Nusantara Medical Science Journal
Volume 7 Issue 1, January - June 2022
P-ISSN: 2460-9757, E-ISSN: 2597-7288
Nationally Accredited Journal, Decree No. 36/E/KPT/2019.

Original Article

MIC and MBC Levels of Combination *Camellia Sinensis* and *Mentha Piperita* Extract Mouthwash Against *Streptococcus Mutans*

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ARTICLE INFO

Keywords:
MIC; MBC; Camellia sinensis; Mentha piperita; Mouthwash;

How to cite:
Setiawati Y., Ramadhani M., Bobsaid J.,Hulwah DOZ. (2022). MIC and MBC Levels of Combination Camellia sinensis and Mentha piperita Extract Mouthwash Against Streptococcus mutans. Nusantara Medical Science Journal, 7(1), 39-49

DOI:
10.20956/nmsj.v7i1.19203

ABSTRACT

Introduction: *Awareness of Indonesian people in maintaining dental and oral health is low, proved by an increase in the percentage of dental and oral health problems by 2.7%. Caries is a dental and oral health problem that occurs in many children. The main cause of dental caries is Streptococcus mutans. To solve this problem, it is necessary to use herbal mouthwash made from a combination of Camellia sinensis and Mentha piperita extract as an antibacterial against Streptococcus mutans.* ***Methods:*** *Mouthwash is made through several processes namely plant determination, extraction, and mouthwash making. Minimal Inhibitory Concentration (MIC) is determined by diluted methods, Minimal Bactericidal Concentration (MBC) is determined by the agar streaking method, and colony tests are calculated using colony counters.* ***Results:*** *The result of plant determination showed the plants in this study were Camellia sinensis and Mentha piperita. At a concentration of 6.25%, no growth of bacteria in each repetition with the number of colonies 0 CFU / ml. While at a concentration of 3.125% found the average number of colonies 13 CFU / ml.* ***Conclusions:*** *Based on good MIC and MBC results,*

mouthwash containing Camellia sinensis and Mentha piperita has been shown to kill and inhibit the growth of Streptococcus mutans bacteria.

1. INTRODUCTION

One of the things that need to be considered in maintaining a healthy body is dental and oral health. However, awareness about the importance of maintaining oral health in Indonesia is still low. This can be proven by the percentage of dental and oral health problems based on Baseline Health Study in 2007 to 2013, an increase of 2.7%, from 23.2% to 25.9%. In addition, there are many kinds of dental and oral health problems, including caries and periodontal. Caries is dental and oral health problem that often occurs in children. Streptococcus mutans bacteria are the primary cause of dental caries, as determined by gram staining (picture of a small-lined coccus) and a sugar fermentation test.[1] *Streptococcus mutans* and streptococci of the mitis species are initial colonizers of the biofilm then form extracellular polymers, which enhance the adherence of other organisms. [2]

According to data from the Baseline Health Study, there has been an increase in the percentage of dental and oral health problems. Study is carried out to be able to overcome these problems, one of which is using herbal plants. Two of the herbal plants have benefits for overcoming dental health problems, namely *Camellia sinensis* (green tea) and *Mentha Piperita* (peppermint). Based on several existing studies, it is known that both are useful as antimicrobials against bacteria in the oral cavity. According to Soegeng Wahluyo's study, green tea contains polyphenolic compounds such as epigallocatechin-3-gallate (EGCG) which can inhibit *S. mutans* bacteria with evidence of a significant decrease in S. mutans growth at 0.5 mg/ml epigallocatechin-3-gallate (EGCG) for 3 minutes.[3] Meanwhile, peppermint leaves contain essential oils that have antimicrobial activity against *Streptococcus mutans* bacteria that cause dental caries.[4]

Mouthwash of green tea and peppermint leaves is made depends on the benefits of both ingredients. Based on study by Otieno et al in 2008, shows that the mixing of several plants (multi-plant extracts) is more effective than single plant extracts because it has higher bioactivity.[5] To determine the effectiveness of this mouthwash, it is necessary to test the MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration). MIC is the lowest concentration to inhibit bacterial growth and one of them is done by dilution method which aims to quantitatively determine antibacterial activity, while MBC is the lowest concentration to kill 99.9% of bacteria and is done by growing bacteria on agar media.[6] The purpose of making this mouthwash is to improve dental and oral health in Indonesia and can be used by various groups.

