, namely the arubighin and theaflavin. The tea that undergoes this enzymatic oxidation is black tea. Tannins contained in tea leaves are condensed tannins, so the presence of condensed tannins is forgotten; the air in the tea leaves will also evaporate. The enzymatic oxidation process by the polyphenol oxidase enzyme can cause many compounds to be lost, one of which is air (Moon et al., 2020).

The water content of black, green, and white tea samples has met the criteria set, which is no more than 8% by BPOM (2014), which is no more than 10%. This is because the analysis is carried out directly when the sample has arrived and does not go through a long storage process that allows the air in the air to be absorbed by the sample (Zayadi et al., 2016). Water content below 10% will have a long shelf life; the decay process by bacteria and fungi can inhibit it, making it more stable and resistant during storage (BPOM, 2014). Water content in simplicia that exceeds 10% can undergo decay and affect texture, taste, and shelf life (Chandra et al., 2013). The difference in water content is also caused by the withering time of each type of tea. This withering process can reduce the water content by 55-70% based on the evaporation process, both by air and exhaled heat (Kusumaningrum et al., 2013). The long withering process will reduce the water content relatively high because the evaporation that occurs is increasing (Praharwati, 2015). The withering process of green tea takes about 5 minutes, while black tea lasts for 6-18 h (Sugiarto, 2013). This is consistent with the study's results, showing that green tea's water content is higher than that of black tea. White tea's water content is lower than green tea's, and this result does not correspond to the theory because the production process is shorter than green tea's, only by withering and drying. This is because there are differences in the processing processes of each factory. Therefore, the results of this study differ from the theory (Fuzi et al., 2017).

Extraction is a process that aims to extract the chemical components contained in a material using a specific suitable solvent. The effectiveness of the extraction depends on the solvent used. Several things must be considered when choosing a solvent, including toxicity, selectivity, polarity, and solvent price (Agustina et al., 2018). The principle of this method is the diffusion of the filtered liquid into the plant cells containing the active ingredient. This diffusion results in a difference in osmotic pressure inside and outside the cell. Due to osmotic pressure, active compounds are forced out (Untari et al., 2014). Extraction is done by maceration using water as a solvent. Water is a readily available, inexpensive, and harmless solvent that is safe for food. However, evaporation takes a long time because it has a higher boiling point than other solvents (Warnasih & Hasanah, 2019). The temperatures during maceration were 70, 100, and 121^{o}C with an immersion time of about 15 minutes. The ratio of

tea and water used is 1:60 b/v. This is because tea is generally made using 70^{o}C (dispenser) or 100^{o}C (boiling water). Autoclave extraction was performed to determine the difference in the extracted antibacterial compounds since catechin compounds dissolve poorly in cold water but well in hot water (Yeni et al., 2014). The duration of brewing there is no special provision for the length of time in brewing. However, if the tea is brewed too quickly, the taste and flavor of the tea will be less visible, while steeping for too long will have a more bitter taste (Shuyuan et al., 2017). Based on the research of Tram (2015), maceration of tea with a ratio of 1:60 w/v produced the highest polyphenols. The ratio of solvent to extract affects the extraction yield of plant bioactivity. The higher the solvent-to-solute ratio, the higher the extraction yield. However, the content of soluble solids will decrease (Vuong et al., 2011).

