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The Minimum Bactericidal Concentration (MBC) of Green Okra (*Abelmoschus esculentus*) Extract Against Root Canal Bacteria *Staphylococcus aureus in vitro*

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ABSTRACT

Introduction: *Staphylococcus aureus* (*S. aureus*) are pathogenic bacteria which can be found as high as 20% embedded within an infected pulp and root canal. Once infected, they must undergo immediate intensive treatment which must be accompanied by irrigation to kill *S. aureus* colonies. NaOCl 2,5% and EDTA 17% are commonly used solutions in root canal treatment. Although common, NaOCl 2,5% and EDTA 17% have been reported to have negative side effects if the periapical tissue is penetrated. Therefore, a natural irrigation solution such as green okra (*Abelmoschus esculentus*) is needed as an alternative because it is abundant in secondary metabolic compounds which serve as an antibacterial agent. This research was meant to establish the Minimum Bactericidal Concentration (MBC) of green okra extract (GOE) against the colonies of *S. aureus*.
Methods: This study was in vitro laboratory experimental research with a Posttest Only Control Group Design. The samples used in this research were, NaOCl 2,5%, EDTA 17%, aquadest, and GOE of 6,25%, 12,5%, and 25% concentrations respectively. MBC was determined by counting the number of colonies that grew on the Petri dish containing Mueller-Hinton agar containing 0,1 ml of *S. aureus* suspension.
Results: The average growth of *S. aureus* colonies in GOE of 6,25%, 12,5%, and 25% concentrations, were 35, 11, and 0 colonies respectively. Aquadest which served as negative control had 177 colonies. Meanwhile, NaOCl 2,5% and EDTA 17% which served as positive control didn't show any signs of *S. aureus* colonies growth.
Conclusions: The MBC of GOE against *S. aureus* bacteria was found at 25% concentration. The GOE of 25% concentration was as effective as NaOCl 2,5% and EDTA 17% in killing *S. aureus* colonies.

Keywords: Minimum bactericidal concentration, green okra, *Abelmoschus esculentus*, *Staphylococcus aureus*, Root canal treatment.

INTRODUCTION

Staphylococcus aureus (*S. aureus*) with a concentration of as much as 20% are found embedded within the infected pulp and root canal of a tooth [1]. The colony of *S. aureus* in the root canal could then enter the blood circulatory system, causing the formation of microthrombus, which could lead to blood

clot [2]. In addition, Bahl et al. (2014) reported that the presence of *S. aureus* plays a part in orofacial infection, such as dentoalveolar abscess [3]. Therefore, the infected tooth root canal needs to undergo root canal treatment (RCT) immediately [4]. RCT consists of 3 stages (triad endodontic), namely biomechanical preparation, sterilization, and obturation. As *S. aureus* could penetrate dentinal tubules, the preparation stages must be accompanied by irrigation [5].

Natrium hypochlorite (NaOCl) and Ethylenediaminetetraacetic acid (EDTA) are commonly used irrigation materials during RCT. NaOCl with a concentration of 2.5% has an excellent antimicrobial property and can dissolve organic tissue (Type-I collagen) [6]. However, Faras et al. (2016) reported that the usage of NaOCl 2.5% as an irrigation material could cause the formation of lesions on the soft tissue of oral cavity, as well as swellings [7]. This is possible because the irrigation of NaOCl into the root canal penetrated the periapical tissue, which resulted in an increase of the permeability of the blood vessels. This in turn, leads to the release of chemical mediator, namely histamine, causing swelling and direct hemorrhage [8]. EDTA 17% is an irrigation material that is used along with NaOCl 2,5% for the treatment of root canal [9]. EDTA 17% has the ability to demineralize and eliminate inorganic components (calcium and ion phosphate) and debris as a result of the smear layer produced during instrumentation [10]. However, Doumani et al. (2017) wrote that any leakage of EDTA 17% into the periapical tissue during root canal preparation could inhibit the functionality of macrophage and changing the inflammation responses to the periapical tissue [11].

