

Antimicrobial Activity, Cytotoxicity and Bioactive Constituents by GC/MS Analysis of Essential Oils

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Abstract: *Objectives:* The study objective was to select natural essential oils that can act as antimicrobial and flavoring agents, to determine the antimicrobial effect, and to evaluate the possibility of its use as a component of mouthwash through toxicity evaluation. *Methods:* Disc-diffusion test, Minimal inhibition concentration (MIC) and Minimum bactericidal concentration (MBC) were measured to evaluate the antimicrobial effect against *Streptococcus mutans* and *Candida albicans* by selecting Cinnamon bark, Lemongrass, Clove bud, Tea tree, Eucalyptus and Girofle (Clove) from natural essential oils commercially available. By analyzing the exact components by gas chromatography mass spectrometry (GC/MS) of six kinds of essential oils, it was possible to identify the major components showing antimicrobial effect. Cell cytotoxicity test was performed using Agar overlay test oil which showed excellent antibacterial effect against *Streptococcus mutans* and *Candida albicans*.

Findings: The antimicrobial effect of *C. albicans* was measured among *S. mutans* and oral flora causing periodontal disease, and Cinnamon bark and Lemongrass oil showed the highest MBC of 3.13 mg/mL (0.313%), followed by Girofle (Clove) and Clove bud oil (MBC: 6.25 mg/mL (0.625%)) showing significant antimicrobial effects. The major components were identified by analyzing the ingredients using GC-MS. Cinnamon bark was identified as cinnamaldehyde and eugenol, and Lemongrass was identified as a major ingredient of antimicrobial activity through the analysis of essential oils, such as citral components, neral and geranial, and Girofle (Clove), eugenol. It was confirmed that information on the concentration of major components can be obtained according to the concentration used and the evaluation of the antimicrobial effect can be accurately performed. The concentration of MBC (0.313%) of cinnamon bark oil measured in this experiment was higher than the recommended concentration of not more than 0.1%. Therefore, the toxicity test was carried out with an agar overlay test according to the concentrations of Lemongrass and Girofle (Clove) essential oils known to be non-toxic and found to be non-toxic at concentrations below 0.313%.

Improvements/Applications: Antimicrobial activity and safety evaluation of Lemongrass and Girofle (Clove) oils were able to determine the optimum concentration for use. These natural oils have an antibacterial effect against *C. albicans* in *S. mutans* and oral flora, and it is expected that further research on other pathogenic bacteria and oral flora of periodontal disease and component analysis and safety evaluation study can be applied to the development of safe and antimicrobial natural mouthwash.

Keywords: Essential oil, *Streptococcus mutans*, *Candida albicans*, Antibacterial, Antifungal, Cytotoxicity

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I. INTRODUCTION

Tooth decay is a disease in which the sugar remaining in the teeth of the oral cavity turns into an acid by the bacteria and corrodes the teeth. *S. mutans* has glucosyl transferase (GTF), a causative agent of dental caries in cells, and forms non-water-soluble glucans from the sugars. Non-water-soluble glucans adhere to the surface of the teeth to induce dental caries [1]. In order to prevent periodontal disease, it is important to remove the bacterial membranes from the teeth. Even if toothbrushes are used to remove plaque, bacteria remain. To compensate for this, studies have been reported that use of mouthwash can reduce the number of bacteria. The initial mouthwash was mainly used for aesthetic purpose of temporarily removing bad breath. However, recently, various kinds of antibacterial and effective substances are applied to the oral cavity to thereby inhibit bacterial formation on the surface of the teeth as well as sterilization [2]. Chlorhexidine attack bacterial cell membranes, causing leaking or sedimentation of cellular constituents and binding saliva mucin to reduce salivary pellicle formation, which interferes with the formation of plaque populations, and It is known to be effective in inhibiting the adsorption of bacteria on the tooth surface by binding to bacteria [3]. However, excessive use of these chemical agents can cause confusion in the oral microorganism gun as well as in the intestinal microorganism gun, resulting in undesirable side effects such as infection by other bacteria or vomiting [4,5]. There is a growing interest in oral remedies that do not have side effects of these drugs, and there is a growing need for research and development of antimicrobial materials using natural products with selective effects on oral bacteria [6]. The largest component of the mouthwash is water. The main component that plays a major role is antimicrobial agents to obtain antibacterial effect and fluoride to prevent dental caries. In addition, alcohol or surfactants are contained in order to dissolve some water-insoluble components, and flavoring agents and the like for imparting a unique flavor are added. At this time, six kinds of natural essential oils capable of acting as antibacterial substances and fragrance were selected, and through the analysis of the antimicrobial effect and components using GC-MS for these essential oils, studies were done on the components that exhibit antibacterial activity, and toxicity evaluation was conducted for the possibility of using mouthwash as an



antimicrobial component.