2. METHODS

This study includes experimental study conducted in some research laboratories to collect primary data. This study concerns health protocols.

Time and place

The study was conducted in July - September 2021 in two places: Materia Medica Batu laboratory and Study Center Faculty of Dentistry Airlangga University.

Plant determination

Green tea (*Camellia sinensis*) and peppermint (*Mentha piperita*) were determined in Laboratorium Materia Medica Batu. The determination process was carried out based on *Serial data terkini tumbuhan obat*,[7] *Flora of Java Vol. I*,[8] *Flora of Java Vol.II*,[9] and *FLORA*.[10]

Plant extraction

The process of extracting green tea leaves and peppermint leaves with 95% ethanol as solvent was carried out by the maceration method. Both leaves were weighed, and the weight of green tea and peppermint leaves were 1276.5 grams and 92.2 grams, respectively. The leaves were washed clean. Then, the green tea leaves were blended with 6380 mL of ethanol, while the peppermint leaves were blended with 461 mL of ethanol. The results of the blender were put in a closed jar and macerated for 3 days. Then, the filtrate was filtered with a clean cloth and evaporated using a vacuum rotary evaporator (with a pressure of 175 mbar) to obtain ethanol extract of green tea leaves and peppermint leaves with a concentration of 100% each. In this process, 200 mL of green tea leaf extract and 45 mL of peppermint leaf extract were obtained.

Mouthwash making

Used tools including pipettes, test tubes, and bottles were washed thoroughly with soap and running water, sterilized by boiling at 100°C for 1 hour, and dried in an oven. 100 mL chocolate bottle was calibrated, then each mouthwash formula was made according to the following formulation:

Table 1. Mouthwash formulation

Material	Concentration Unit	F1	F2	F3	F4	F5	FA	FB
NaCl	b/v	1%	1%	1%	1%	1%	1%	1%
Benzoic acid	b/v	0.5%	0.5%	0.5%	0.5%	0.5%	0.5%	0.5%
Sorbitol	b/v	5%	5%	5%	5%	5%	5%	5%
Peppermint oil	v/v	1%	1%	1%	1%	1%	1%	1%
A mix of green tea and peppermint extract (1:6)	v/v	20%	40%	60%	80%	100%	-	-
Green tea extract	v/v	-	-	-	-	-	20%	-
Peppermint extract	v/v	-	-	-	-	-	-	20%
Aquades	ad	ad 100 ml				-	ad 100 ml	

Sterilization of laboratory equipment

Sterilization of laboratory equipment was carried out using an autoclave at a temperature of 121 ° C for 30 minutes.

Isolation and identification of *Streptococcus mutans* bacteria

Bacteria isolated from volunteer's plaque by dredging in moderation or saliva collected and taken 1 ml and then inserted in the medium BHIB (Brain-Heart Infusion Broth),

incubated for 48 hours at a temperature of 37 ° C anaerobically, grown on TYC (Tryptone, Yeast extract, Cystine), then identified through staining grams (picture of small-lined coccus) and sugar fermentation test.

Bacterial suspension making

Samples of *S. mutans* bacteria in the BHIB medium were taken using a micropipette and dripped into another test tube that has contained a sterile BHIB medium. The bacterial suspension on the test tube compared to the standard 0.5 McFarland.