The yield of the extraction results is shown in Figure 2. Based on Rohadi et al. (2019), the extract produced by maceration of tea at a temperature of 60oC yielded 14.80% in black tea, 6.72% in green tea, and 2.24% in white tea. High solvent temperature can increase extraction efficiency because heat can increase cell wall permeability, solubility, and dispersion of extracted compounds and reduce solvent viscosity. However, high temperatures can also degrade polyphenolic compounds (Maslukhah et al., 2014). Differences in yield results were attributed to solvent type, material weight to solvent volume ratio, extraction time, temperature, and particle size (Sabathani et al., 2018). Temperature optimization is a way to get the best temperature conditions. The optimal temperature is chosen by testing the antibacterial drug against *Escherichia coli* and *Staphylococcus aureus*. *Escherichia coli* is a gram-negative bacterium always present in humans' and animals' digestive tracts (Melliawati, 2009). These bacteria are facultative anaerobes, live in the range of 20-40^{o}C, and are optimum at 37^{o}C (Yogi Rabani RS & Elza Fitriani, 2022). *Staphylococcus aureus* is a Gram-positive bacteria found in various parts of the human body, such as skin, hands, digestive tract, and hair (Kurniadi et al., 2017). These two bacteria were chosen because they cause diarrhea (Omwenga et al., 2011). Diarrhea can occur due to more than one mechanism. Diarrhea caused by bacterial infection has at least two mechanisms: increased intestinal secretions and decreased absorption in the intestine. Bacterial infections cause inflammation and release toxins. The mechanism of diarrhea caused by bacteria includes attachment of bacteria to epithelial cells with or without mucosal damage, mucosal invasion, and production of enterotoxins or cytotoxins. One bacterium may use one or more mechanisms to impair intestinal mucosal defenses (Muttaqin et al., 2018).

The disc diffusion method is often used to test the antibacterial activity of plant extracts. This method can predict the antibacterial activity of an extract solution at a given concentration based on the zone of inhibition of an agar test medium. Disc diffusion's advantages are that the test is simple and the cost is low (Ouedrhiri et al., 2016). Antibacterial testing in this study was intended to select the optimal temperature during maceration for the formulation test of the three teas. This test uses a concentration of 60,000 ppm, which is the result of research by Rohdiana et al. (2013), which used a concentration of 60,000 ppm showed the highest inhibition zone, the inhibition zone in green tea Tong Tji 9.4 mm and black tea 7.8 mm. An antibacterial agent is a compound that can kill or inhibit the growth and reproduction of bacteria. The antibacterial substance is usually present in the body as a secondary metabolite (Sartika et al., 2013). Antibacterial activity was indicated by the inhibition zones of *E. coli* (Figure 4) and *S. aureus* (Figure 5) produced by the three types of tea with different maceration temperatures. The tea with the highest inhibition zone in the E. coli test was white tea, with an inhibition zone of 17.03 mm at 100oC maceration temperature. The highest inhibition zone of S. aureus was white tea, with an inhibition zone of 11.00 mm and a maceration temperature of 100 C. This optimal temperature (which produces the highest zone of inhibition) was chosen to test the antibacterial agents of three types of tea preparations. A positive control in the form of chloramphenicol and a negative control in the form of distilled water was used in this test.

Chloramphenicol is a broad-spectrum antibiotic effective against Gram-positive and Gram-negative bacteria (Prisinda et al., 2021).

Maceration at 100^{o}C showed the highest inhibition zone. This is presumably because antibacterial compounds such as polyphenols (catechins) are soluble in water solvents. The inhibition zone at the maceration temperatures of 70^{o}C and 121^{o}C was smaller than at 100^{o}C. According to Suzuki et al. (2003) and Wazir et al. (2011), maceration with higher temperature, higher total phenol, and high temperature can increase phenolic solubility and release bound phenolic compounds caused by damage to cell elements. However, temperatures that are too high can cause polyphenols (catechins) to epimerize at high temperatures (Rohdiana & Alghifari, 2015). According to research by Rohdiana et al. (2013), the polyphenol content soaked for 3 minutes was higher at 75^{o}C than at 95^{o}C. This is thought to be due to catechin compounds' degradation, epimerization, and oxidation. This epimerization occurred at a temperature of 120^{o}C (Nurjanah et al., 2016). The diameter of the zone of inhibition produced by white tea was greater than that of green and black tea. This is due to differences in the concentrations of antibacterial compounds in the three types of tea. According to Fakheri et al. (2015), the content of phenolic compounds in white tea and green tea is higher than in black tea. This happens because of the short processing and the absence of oximatic process. The phenolic compounds (catechins) in black tea will be oxidized during the oximatic process to produce two primary pigments: the aflavins and the arubigins (Mitrowihardjo et al., 2012). Almajano et al. (2008) showed a higher yield of antibacterial activity of unfermented tea than that of fermented tea. High antibacterial activity was observed in samples with high phenol content (Fakheri et al., 2015).

