Antibacterial Activity Of Combination Of Rosella Flower Ethanol Extract (*Hibiscus sabdariffa* L.) And Chlorhexidine Gluconate 0.2% Against *Streptococcus mutans* ATCC 25175

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Abstract. Dental caries is a problem in the human oral cavity due to the buildup of plaque, which is caused by bacteria such as *Streptococcus mutans*. Nevertheless, mouthwash may result in a parched mouth and numbness in the oral cavity. Antibacterial properties are therefore recognized in the rosella flower plant (*Hibiscus sabdariffa* L.). This research was conducted to ascertain the antibacterial activity of the combination of Ethanol Extract of Rosella Flowers (EER) and chlorhexidine gluconate (CHG) 0.2% in inhibiting the growth of *S. mutans* ATCC 25175 bacteria. The maceration method was employed in the extraction procedure, and the agar well diffusion method was followed for antibacterial testing. The concentrations of EER were 5%, 10%, and 20%. The combination of EER 5% and CHG 0.2% was made in comparison series of (1) 1:3, (2) 1:1, and (3) 3:1. Furthermore, the diameter of the inhibitory zone (DZI) and the calculation of Combination Index (CI) were analyzed. The EER at 5%, 10%, and 20% revealed DZI of 12.17 ± 1.04, 21 ± 0.87, and 32.17 ± 0.29 mm, respectively. Then, the combination series of EER 5% and CHG 0.2% of (1) 1:3, (2) 1:1, and (3) 3:1 uncovered DZI of 32.17 ± 0.58; 31.67 ± 1.89; 27.67 ± 0.76 mm, respectively, categorized as very strong. The CI showcased that a combination was antagonistic. The chemical compounds of EER 5% increased the DZI towards *S. mutans* ATCC 25175 in combination with CHG 0.2%.

Key word: antibacterial activity, dental caries, rosella flower, *Streptococcus mutans*, chlorhexidine

1 Introduction

According to 2015 data, the global prevalence of untreated dental caries in permanent teeth was approximately 34.1% (general health). The Indonesian Ministry of Health (2018) reported that approximately 57.6% of the population was affected by dental and oral health issues. The development of caries constitutes one of the issues associated with dentition. The accumulation of dental plaque that is not cleansed on a regular basis is the cause of this caries [1]. The enamel, which is the outermost part of the tooth surface, is the source of dental caries. The enamel surface will undergo crystalline demineralization as a result of the production of organic acids from the metabolism of carbohydrates (glucose) by bacteria. The end result of glucose metabolism is lactic acid, which is involved in the formation of dental caries. If bacterial activity remains constant, dental caries will develop in the dentin layer at a deeper level. An example of an activity that can reduce the occurrence of dental caries is brushing the teeth. Glucose in the teeth can be lost due to cleaning the teeth or the presence of saliva; the biofilm formed by the acid will become neutral again (buffer action). If the dentin layer of the teeth is not treated or filled, it can interfere with the nerves in the teeth and make sufferers sick [2]. One of the bacteria that can contribute dental caries is *Streptococcus mutans* especially in acid environments [3].

Additionally, chlorhexidine gluconate (CHG) is bacteriostatic in low concentrations and bactericidal in high concentrations [4]. Chlorhexidine can control dental plaque bacteria by working on the bacterial cytoplasmic membrane. Nevertheless, it induces unpleasant side effects, including a burning sensation and numbness in the oral cavity, as well as a dry mouth, which renders it less convenient to use [5].

Furthermore, the rosella plant has the potential to alleviate fever, toothache, and urinary tract disorders [6].

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Rosella plants can also function as cytotoxic agents for skin disorders such as eczema (antimicrobial or antiseptic) [7], [8]. Based on prior research, ethanol extract of rosella flower petals (EER) has the capacity to either inhibit bacterial proliferation or function as an antibacterial. The ethanol extract rosella at a concentration of 30% in gel formulation has an inhibition zone against *S. mutans* of 10.72 ± 0.540 mm [9]. As such, the combination of EER and CHG is expected to determine the effectiveness of the combination of chemical drugs with herbal medicines, reducing the dose of CHG 0.2% as an antibacterial or lowering the uncomfortable effects caused by using CHG.