Solid media making (agar)

The solid medium used in this study was TYC agar media (Tryptone, Yeast extract, Cystine). 249.99 grams of TYC agar base dissolved in 1000 ml of aquades in the Erlenmeyer squash and heated until dissolved. Agar was sterilized using autoclaves at 121 °C for 15 minutes and cooled until the temperature reaches 50 ° C. Agar was mixed until flat and put into a petri dish. TYC agar media was used in the testing of the MBC and colony count.

Liquid media making

Liquid media was used in the form of BHIB (Brain Heart Infusion Broth). 37 grams of BHI broth base dissolved in 1000 ml of aquadest in Erlenmeyer squash, heated to dissolve, and put into a test tube as needed. Continued with sterilization using autoclave at a temperature of 121 ° C for 15 minutes. The finished BHIB media was used in MIC testing.

Minimum Inhibitory Concentration testing

MIC is the lowest concentration for inhibiting bacterial growth and one of them is done by dilution method that aims to quantitatively determine antibacterial.[6] The testing was done by dilution method. 12 sterile test tubes were provided. Tube 1-10 were the treatment tube, tube 11 was positive control (amoxicillin), and tube 12 was negative control (medium and bacterial suspension). Tube 1 was filled with a mouthwash solution with a concentration of 100% as much as 2 ml. Tubes 2-12 were filled with a medium of BHIB as much as 1 ml. A mouthwash solution of 1 ml from tube 1 was taken using a micropipette to be transferred to tube 2, then homogenized so that the concentration of mouthwash substances in tube 2 is half of tube 1. So on until tube 10. Tubes 1-10 and 12 each were added bacterial suspension with a turbidity of 0.5 Mcfarland as much as 1 ml. All the tubes were then placed in an incubator at a temperature of 37° for 48 hours and observed the absence of bacterial growth through the turbidity level of the solution.

Minimum Bactericidal Concentration testing

MBC is the lowest concentration for killing 99.9% of bacteria and is done by planting bacteria in agar media.[6] The testing was done by streaking method. The solution on the test tube that had been incubated for 48 hours at a temperature of 37 °C for MIC testing was taken using ose and streaking on the TYC agar medium. A petri dish divided into 12 parts and each part contained solution from the tubes. Furthermore, it was incubated at a temperature of 37 °C for 48 hours and observed the absence of bacterial growth.

Colony count test

TYC agar media was provided on the petri dish. The solution on the test tube for dilution test (which has undergone an incubation process at 37 °C for 48 hours) was taken using micropipette, dripped over TYC agar media, flattened using a spreader, and incubated at 37 °C for 48 hours. Colonies were calculated using colony counters. In this study, repeated colony tests twice.

Data processing

The data obtained was converted into table form, then processed using software Microsoft Excel 2010 on the computer. The process of processing the data includes editing, coding, data entry, cleaning, and verification.

3. RESULTS

Plant determination

The determination of *Camellia sinensis* (green tea) was carried out based on FLORA with the results of the determination: 16-2b-3b-4b-6b-7b-9b-106-116-120-136-14a-15a-109a-110b-111b-112a-113b-116-1196-1206-128b-129b-135b-136b-139b-140b-142b-143b-146b-154b-155b-156b-162b-163b-167b-169b-171b-177b-179a-180b-1826-1836-184a-1.[10] Based on this result, it can be concluded that the plant sample used in this study was really *Camellia sinensis* (green tea).

Table 2. Determination result of *Camellia sinensis*

Kingdom	Plantae
Subkingdom	Tracheobionta
Super Division	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Dilleniidae
Ordo	Theales
Famili	Theaceae
Genus	Camellia
Species	*Camellia sinensis* (L.) Kuntze
Synonym	*Camellia bohea* (L.) Sweet; *Camellia chinensis* (Sims) Kuntze; *Thea sinensis* L.
Common Name	Green tea
Determination Key	lb-2b-3b-4b-6b-7b-9b-10b-11b-12b-13b-14a-15a-109a-110b-111b-112a-113b116a-119b-120b-128b-129b-135b-136b-139b-140b-142b-143b-146b-154b-155b 156b-162b- 163b-167b-169b-171b-177b-179a-180b-182b-183b-184a-1.