The resulting inhibition zone was higher in Escherichia coli bacteria compared to Staphylococcus aureus. This happens because Staphylococcus aureus is a bacteria. The cytotoxicity or bioactivity test was performed using the shrimp *Artemia Salina* Leach larvae. The shrimp mortality test is often used in bioassays and preliminary tests of compounds with bioactive content and pharmacological effects (Meyer et al., 1982). Larval mortality was measured as lethal concentration 50 (LC50) in g/ml. This LC50 value indicates a concentration that can kill as much as 50% of the shrimp larvae population. This concentration value is obtained by calculating probit analysis. The LC50 values produced by black, green, and white tea were 1762.69 g/mL, 1463.24 g/mL, and 1243.38 g/mL (Table 3). The difference in the LC_{50} value may be due to differences in the bioactive compounds extracted from the sample (Theresia et al., 2019). The highest LC_{50} value was then used to test the tea extract formula for antibacterial activity. The toxicity level of the extract was classified as highly toxic with an LC50 of 30 ppm, toxic with an LC50 of 31 ppm, 1000 ppm, and non-toxic with an LC50 of 1000 ppm (Kaban et al., 2016). Samples that have this bioactivity can be seen from shrimp larvae's death. The LC50 value is a concentration that can kill 50% of the test animal population. This toxicity test has a broad pharmacological spectrum with a reliable, rapid, and inexpensive procedure (Mclaughlin et al., 1998).

One way to determine the potential of a compound as an alternative to new drugs is to conduct a biological toxicity test. The principle of the toxicity test is that bioactive components are always toxic in high doses and drugs in low doses. The toxicity of the extract results from the concentration of compounds in the extract (Agustini & Setyaningrum, 2017). The toxicity test was performed on 4-hour-old larvae. Larvae at this age have a low resistance to the environment. If it is more than 48 h, other factors such as oxygen content, nutritional needs, and light also influence. Two ways can cause shrimp larvae's death are inhalation and diffusion. Inhalation is the entry of toxic substances into the body through inhalation, while diffusion occurs through thin tissue of larval skin (Sukardiman., 2004). The formula for extracts of black tea, green tea, and white tea uses a total concentration of 1800 ppm, where this value is derived from the LC_{50} value. The test was carried out by testing each tea with a concentration of 1800 ppm, while for a mixture of two types of tea using a concentration ratio of 900 ppm: 900 ppm

and for a mixture of three types of tea using a concentration ratio of 600 ppm: 600 ppm: 600 ppm. The tea extract used is tea from maceration with a temperature of 100ºC. This test was carried out on *Escherichia coli* and *Staphylococcus aureus* bacteria. Chloramphenicol at a concentration of 1800 ppm was used as a positive control. Zone of inhibition results were then analyzed using Design Expert 10.0® software and the Simex centroid design-mixture design method. Antibacterial activity tests of black tea, green tea, and white tea extract formulations are shown in Figures 6 and 8. The antibacterial test results showed that single extracts and mixed extracts had a zone of inhibition against both *E. coli* and *S . aureus*. Based on Duncan's analysis, there was a significant difference ($p<0.5$) in each mixture extract and single extract as an antibacterial agent. Duncan's test results showed the homogeneity of the sample's ability to inhibit bacterial growth. The homogeneity is shown in Figure 6 and Figure 8 with superscript letters; the same letters indicate a homogeneous response from each sample.