2 Methods

The tools utilized in this research included analytical scales (Bell engineering®), blender (Tefal®), stirring rod, jar, stainless spoon, filter paper (Whatman), water bath (Memmert®), vortex (Thermo scientific®), autoclave (Hirayama®), glassware (Iwaki®), petri dish (Onemed®), tube, cotton swab (Sakamed®), bunsen, test tube (Iwaki®), 1.5mL Eppendorf tube (Onemed®), Laminar Air Flow (LAF) (Biobase®), micropipette (Scorex acura 825®), micropipette tip (Kairos®), incubator (Memmert®), hotplate magnetic stirrer (Thermo scientific cimarec®), ruler (Butterfly®), caliper, universal pH stick, aluminum foil (Klin Pak®), frying pan, mask (Onemed®), and handscoon (Onemed®).

In addition, the materials used in this research encompassed rosella flower petals, *Streptococcus mutans* ATCC 25175, chlorhexidine gluconate 0.2% (Minosep®), ethanol 70% p.a (Genera labora), distilled water, TSA media or Tryptic Soy Agar (Oxoid®), NA media or Nutrient Agar (Merck®), NB media or Nutrient Broth (Himedia®), 2% HCl, 2% NaOH, 2N HCl, Dragendorff's reagent, Mayer's reagent, anhydrous acetic acid, and concentrated H_2SO_4, $CHCl_3$.

2.1 Plant determination

Plant determination was carried out at the Biology Laboratory, Faculty of Applied Science and Technology, Universitas Ahmad Dahlan.

2.2 Extract

Rosella flower simplicias were collected from Wonogiri, Central Java. Simplicia was mashed using a blender. Four hundred grams of rosella powder was put into a jar, and 4 L of 70% p.a. ethanol (1:10) was added for 5 days. Then, the filtrate was filtered from the filter and re-macerated with 4 L of 70% ethanol for 3 days. The results of the maceration were concentrated using a water bath at a temperature of 50°C to produce a thick extract.

2.3 The phytochemical screening

Staining reagents were performed, such as NaOH for flavonoid identification, the Dragendroff reagent for alkaloid, distilled water for saponin, FeCl 1% for tannin, and $CHCl_3$ for terpenoid.

2.4 Antibacterial test

Bacterial activity was identified using the agar well diffusion method. Media Tryptic Soy Agar was used to culture bacteria. The bacteria were inoculated using nutrient broth and incubated at 37°C for ±24 hours, aseptically. The colony of bacteria was compared to McFarland standard 1 to get an estimated bacterial density of $3x10^8$ CFU/mL [10].

First, a stock solution was made with a concentration of 20%, 1 g of ethanol extract of rosella flower petals dissolved in 5 mL of sterile distilled water. Next, the stock solution was diluted to 5% and 10%. The grade series sample solution had been tested for antibacterial activity, and the appropriate grade was then selected to be combined with CHG 0.2%. Each combination was made into a comparison of EER and CHG by comparison of (1:3), (1:1), and (3:1), respectively, until the final volume of 1 mL. The sterile distilled water solution served as the negative control, while CHG 0.2% was the positive control. Following that, 30 µL of the sample was put into each well. This test was done in triplicate for each comparison series. The petri dishes were placed in an incubator for ± 24 hours at a temperature of 37°C. In addition, the DZI, or diameter zone, of the inhibitor is the region of the well that is either sterile or does not support the growth of bacteria. This is where antibacterial activity was observed. The strength of antibacterial activity is described in **Table 1.**

Table 1. Category of antibacterial strength [11]

Diameter zone of inhibition (mm)	Category
>20	Very strong
10-20	Strong
5-10	Medium
< 5	No response