Meanwhile the determination of *Mentha piperita* (peppermint) was carried out based on *Serial data ilmiah terkini tumbuhan obat*,[7] *Flora of Java Vol. I*,[8]and *Flora of Java Vol.II*,[9] with the results of the determination: 1b-2b-3b-4b-126-136-146-176-186-19b-206-21b-22b-23b-24b-25b-26b-27a-28b-29b-306-31b-403b-404b-405b-414a-415b-451a-452b-

453a-454a-455b-456b-457a1b-2b-3a-4c-5b-7b-8c-11a-12a-136-150-200-21b-236-24b. Based on this result, it can be concluded that the plant sample used in this study was really *Mentha piperita* (peppermint).

Table 3. Determination result of *Mentha piperita*

Kingdom	Plantae
Subkingdom	Tracheobionta
Super Division	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Asteridae
Ordo	Lamiales
Famili	Lamiaceae
Genus	Mentha
Species	Mentha piperita L.
Common Name	Peppermint
Determination Key	lb-2b-3b-4b-12b-13b-14b-17b-18b-19b-20b-21b-22b-23b-24b-25b-26b-27a-28b 29b-30b-31b-403b-404b-405b-414a-415b-451a-452b-453a-454a-455b-456b-457a lb-2b-3a-4c-5b-7b-8c-11a-12a-13b-15c-20b-21b-23b-24b

Minimum Inhibitory Concentration (MIC)

Ten test tubes containing different concentrations were then viewed as the results of the diluted test. Then, the color was matched with positive and negative controls.

Picture 1. MIC Test Results with Dilution Method. Tube 1 (100%), 2 (50%), 3 (25%), 4 (12.5%), 5 (6. 25%), 6 (3.125%), 7 (1.56%), 8 (0.78%), 9 (0.39%), 10 (0.19%), control (+), and control (-)

Table 4. MIC Test Results with Dilution Method

Concentration	Diluted Test Result
0.19%	Turbid
0.39%	Turbid
0.78%	Turbid
1.56%	Turbid
3.125%	Turbid
6.25%	Turbid
12.5%	Turbid
25%	Turbid
50%	Turbid
100%	Turbid
Control (+)	Clear
Control (-)	Turbid

From table 4 it is known that the turbidity above is caused by the murky color of the mouthwash and the deposits of substances contained in it. As a result, MIC cannot be determined from the dilution method so colony tests are required to find out and ascertain MIC levels.

Minimum Bactericidal Concentration (MBC)

From the diluted tube earlier, streaking was done on the agar plate to determine MBC levels. Then viewed from each concentration whether there was bacterial growth or not in four repetitions.

Table 5. MBC Test Results

Concentration	Bacterial Growth In Each Repetition

	I	II	III	IV
0.19%	+	+	+	+
0.39%	+	+	+	+
0.78%	+	+	+	+
1.56%	+	+	+	+
3.125%	+	+	+	+
6.25%	-	-	-	-
12.5%	-	-	-	-
25%	-	-	-	-
50%	-	-	-	-
100%	-	-	-	-
Control (+)	-	-	-	-
Control (-)	+	+	+	+

Based on the results of MBC testing in table 5, at a concentration of **6.25%** known that no growth of bacteria at each repetition. So, MBC for the mouthwash is at a concentration of 6.25%.

Colony count

The number of bacterial colonies was calculated using the colony counter. In this study, we used two repetitions.