The zone of inhibition generated from each sample is different. A single sample of white tea showed the highest inhibition zone in the test against E. coli. The three-tea mixture sample showed the highest zone of inhibition in the test against S. aureus. All samples tested against E. coli and S. aureus had lower antibacterial activity than positive controls. This indicates that the sample has lower antibacterial activity than antibiotics (chloramphenicol), even at the same concentration. Using the simple centroid design method, the resulting inhibition zones were analyzed using Design Expert 10.0. The input data results will provide a polynomial model that is in accordance with the results of the response measurements used. A polynomial model can be determined based on the value of the standard deviation, the estimate of the sum of squares, and the prediction of R2. A good polynomial model has a low standard deviation value and prediction of the number of squares and a high predictive value of R2 (Abdullah & Chin, 2010). Based on the regression coefficient of the test on E. coli from the three extracts used, it showed that white tea (9,498) had higher inhibitory activity than green tea (9.085) and black tea (8.525). Based on the regression coefficient of the test on S. aureus from the three extracts used, it was shown that white tea (9.211) had higher inhibitory activity than green tea (8.718) and black tea (8.592). The coefficient in the equation obtained has a positive value. This shows that all components are complementary (Abdullah & Chin, 2010). Contour plots describe the proportion of the best mix or mixed model to the response (Sahin et al., 2016). The contour plot displays the response region generated by the sample and the upper and lower limits of the resulting inhibition zone. The response area on the contour plot is limited by the upper and lower limits, represented by different colors. In this analysis, the color for the upper limit is red, and the lower limit is blue. The blue-to-red color indicates an increasing response from the resulting inhibition zone.

Figure 7 shows the green-to-yellow color in the area around the white tea. This indicates that the antibacterial activity against *E. coli* was not significantly different. However, white tea samples produced the best response, which could be seen from the yellow color in the white tea area. Figure 7 shows almost the same color, namely green to yellow. These results indicate that the response is similar against S. aureus. The inhibition response area analysis results of each sample showed no significant antibacterial activity ($p<0.05$) against the simplex centroid design model. This can be seen in ANOVA Tables 3 and 4, generated from the linear model showing $p>0.05$. The single extract of white tea inhibited the growth of E. coli and S. aureus very well. The mixed extract of the three types of tea inhibited S. aureus well, while E. coli, it was pretty good, although it was inferior to the white tea extract. Mixture design creates a system consisting of a mixture of components whose total number is constant. The resulting response is a function of the relative proportion of each component in the system. Mixture design can use two or more components. Increasing the number of components will increase the dimensions of space used to describe the mixture. The simplest object that represents the dimensions of the mixture is called a simplex. Simplex-lattice, simplex-centroid, or simplex-centroid with axial design can be used in mixture designs (Şahin et al., 2015). The simplex-

centroid design is mixed with a dot pattern on the sides. This method pays attention to the effect of single components and two-component combinations and studies three-component combinations (Şahin et al., 2015).

Greenwell and Rahman (2015) and Rahman (2016) state that tea extraction with water as a solvent can extract phenolic compounds, alkaloids, flavonoids, steroids, saponins, and tannins. The presence of antibacterial activity is due to phenol and flavonoids, which are the main components of antimicrobial agents. These compounds can damage the cell walls of bacteria. Damage to the cell wall can kill bacteria (Pillet et al., 2016). Other compounds capable of acting as antibacterial are alkaloids (Devi et al., 2012). Phenol compounds can work by denaturing cell proteins and damaging cell membranes. Depending on the concentration used, phenol compounds can be bactericidal or bacteriostatic (Arifin et al., 2017). The mechanism of action of phenol as an antibacterial agent poisons the protoplasm, damages and penetrates the cell wall, causing cell leakage, and precipitates bacterial cell proteins at high concentrations, while at low concentrations, inhibits enzyme synthesis. According to Arshad and Batool (2017), phenolic compounds can break through cell walls to break the cross-links of peptidoglycans. By breaking through the cell wall, phenolic compounds cause the cell to leak nutrients by damaging the hydrophobic bonds of components that make up cell membranes, such as proteins and phospholipids, and dissolving the binding hydrophobic components, which increases membrane permeability. Damage to this membrane will inhibit the activity of biosynthetic enzymes needed in bacterial metabolism.