Six pure essential oils commercially available were selected for the study as shown in table 1.

II. MATERIALS AND METHODS

2.1. Essential oils

Table 1. List of essential oils

Common name	Botanical name	Part	Purity	Manufacturers
Cinnamon bark	<i>cinnamomum zeylanicum</i>	Bark	100%	Neumond, Germany
Lemongrass	<i>Cymbopogon flexuosus</i>	Leaf	100%	Neumond, Germany
Clove bud	<i>Syzygium aromaticum/ (Eugenia caryophyllus)</i>	Flower, Bud, Leaf	100%	Neumond, Germany
Tea tree	<i>Melaleuca alternifolia</i>	Leaf	100%	Huile & Co., Korea
Eucalyptus	<i>Eucalyptus radiata</i>	Leaf	100%	Huile & Co., Korea
Girolfle	<i>Eugenia caryophyllus (Clove)</i>	Flower	100%	Le Chatelard 1802, France

2.2. Antibacterial and antifungal effects test

2.2.1. Bacterial and yeast strains

The antimicrobial properties were tested based on the inactivation of the bacteria and yeast strains. The bacteria and yeast strains used were Streptococcus mutans ATCC25175 (S. mutans) and Candida albicans KCTC27253 (C. albicans). S. mutans is a normal resident the oral cavity and contributes to tooth decay. S. mutans were cultured in anaerobic agar plate medium (BHI; Difco Lab., Detroit, MI, USA) agar plates. S. mutans were prepared by growing anaerobically to the stationary phase and sub cultured in BHI broth to the mid logarithmic growth phase in a test tube at 37°C.

C. albicans were cultured on yeast extract peptone dextrose broth agar (YEPD; 5 g yeast extract, 10 g peptone, 10 g dextrose, 500 mL distilled water, 10 g agar) plates. C. albicans was incubated in a fixed bed under aerobic conditions and then cultured in a YEPD broth medium from a test tube at 37 °C until the mid logarithmic growth phase [7].

2.2.2. Disc-diffusion test

The antimicrobial effects of the essential oils were determined using a paper disc diffusion method to test the effect of essential oils. The essential oils were made with culture medium at the concentration 2.5%, 1.25%, 0.625%, 0.313%, 0.156% and 0.078% (v/v). The disc of 8 mm diameter and 1.5 mm thickness (Advantec, Tokyo, Japan) was soaked with 50uL of each concentration of the solution, and then placed onto BHI agar and YEPD agar plates, which were treated on the surface agar with 200 µL of 108 cfu/mL suspension for for S. mutans and C. albicans.

The inoculated agar plates were then incubated at 37°C for 48h for S. mutans and at 37°C for 24h for C. albicans. The antimicrobial effect was evaluated the diameter of inhibition zones in millimeters, using digital caliper (Mitutoyo, Japan) and the mean of 3 evaluations of the diameter of each inhibition zone for each disc was determined [8]. The test was repeated twice for accuracy.

2.2.3. Minimal inhibition concentration (MIC)

The Minimum Inhibitory Concentration (MIC) was determined by using the broth serial dilution method (Figure 1). Serial dilutions of the high concentration were made in a sterile test tube containing BHI and YEPD broth medium + Tween 80 (0.5% v/v) to prepare a concentration range from 0.78 to 25 mg/mL. [9]

The diluted samples (180 µL) were transferred to 96-well

microplate. The control group consisted of 180 µL of culture medium (BHI and YEPD) containing Tween 80. And then, 20 µL of the culture suspension (106 viable cells/mL) of S. mutans and C. albicans were added to all wells of the microplate. The final volume of each well was 200 µL. After all sample of well plate with the control groups were incubated at 37°C for 48 h in CO2 incubator, the turbidity of the medium was visually observed [10-12]. The MIC was determined as the lowest concentration of non-turbid essential oil with inhibited microbial growth. The tests were performed in duplicate.