The antibacterial activity of the combination of EER and CHG was calculated using the Combination Index (CI) formula [12].

$$CI = \frac{DZIa + DZIb}{DZIab} \quad (1)$$

Note:
DZIa : Diameter zone of inhibitor of the mean of EER
DZIb : Diameter zone of inhibitor of the mean of CHG
DZIab : Diameter zone of inhibitor of the mean of combination of EER and CHG

A CI value of less than 1 indicates that each sample produces a lower bacterial inhibitory effect than the combination or is said to have a synergistic effect. In comparison, a CI value of more than 1 suggests that the combination of samples has a lower bacterial inhibitory effect than each individual sample or is considered to have an antagonistic effect [13].

3 Result and discussion

The yield of extract was 49.16 %. The phytochemical screening based on the staining reagent is presented in **Table 2**, while the antibacterial activity is shown in **Tables 3 and 4**. Based on the experiment, EER contains flavonoids, alkaloids, saponins, tannins, and triterpenoids. Flavonoids as antibacterials have a mechanism of action by inhibiting cell wall function and bacterial energy metabolism. This compound can inhibit cell wall function by binding to extracellular proteins to form complex compounds that will damage the cell wall and cause intracellular proteins to be pulled out of the cell. Then, flavonoids can inhibit the use of oxygen in bacteria, so they cannot produce sufficient energy for cell development[14]. Alkaloids are antibacterials, as they interfere with the formation of peptidoglycan in cells, resulting in imperfect cell wall formation. As a study [15] revealed, *Peganum harmala* L flower contains alkaloids and shows antibacterial activity toward *Candida albicans* and Gram-positive bacteria (*Micrococcus luteus* and *Staphylococcus aureus*).

Table 2. The phytochemical screening of EER

Test	Reagents	Reference [16]	Result	Note
Flavonoid	NaOH 2%	Yellow	Yellow	+
Alkaloid	HCl 2N + Dragendorff	Orange precipitation	Orange precipitation	+
	HCl 2N+ mayer	White precipitation	Not visible	-
Saponin	HCl	Stable foam for 10 minutes	Stable foam 2 cm for > 20 minutes	+
Tannin	$FeCl_3$ 1%	Dark blue or blackish	Blackish brown	+
Terpenoid	$CHCl_3$ + Anhydrous acetic acid + H_2SO_4 concentrated	Brown color (triterpenoid)	Brown color	+
	$CHCl_3$ + Anhydrous acetic acid + H_2SO_4 concentrated	Blue green color (steroid)	Brown color	+

Note : + : secondary metabolites present, - : secondary metabolite absent

Saponins, as antibacterials, work by reducing the surface tension between cells or increasing the permeability of cell walls, resulting in leaks and causing intracellular compounds to come out of the cells. Saponin will diffuse into the cell through the fragile cell wall and then bind to the cytoplasm. Therefore, the cytoplasm will come out of the bacterial cell and cause cell death [17]. Water extract of *Jatropha curcas* flower that contains saponin showed a 21.5 ± 2.12 mm inhibition zone against *S. typimuruim* [18]. Besides, tannin has an antibacterial mechanism of action in the form of inactivating bacterial enzymes and causing cell lysis. Tannin compounds will inhibit or inactivate the reverse transcriptase and DNA topoisomerase enzymes, which will disrupt protein transport in bacteria so that cells do not form properly. Tannins damage cell walls because they target polypeptides, resulting in imperfect cell wall formation. As a result, bacteria will die due to the osmotic pressure that occurs [14]. In addition, triterpenoids react with transmembrane proteins in the outer cell walls, thus forming strong polymer bonds and causing cell death [19].