Natural plants were utilized with the hope of being safer, while having minimal side effects when compared to chemical products. As an example, green okra (*Abelmoschus esculentus*) as an alternative material for the irrigation of RCT [12]. Based on previous phytochemistry research, the extract of green okra with 100% concentration contains several antibacterial compounds such as alkaloids (6.88%), flavonoids (5.01%), tannins (3.81%), saponins (4.02%), and terpenoids (2.95%), which could be beneficial in reducing or even eliminating the bacteria colonies found within the infected root canal, namely *S. aureus* [13].

The previous research regarding the inhibition zone of green okra extract (GOE) to *S. aureus* were done by Vitasari et al. (2022). The results exhibit that GOE 6.25% has an inhibition zone of 14.51 mm [14]. Another research done by Lutfi et al. (2020) shows that GOE has an antibacterial effect towards *Aggregatibacter actinomycetemcomitans* (*Aa*). The research proved that minimal bactericidal concentration (MBC) of GOE to *Aa* is at 6.25% [15]. MBC is the lowest minimum concentration that could eliminate ≥99.9% of the bacteria population [17]. The goal of this research is to establish the MBC of GOE to *S. aureus*, as well as its effectiveness when compared to NaOCl 2.5% and EDTA 17% towards S. aureus of GOE to *S. aureus*, as well as its effectiveness when compared to NaOCl 2.5% and EDTA 17% towards *S. aureus*.

MATERIALS & METHODS

Study design and samples

This laboratory experimental research was conducted in vitro with a post-test only control group design research method 15. This research used six sample groups: GOE with concentrations of 6.25%, 12.5%, 25%, NaOCl 2.5% and EDTA 17% as positive controls, and aquadest as a negative control. According to Federer's formula, the experiment must be repeated 4 times.

Plant extracts and concentration

The necessary material of fourteen kilograms ripe fresh dark green okras with 5-7 days of harvest were acquired from PT Mitra Tani Dua Tujuh, Jember. The green okra was identified in a plant laboratory, Agriculture Production, Jember State

Polytechnic. After the green okras were identified, they were washed and cut into small pieces. Thereafter, the green okras were wind-dried in a shaded area from the sun for 12 days. Once dried, they were smoothened into powder and sifted with 40 mesh sieves. Ethanol 96% was used as a solvent, with a 1:5 gr/ml ratio of green okra powder to ethanol. They are then macerated for 3x24 hours in room temperature. After 3x24 hours, the extract was filtered with a filter paper. A rotary vacuum evaporator was used to concentrate the filtrate at 40°C, until a viscous extract with 100% concentration was obtained. The 100% concentration of green okra extract was then diluted with serial dilution method using Mueller-Hinton-Broth (MHB) as a solvent. Dilution was done to obtain a concentration variety of GOE 6.25%, 12.5%, and 25% [15].

Microbial assay

S. aureus strain ATCC 6538 were acquired from Laboratory Research Center, Dental and Oral Hospital, Airlangga University. One ose of *S. aureus* were retrieved from a culture medium and inserted into reaction tubes containing 5 ml of MHB. The tubes were incubated at 37°C for 24 hours [17]. Thereafter, the bacteria were standardized according to 0.5 McFarland (1.5 x 10^8 CFU/ml) standard[15]. GOE tubes with 6.25%, 12.5%, 25%, NaOCl 2.5%, EDTA 17%, and aquadest concentration were each added with a 0.1 ml suspension of *S. aureus*. GOE with the lowest concentration without any bacterial growth and clear solution is MBC [16].

MBC tests were done with solid dilution method. Using sterile micropipettes, 0.1 ml of the test results were taken per suspension [17]. Bacteria suspension on the micropipettes were dropped onto Mueller Hinton Agar mediums, which have been prepared on Petri dishes and were spread throughout with a spreader. The mediums were then incubated at 37°C for 24 hours. The MBC-test results were established with the lowest concentration where ≥99,9% of the *S. aureus* colonies were killed. Calculations were done through direct visual observation by three people. Observation results were then noted.