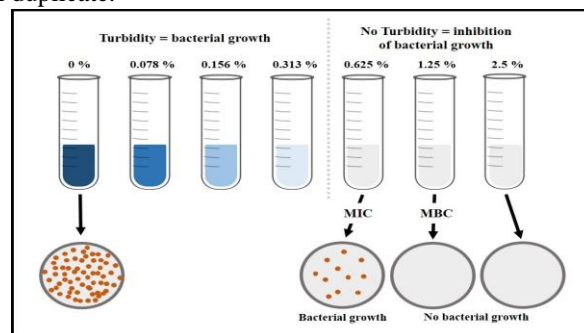


Figure 1. Diagram of the broth dilution method.

2.2.4. Minimum bactericidal concentration(MBC)

To determine the minimum bactericidal concentration (MBC) of essential oils, samples were selected that did not show microbial growth on the broth medium in previous experiments. Each sample were treated with 100 mL onto the surface of BHI and YEPD agar medium and incubated at 37°C for 48 h in CO2 incubator, the colony growth was observed for S. mutans and C. albicans [10-12]. The MBC was measured as the lowest concentration of essential oil tested able to inhibit microbial growth on agar plate, indicating 99.5% killing of the original inoculum. The tests were executed in duplicate.

2.3. Cell cytotoxicity test

2.3.1. Cell cultures

L929 mouse fibroblast cells (L929 cells) were cultured in RPMI-1640 (GIBCO Invitrogen, Indianapolis, IN, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (GIBCO Invitrogen, Indianapolis, IN, USA), solution containing penicillin (100 IU/mL) and streptomycin (100µg/mL) (Sigma Aldrich, St.



Louis, MO, USA). Cultures were performed at 37°C in a humidified incubator under 5% CO₂ atmosphere. After the confluent cell monolayer covered 80% of the cell culture dish, cells were trypsinized and harvested cells were used for cytotoxicity tests [13].

2.3.2. Agar overlay test

The biocompatibility of the essential oils was determined by testing cytotoxicity based on the agar overlay test. For the test, 10 mL of L929 cell suspensions of about 3 × 10⁵ cell/mL were seeded into a 10 cm diameter cell culture dish and incubated for 24 h at 37°C under a 5% CO₂ atmosphere. After L929 cell were covered approximately 80% of the cell culture dish, the liquid medium was removed. Then, 10 mL of RPMI-1640 medium containing 5% FBS and 2% agar (Difco Lab., Detroit, MI, USA) mixed solution (RPMI : agar = 5 : 5) was dispensed into the cells cultured dish. After the mixture of agar and medium was solidified, 5 mL of FBS and Neutral Red mixed solution (0.01% in phosphate-buffered saline, Sigma Aldrich, St. Louis, MO, USA) was injected into the agar medium for staining the cells and stored at 37°C for 15 min. Excess Neutral red dye was then removed and the test specimens were placed on the agar medium surface and incubated for 24 h at 37 ° C under a 5% CO₂ atmosphere. After 24 h of incubation, cell photographs were observed with a microscope. The cell cytotoxic was evaluated by analyzing the decolorized zones and cell lysis around and/or under the specimens according to ISO7405 and ISO10993-5 [13-17]. Each test was performed three times.

2.4. Gas chromatography mass spectrometry(GC/MS)

GC/MS was executed using Shimadzu QP 2010 Ultra (Japan). The GC/MS analysis conditions were maintained at oven temperature of 40°C for 2 minutes, elevated temperature of 10°C per minute and maintained at 260°C for 10 minutes, and GC column used DB-5MS (0.25 mm diameter, 0.25 µm thickness and 30m length). Carrier gas was helium and total flow was analyzed at 1 mL/min. The injector and interface temperature was set at 260 ° C, and the temperature of the ionurce was set at 200 ° C. SCAN mode was used to analyze in the range of 50 - 550 m/z. For the component identification of the mass spectrometry spectrum analyzed by GC/MS analysis was performed using a library of mass spectra. (Wiley Registry of Mass Spectra Data, 2000) [8].

III. RESULTS AND DISCUSSION

3.1. Antimicrobial activity of Essential oils

The antimicrobial effect of essential oils on *S. mutans* is shown in Figure 2. The diameter of the inhibition zone evaluated in Figure 2 was measured and shown in Table 2. From these results, it was found that cinnamon bark, lemongrass, clove bud and girofle (clove) were superior to tea tree and eucalyptus against *S. mutans*. Figure 3 shows the antimicrobial effect against *C. albicans*. The diameter of the inhibition zone measured for each essential oil was measured and shown in Table 3. Cinnamon bark showed the highest antimicrobial activity against *C. albicans*, followed by lemongrass, clove bud, and girofle (clove), and no antimicrobial activity was observed at tea tree and eucalyptus concentrations below 25 mg/mL.