Table 3. The diameter zone of inhibition (DZI) of EER towards *S. mutans* ATCC 25175

Treatment	Dimeter zone of inhibition (mm)			Mean ± SD (mm)	Description
	R1	R2	R3		
Positive control	24.5	31	26.5	27.33 ± 3.33	Very strong
Negative control	0	0	0	0	None
EER 5 %	12.5	11	13	12.17 ± 1.04	Strong
EER 10 %	22	20.5	20.5	21 ± 0.87	Very strong
EER 20 %	32	32.5	32	32.17 ± 0.29	Very strong

Note: Positive Control: 0.2% Chlorhexidine, Negative Control: sterile distilled water, R: replication, SD: Standard Deviation

The antibacterial activity of EER concentrations 5%, 10%, and 20% exhibited significant differences ($p<0.05$) based on statistical analysis using Kruskal-Wallis. Since the concentration EER 5% showed strong activity, the authors continued testing the combination with CHG 0.2 %. The combination of EER and CHG 0.2% was used to determine the resulting antibacterial activity and ascertain whether it gave synergistic or antagonistic activity. The synergistic activity is indicated by the results of a larger inhibition zone diameter compared to the single use. In contrast, the antagonistic activity is indicated by the results of DZI, or the therapeutic effect provided is smaller than single use [20].

Table 4. The diameter zone of inhibition (DZI) combination of EER 5% and CHG 0,2% against *S. mutans* ATCC 25175

Treatment	Dimeter zone of inhibition (mm)			Mean ± SD (mm)	Description
	R1	R2	R3		
Positive control	28	32	29	29.67 ± 2.08	Very strong
Negative control	0	0	0	0	None
EER 5 %: CHG 0.2% 1:3	32.5	32.5	31.5	32.17 ± 0.58	Very strong
EER 5 %: CHG 0.2% 1:1	33	32.5	29.5	31.67 ± 1.89	Very strong
EER 5 %: CHG 0.2% 3:1	27.5	28.5	27	27.67 ± 0.76	Very strong

Note: Positive Control: 0.2% Chlorhexidine, Negative Control: sterile distilled water, EER 5% : Ethanol extract of Rosella 5%, 0.2 % CHG: 0.2% Chlorhexidine, SD: Standard Deviation.

The Kruskal-Wallis statistical analysis revealed significant differences ($p<0.05$) in the antibacterial activity of the combination of EER 5% and CHG 0.2%. The synergistic or antagonistic activity of EER 5% and CHG 0.2% was determined through a Combination Index (CI) calculation. The results of the Combination Index (CI) demonstrated 1.30, 1.32, and 1.51 for a combination of EER 5%: CHG 0.2% 1:3, 1:1, and 3:1, respectively, indicating that the combination of three level comparisons showed antagonistic activity. However, once the single EER 5% was compared, it gave greater DZI on the combination with CHG 0.2% even at the low portion of EER 5 % (1:3). Related to that, chlorhexidine (CHG) 0.2% can kill bacteria in the mouth by binding between the positive charge of CHG (which is cationic) and the negative charge of phosphate particles in the bacterial cell walls so that CHG can penetrate the cytoplasm and cause cell death [5], [21]. Meanwhile, EER contains secondary metabolite compounds, such as flavonoids, saponins, tannins, alkaloids, and triterpenes. These compounds have antibacterial properties with the same mechanism of inhibiting bacterial growth.

Furthermore, it is recognized that CHG 0.2% and EER possess a multifaceted antibacterial mechanism; consequently, there is the potential for an antagonistic mechanism. This might be influenced by the type or characteristics of the bacteria used in the research. In addition, CHG is not compatible with several compounds, such as chelating agents (tartaric acid, citric acid, phenolic compounds, especially tannins). Based on research [6], rosella calyx extract contains quite high levels of organic acids, such as malic acid and citric acid. Chemical compounds of *H. sabdariffa* L have the potential to be developed in the field of dentistry [22]. Nevertheless, the interaction of EER in combination with CHG should be explored more for the effectivity and toxicity.

4 Conclusion

The ethanol extract of rosella flower (EER) contains flavonoids, alkaloids, saponin, triterpenoid, and tannin. The antibacterial activity of the combination of EER and CHG, at 0.2%, exhibited strong activity, yet the combination was antagonistic based on the CI index.

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