Flavonoids can damage the membranes of bacterial cells. These flavonoids will form a complex compound between groups of flavonoid compounds with extracellular proteins in cells. In addition, it can also inhibit the energy metabolism process in bacterial cells by inhibiting the bacterial cell respiration system (Asif, 2017). Alkaloid compounds have a blocking mechanism that disrupts the peptidoglycans of bacterial cells, so the cell wall layer is not fully formed and causes cell death. Othman et al. (2019) state that the alkaloid compound has a nitrogen-containing base group that reacts with and affects bacterial DNA. This reaction causes changes in the structure and composition of amino acids, which causes damage and promotes bacterial cell lysis. A positive control antibiotic in the form of chloramphenicol at a concentration of 1800 ppm was used in the antibacterial activity test. Chloramphenicol is a broad-spectrum antibiotic effective against gram-positive and gram-negative bacteria (Prisinda et al., 2021). In addition, distilled water was used as a negative control. Aquadest is also a solvent used to prepare test solutions when this aquadest cannot prevent bacterial growth. Jannata et al. (2014) classified an inhibition zone of 5–10 mm as a low antibacterial agent. Based on how they work, antibacterials are categorized into bactericidal and bacteriostatic. Bactericidal is an antibacterial that works to kill bacteria, while bacteriostatic works to inhibit their growth. Some antibacterials will be bacteriostatic in low concentrations and bactericidal in high concentrations (Dwicahyani et al., 2018).

The zone of greatest inhibition was shown in Escherichia coli bacteria. Gram-positive inhibition response is stronger than gram-negative. According to Panawala (2017), this may be due to differences in the cell wall composition of Gram-positive and Gram-negative microbes. Gram-positive cell walls contain many teichoic and tectonic acid compounds and polysaccharide molecules. Navarre and Schneewind (1999) state that these components protect cells from enzyme lysis activities, while other substances determine cell reactions in Gram staining. Silhavy et al. (2010) wrote that Gram-positive bacteria contain thicker peptidoglycan than Gram-negative bacteria. The porin protein determines the permeability of the outer membrane of Gram-negative bacteria. Porins are the gateway to several polar molecules. Hydrophilic molecules, more minor than the diameter of the porin, will enter through the porin (Ude et al., 2021). The effectiveness of antibacterial compounds is based on the zone of inhibition around the disk paper, which is saturated with a specific concentration of the substance. The resulting zone of inhibition varies greatly depending on the dispersion of the

substance, the type of medium, and many other factors (Kowalska-Krochmal & Dudek-Wicher, 2021). Trianes et al. (2022), inhibition zone formation is influenced by incubation temperature, time, and microbial concentration. Therefore, the growth inhibitory efficiency of the agar medium formed during this study was also affected by temperature and incubation time, as well as the concentration of microbes that produced inhibitory zones for these two bacteria. Also, the diameter of the inhibition zone is not always directly proportional to the increase in sample concentration because there are differences in the diffusion of antibacterial compounds in the agar medium, and their activity is affected by their types and concentrations (Wang et al., 2022).

4. Conclusion

Black tea, green tea, and white tea water extracts had antibacterial activity at all maceration temperatures (70^{o}C, 100^{o}C, and 121^{o}C). The most optimal temperature that produces the highest inhibition zone is the maceration temperature of 100^{o}C. The zone of inhibition against *Escherichia coli* was 17.033 mm from white tea, and against *Staphylococcus aureus*, it was 11.000 mm from white tea. The formula test against E. coli showed the highest performance for white tea extract samples with an inhibition zone diameter of 9750 mm. In contrast, the S. aureus test showed the highest inhibition results for white tea, tea, and black tea in the 9.65mm blocking range. The simple centroid design analysis results showed that the linear model results were not significant in the antibacterial test of *E. coli* and *S . aureus*.

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