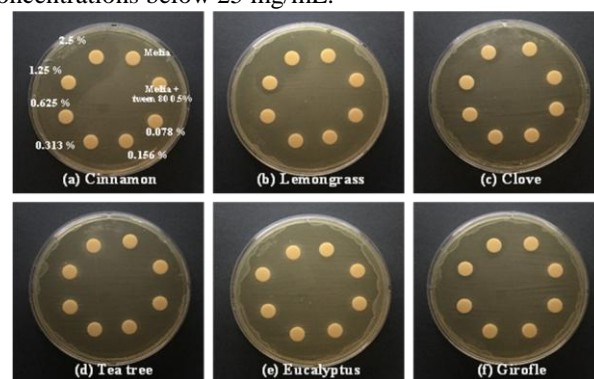


Figure 2. Inhibition zones evaluated by paper disc diffusion test for essential oils against Streptococcus mutans

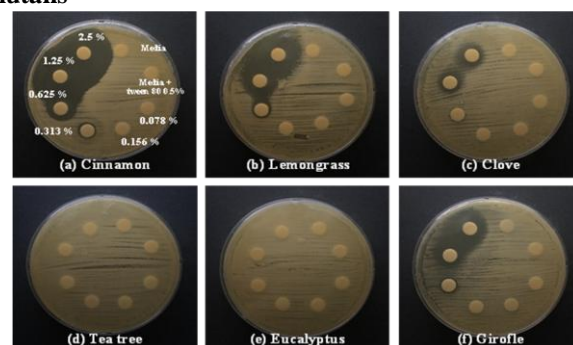


Figure 3. Inhibition zones evaluated by paper disc diffusion test for essential oils against Candida albicans

Table 2. Antimicrobial effect of the six essential oils against Streptococcus mutans by paper disc diffusion test (Mean±SD)

Concentration (mg/mL)	Diameter of inhibition zone (mm)					
	CN	LG	CL	TT	ET	GF
25	25.88±0.4	19.56±0.1	16.16±0.4	9.63±0.0	9.47±0.1	-
12.5	22.81±0.2	16.52±0.2	13.52±0.6	7	2	15.80±0.25
6.25	12.51±0.4	10.16±0.1	9.88±0.10	-	-	12.16±0.21
3.13	3	0	0	-	-	11.04±0.11
1.56	-	-	-	-	-	-
0.78	-	-	-	-	-	-

-, No inhibition zone

CN: Cinnamon bark, LG: Lemongrass, CL: Clove bud, TT: Tea tree, ET: Eucalyptus, GF: Girofle



Table 3. Antifungal effect of six essential oils against *Candida albicans* by paper disc diffusion test(Mean±SD)

Concentration (mg/mL)	Diameter of inhibition zone (mm)					
	CN	LG	CL	TT	ET	GF
25	35.32±1.45	28.68±0.38	16.56±0.41	-	-	21.48±0.25
12.5	30.98±0.63	23.28±0.50	14.74±0.16	-	-	17.91±0.83
6.25	17.06±0.84	12.05±1.05	9.12±0.12	-	-	11.31±0.32
3.13	11.34±0.50	-	-	-	-	-
1.56	-	-	-	-	-	-
0.78	-	-	-	-	-	-

-, No inhibition zone

CN: Cinnamon bark, LG: Lemongrass, CL: Clove bud, TT: Tea tree, ET: Eucalyptus, GF: Girofle