Table 6. Results of *S. Mutans* Colony Count On TYC Media From Diluted Test Tube

Concentration	**Number of colonies per repetition (CFU/ml)**		**Sum**	**Mean**
	I	**II**		
0,19%	120	133	253	126.5
0,39%	79	86	165	82.5
0,78%	54	56	110	55
1,56%	33	37	70	35
3,125%	14	12	26	13
6,25%	0	0	0	0
12,5%	0	0	0	0
25%	0	0	0	0
50%	0	0	0	0
100%	0	0	0	0
Control (+)	0	0	0	0
Control (-)	156	158	314	157

In addition to knowing the exact number of bacterial colonies, this test can also tell the level of MIC and MBC. Based on the table above, as the concentration of mouthwash increases, the number of colonies also decreases. MIC is obtained at a concentration

of **3.125%**. MIC is the lowest concentration that can inhibit bacterial growth by as much as 90%. The colony data confirmed the previous MBC test, which was at a concentration of **6.25%** because MBC is the lowest concentration capable of killing bacteria by 99.9%.[11]

4. DISCUSSIONS

In the test result (table 5) at a concentration of 6.25%, there is no bacterial growth in each repetition. It is then known that MBC is at a concentration of 6.25%, which means that mouthwash with a concentration of 6.25% can already kill 99.9% of *S.mutans* bacteria. MBC level in this study is relatively good compared to existing MBC studies in killing *Streptococcus mutans* which were conducted by Nardi et al, their study concerned about ozonated olive oil in caries prevention, with the MBC result at a concentration of 12.5%.[12] Another study related to this topic was conducted by Rostikawati and Supratman with MBC result at a concentration of 15% using *Physalis angulata* l. ethanol extract.[13] Rollando's study with MBC yielded a 20% concentration using *Massoia aromatica*.[14] Martins et al also conducted a study with MBC results at a concentration of 47.6 percent using red propolis.[15]

In addition to MIC and MBC, the study also conducted a Colony test. From the colony tests that have been done, it can be known the number of bacterial colonies at each concentration. In this colony test study can also find out the level of MIC that has not been determined in the previous test, which is at a concentration of 3.125%. The level of MIC in this study is relatively good compared to mouthwash MIC result in previous studies, including studies conducted by Nardi et al with MIC results at a concentration of 3.125% using ozonated olive oil,[12] Sinuraya's study with MIC results at a concentration of 5% using *Terminalia catappa* L ethanol extract,[16] Septiyaningrum et al's study with MIC results at a concentration of 7% using *Cymbopogon nardus* L extract,[17] and Ningrum's study with MIC results at a concentration of 12.5% using *Mikania micrantha* extract.[18] Fitri and Humairoh also conducted a study with MIC results at a concentration of 20% using guava leaf extract.[19] Because of distinctions in the ingredients used to kill *Streptococcus mutans*, the MIC and MBC results in this study differed from previous studies. Mouthwash in this study constituted of two ingredients, whereas previous studies constituted of one. *Camellia sinensis* contains a high catechin content. *Mentha piperita* contains menthol, which creates a synergistic effect with this function. As a consequence, it has enhanced antibacterial activity. In addition, the colony test also confirmed MBC results from the previous test at a concentration of 6.25%.

5. CONCLUSION

The level of MIC mouthwash made from a combination of *Camellia sinensis* extract (green tea) and *Mentha piperita* (peppermint leaves) is located at a concentration of **3.125%,** and MBC is located at a concentration of **6.25%.** The MIC and MBC results are found to be good based on these findings. Mouthwash with a minimum concentration of 3.125% can inhibit the growth of bacteria. Meanwhile, mouthwash with a concentration of 6.25% can kill the bacteria. This study is limited to only two plants, so it is expected that further study using mouthwash based on two other plants or more. Study on bacteria other than *Streptococcus mutans* is also needed to determine the levels of MIC and MBC mouthwash against other bacteria.

ACKNOWLEDGMENTS

Praise and gratitude author prayed to the presence of God Almighty that this scientific article can be resolved properly. We also thank all those who have helped in the preparation of this article, in particular to the Ministry of Education and Culture, Study and Technology, all the lecturers who support PKM Airlangga University, Materia Medica Batu laboratory, and Study Center Faculty of Dentistry Airlangga University.

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Conflict of Interest Statement:

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.