RESULT

The incubation results of the MBC tests against *S. aureus* after 24 hours at 37°C can be seen at Figure 1.

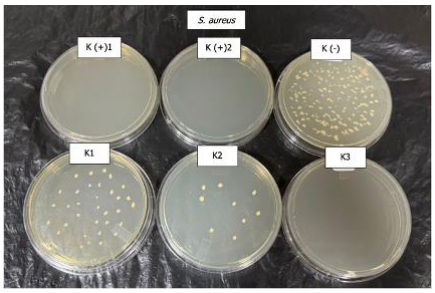

Figure 1. Colony growth K (+)1=NaOCl 2,5%, K (+)2=EDTA 17%, K (-) =Aquadest, K1=GOE 6,25%, K2= GOE 12,5%, K3= GOE 25%.

Based on the picture above, GOE with concentrations of 6.25%, 12.5%, and aquadest still show the growth of *S. aureus* colonies in each Petri dish. On the contrary, GOE with a concentration of 25% did not show any signs of growth of the *S. aureus* colonies, equivalent to NaOCl 2.5% and EDTA 17% The average growth of *S. aureus* colonies in GOE of 6.25%, 12.5%, and 25% concentrations, were 35, 11, and 0 colonies respectively. NaOCl 2.5% and EDTA 17% which served as positive control were 0. Meanwhile, Aquadest which served as negative control had the most colonies growth, 177 colonies. The results are illustrated in Table 1. below.

Table 1. Average colony growth of *S. aureus*

Samples	Average colony growth
Aquadest	177
GOE 6.25%	35
GOE 12.5%	11
GOE 25%	0
NaOCl 2.5%	0
EDTA 17%	0

GOE appeared as a dark green solution, where observation on its opacity was not possible. The results of the MIC test were

determined by a decrease in the number of colonies by 90% of the number of bacteria in the original inoculum (Aquadest). This meant that there should be a 10% maximum of 177 colonies, which equals to 18 colonies in a Petri dish to be considered as the MIC. GOE with a concentration of 12.5% had only 11 bacterial colonies. This shows GOE with a concentration of 12.5% is the MIC. The MBC can be determined by counting the colonies that grew on each Petri dish. GOE that eliminated □99,9% of the *S. aureus* bacteria of the original inoculum is the MBC. This means that there must be a maximum of 0.01 x 177 = 0,177 bacteria in a Petri dish to be considered as the MBC. GOE with a concentration of 25% had 0 colonies in the Petri dish so this means that it is MBC.

The MBC result was analyzed by the Mann-Whitney test to determine which study group had significant differences. The Mann-Whitney test results showed significant differences (a<0.05) in all study group, except GOE 25% with NaOCl 2,5%, GOE 25% with EDTA 17%, and NaOCl 2,5% and EDTA 17%.

Table 2. Mann-Whitney test result

Samples	GOE 6.25%	GOE 12.5%	GOE 25%	NaOCl 2.5%	EDTA 17%	Aquadest
GOE 6.25%		0,020*	0,014*	0,014*	0,014*	0,021*
GOE 12.5%			0,013*	0,013*	0,013*	0,020*
GOE 25%				1,000	1,000	0,014*
NaOCl 2.5%					1,000	0,014*
EDTA 17%						0,014*
Aquadest						

*** = significant differences**

DISCUSSION

The results of this study showed that GOE with a higher concentration could eliminate *S. aureus* colonies. It is proven that 25% of the results had no colony growth. This is probably because the lower the concentration of GOE, the lower the secondary metabolite compounds contained in it. Research by Vitasari et al. (2022) proved that the concentration of GOE will be directly proportional to the content of secondary metabolite compounds and their antibacterial activity. The higher the concentration of extract used, the more secondary metabolite compounds contained in it, so that it is able to inhibit and eliminate the growth of *S. aureus* colonies [14].