3.2. Determination of MIC and MBC of essential oils

MIC values were measured by broth dilution assay. The MIC results for the six essential oils are shown in Table 4. The MIC of Cinnamon bark and Lemongrass against *S. mutans* was the highest at 3.13 mg/mL, followed by Clove bud and Girofle (Clove) at 6.25 mg/mL. Tea tree and Eucalyptus were measured at 25 mg/mL. Essential oil ingredients have been reported to reduce pathogenicity by blocking populations of bacteria involved in the early development of plaque, delaying bacterial replication, and reducing the amount of plaque [18]. The essential oils used in this study were also found to have antimicrobial activity in the oral microorganism *S. mutans*. *S. mutans* is found in human dental caries lesions and produces organic acids by decomposing monosaccharides and fructose to cause dental decay and tooth dental caries, and it is known that lactic acid is actively produced in an acidic environment and causes dental caries [1]. The values of MIC and MBC measurements of the six essential oils on *S. mutans* were shown in Table 4. *S. mutans* The MIC of Cinnamon bark and Lemongrass oil was 3.13 mg/mL for *mutans*, and the MIC of Clove bud and Girofle (Clove) oil was 6.25 mg/mL for Tea tree and Eucalyptus it was 25 mg/mL. The MBC of Cinnamon bark and Lemongrass oil against *S. mutans* was 3.13 mg/mL or less, the MBC of Clove bud oil was 12.5 mg/mL, the MBC of Girofle (Clove) oil was 6.25 mg/mL, and the tea tree and Eucalyptus were more than 25 mg/mL. Figure 4 shows the results of MBC measurement of 6 essential oils for *S. mutans*.

C. albicans belongs to a normal oral microorganism group, which is known to be present in the oral cavity in an uninfected state in a population of 30 to 50% [19]. However, due to the lowering of human immunity, the increase of oral

glucose concentration, and the lowering of saliva pH, if an environment for proliferation is established, infection by bacteria will occur. Therefore, the conditions in the tooth cavity that can inhibit the growth of these bacteria are very important for maintaining oral health. The results of the MIC and MBC measurements for the six essential oils on *C. albicans* were also shown in Table 4. The MIC of Cinnamon bark, Lemongrass, Clove bud and Girofle oil against *C. albicans* was 3.13 mg/mL and Tea tree and Eucalyptus was measured as 25 mg/mL. The MBC of Cinnamon bark and Lemongrass oil against *C. albicans* was measured to be less than 3.13 mg/mL, and the MBC of Clove bud and Girofle oil was measured to be 6.25 mg/mL and Tea tree and Eucalyptus was more than 25 mg/mL. Figure 5 shows the results of MBC measurement of 6 essential oils against *C. albicans*.

Table 4: MIC and MBC of the six essential oils

Essential oils	Concentrations (mg/mL)			
	<i>Streptococcus mutans</i>		<i>Candida albicans</i>	
	MIC	MBC	MIC	MBC
Cinnamon bark	3.13	6.25	3.13	<3.13
Lemongrass	3.13	6.25	3.13	<3.13
Clove bud	6.25	12.5	6.25	6.25
Tea tree	25	>25	>25	>25
Eucalyptus	25	>25	25	>25
Girofle (clove)	6.25	6.25	3.13	6.25

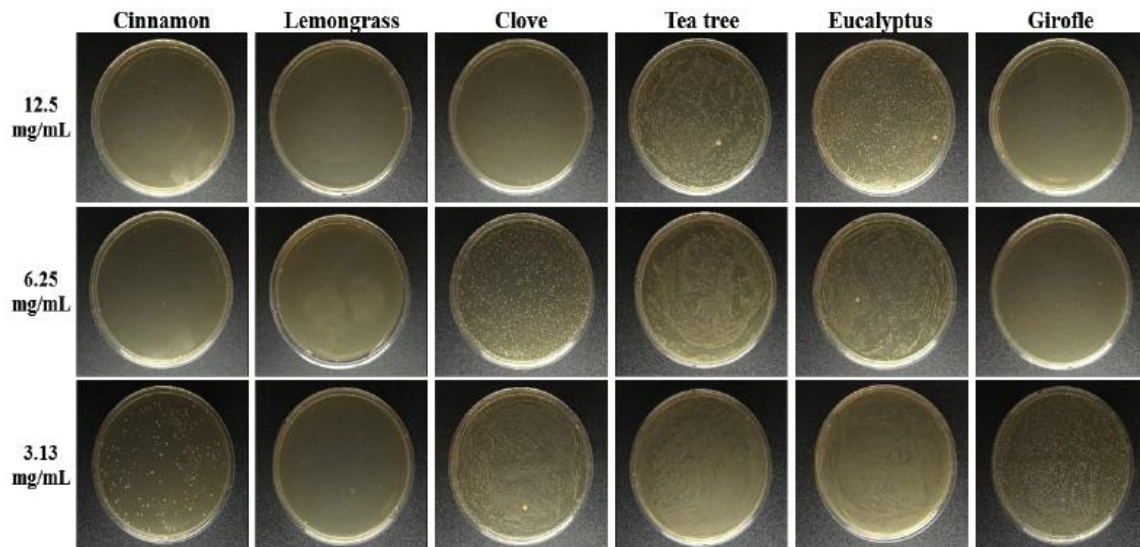


Figure 4. MBC values of selected essential oils (3.13 to 1.25 mg/mL) against *Streptococcus mutans*.

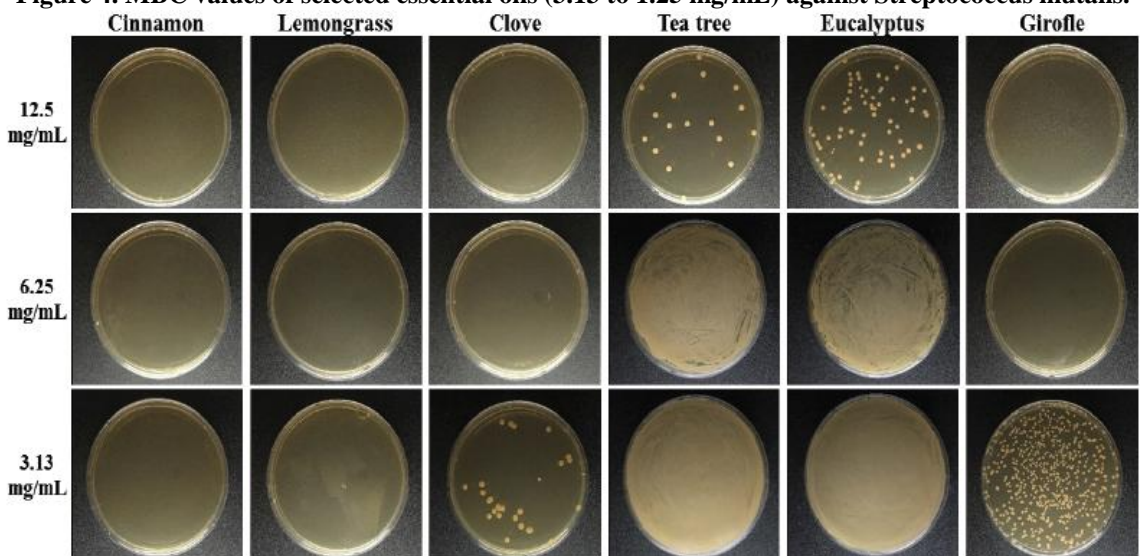


Figure 5. MBC values of selected six essential oils (3.13 to 1.25 mg/mL) against *Candida albicans*

3.3. Chemical constituents of essential oils by GC/MS

The chemical ingredients of the six essential oil were analyzed using GC/MS. Figure 6 is the GC/MS total ion chromatogram (TIC) of Cinnamon bark oil. The Cinnamon bark oil mainly contained cinnamaldehyde (54.22 %) and eugenol (18.99 %) that elutes at 11.74min and 12.74min. Other components identified were 1,8-cineole (2.20%, 7.86min), linalool (5.72%, 8.95min), caryophyllene (6.51%,

13.52min), cinnamyl acetate (7.17%, 13.91min) and benzyl benzoate (2.73%,17.70min). In previous studies, cinnamaldehyde, one of the major components of cinnamon bark oil, was the best antimicrobial effect against *S. mutans*, followed by eugenol [20, 8]. Although cinnamon bark oil is an antibacterial agent, it can be used as a food additive for people, but there is a case report of allergic contact dermatitis and stomatitis [21, 22].

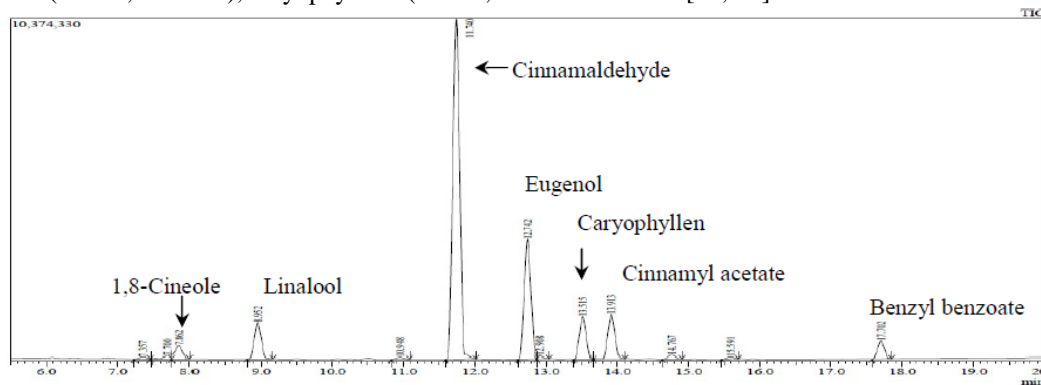


Figure 6. GC/MS TIC of Cinnamon bark oil (*Cinnamomum zeylanicum*, Germany)