Green okra extract with a concentration of 100% contains several secondary metabolites such as alkaloids, flavonoids, saponins, tannins, and terpenoids [18]. Alkaloids are secondary metabolite compounds with the largest percentage (6.88%). Alkaloids can damage bacterial DNA and RNA, inhibit the synthesis of bacterial proteins and nucleic acids, and efflux pumps, thereby preventing the resistance of *S. aureus* bacteria and increasing the antibacterial effect [18, 19]. Flavonoids (5.01%) contained in GOE have hydroxyl (polar) groups that can damage the *S. aureus* cell wall layer of by forming protein complexes in the bacterial peptidoglycan layer and damaging the cell membrane.

Saponins (4.02%) work with saponification reactions, namely the reaction between fats or triglycerides with alkalis (NaOH and KOH) which can reduce the surface tension of the *S. aureus* bacterial cell wall and increase the permeability of the cell membrane so that antibiotic compounds can enter the cell [18, 20]. Tannins (3.81%) are able to form hydrophobic complexes with proteins, polysaccharides, and nucleic acids, inactivating the enzymes and cell wall protein transport, thus disrupting the growth of *S. aureus* bacteria. Terpenoids (2.95%) can make changes in ion channels (Na^+, K^+, Ca^{2+}, or Cl^-) in the cell membrane of *S. aureus* bacteria [21]. In addition, terpenoids

will bind to transmembrane proteins (porins) on the outer membrane of the bacterial cell wall by forming polymeric bonds that result in impaired permeability of the bacterial cell wall resulting in rupture of the *S. aureus* bacterial cell wall. All compounds contained in GOE work synergistically and strengthen each other so that they can inhibit and even kill bacterial growth [22].

The Mann-Whitney test results prove that GOE with 25% concentration has no significant difference when compared with NaOCl 2.5% and EDTA 17%. This is evidenced by the absence of colony growth of *S. aureus* bacteria in the Petri dish tests. Therefore, it can be said that 25% GOE has the same effectiveness as NaOCl 2.5% and EDTA 17% in killing *S. aureus* colonies. However, GOE is a natural ingredient, resulting in the need of a greater concentration to match the effectiveness of NaOCl 2.5% and EDTA 17%. GOE in this study has not been purified and fractionated, so that it was not specialized as an antibacterial agent because it still has many other compounds in it such as dyes (chlorophyll, carotenoids, and cyanine) and food substances (alpha cellulose, hemicellulose, lignin, pectin, fat, and wax) which are likely to affect the growth of *S. aureus* bacteria [23].

On the other hand, NaOCl 2.5% is a pure solution that was specialized as a broad-spectrum antibacterial. It has antibacterial reactions in the form of saponification, chloramination, and neutralization. The saponification reaction of NaOCl can degrade fatty acids, converting them into fatty acid salts (soap) and glycerol (alcohol), thus causing damage to the bacterial cell membrane. In addition, there is also a chloramination reaction, which is a reaction between hypocaloric acid and NaOCl 2.5% that interacts with organic matter resulting in degradation and hydrolysis of amino acids. Furthermore, NaOCl 2.5% will also neutralize amino acids in the bacterial cell wall into salt and water during the neutralization reaction [24]. In contrast to NaOCl 2.5%, EDTA as a chelating agent works by reducing the availability of cations (Mg^{2+} and Ca^{2+}) so that it could destabilize bacterial cell membranes [25].

The limitations of this research are this study relies on crude extracts, which may contain varying concentrations of active compounds, potentially affecting reproducibility. Moreover, this study is conducted under laboratory conditions, which may not fully replicate the complex oral environment. For future research, the researcher suggests performing fractionation on the green okra fruit extract to obtain a purified green okra fruit extract that contains only antibacterial components.

CONCLUSION

Based on this study, it can be concluded that MBC of the GOE against *S. aureus* is at 25% concentration. In addition, GOE with 25% concentration has the same level of effectiveness with NaOCl 2.5% and EDTA 17% in eliminating *S. aureus* colonies.

Declaration by Authors

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