Figure 7 is the GC/MS TIC of Lemongrass oil. The Lemongrass oil predominantly contained neral (Citral b,

cis-Citral) (34.35 %) and geranial (44.78 %) that



elutes at 11.09min and 11.50min. Other components identified were limonene (3.76%, 7.75min), geranyl acetate (3.71%, 12.91min), caryophyllene (3.02%, 13.52min). These citral components of Lemongrass essential oil have been reported to exhibit antibacterial activity [11, 23].

Figure 8 is the GC/MS TIC of Clove bud oil. Figure 11 is the GC/MS TIC of Girofle (clove) oil. Clove bud and Girofle (Clove) oils had the same main ingredients but different contents of ingredients. The antimicrobial activity of the oil was also different depending on the content of the major components. The main component of Clove bud and clove essential oil is eugenol and MBC for *S. mutans* of Girofle (clove), which has high eugenol content, was found at lower concentration (Table 4). The Clove bud oil predominantly contained eugenol (76.43%) and eugenyl acetate (16.28 %) that elutes at 12.76min and 14.77min. Other components identified were caryophyllene (5.49%, 13.52min), caryophyllene oxide (1.20%, 15.60min). The Girofle (clove) oil predominantly contained eugenol (83.57%) and eugenyl acetate (11.29 %) that elutes at 12.76min and 14.77min. Other components identified were caryophyllene (3.62%,

13.52min), caryophyllene oxide (0.96%, 15.60min). There has been a previous study that these Clove oils have a medical and biochemical activity and are useful natural oils widely used in the pharmaceutical, perfume and flavoring industries [24].

Figure 9 is the GC/MS TIC of Tea tree oil. The Tea tree oil mainly contained terpinen-4-ol (43.36 %) and γ -terpinen(21.31 %) that elutes at 10.26min and 8.23min. Other components identified were α -terpinen(10.41 %, 7.53min), α -pinene(2.53%, 6.09min), ρ -cymene(4.13%, 7.70min), 1,8-cineole (3.99%, 7.87min), α -terpinolene(3.32%, 8.68min), α -terpineol(3.28%, 10.49min)[25].

Figure 10 is the GC-MS total ion chromatogram of Eucalyptus oil. The Eucalyptus oil mainly contained 1,8-cineole (82.76 %) that elutes at 7.87min. Other components identified were α -pinen(1.98%, 6.09min) limonene (2.20%, 7.86min), terpinen-4-ol (1.31%, 10.26min), α -Terpineol (7.03%, 10.49min). The major component of Eucalyptus oil is 1,8-cineole, and there has been a report on the antimicrobial effect measurement [10,26].

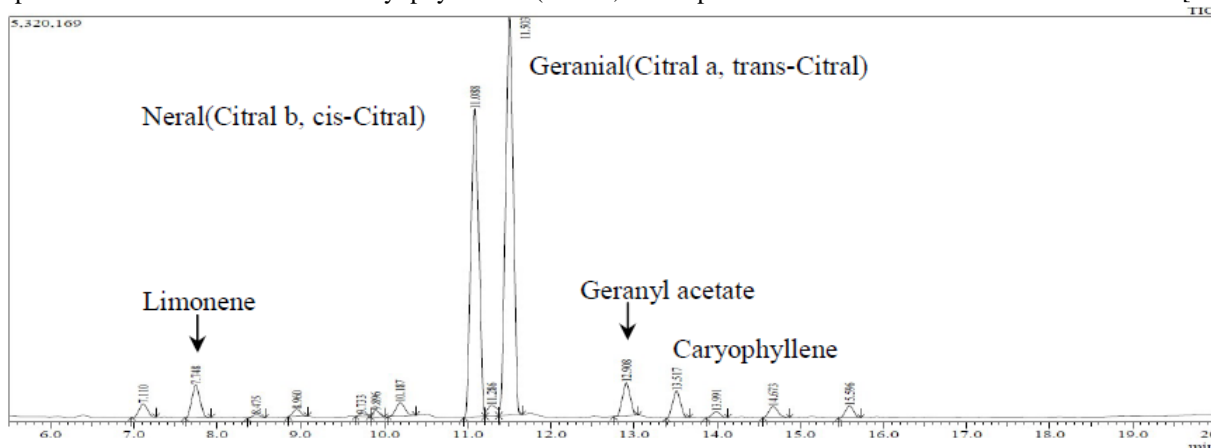


Figure 7. GC/MS Total Ion Chromatogram(TIC) of Lemongrass oil (*Cymbopogon flexuosus*, Germany)

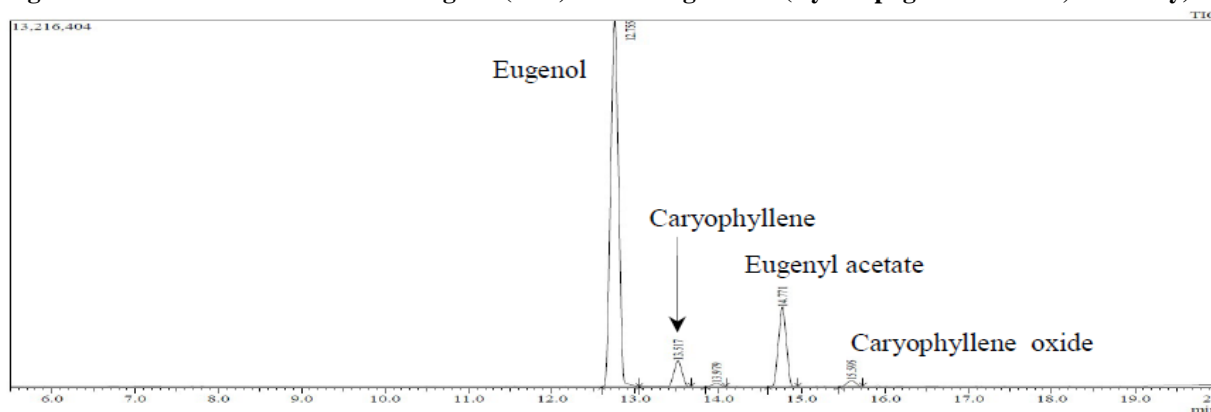


Figure 8. GC/MS TIC of Clove bud oil (*Syzygium aromaticum*, Germany)

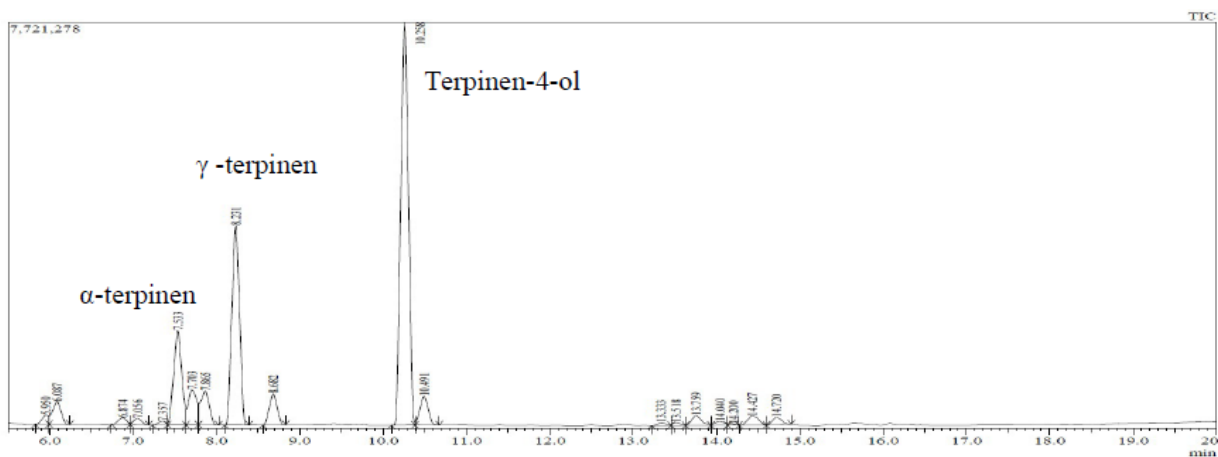


Figure 9. GC/MS TIC of Tea tree oil (*Melaleuca alternifolia*, Korea)

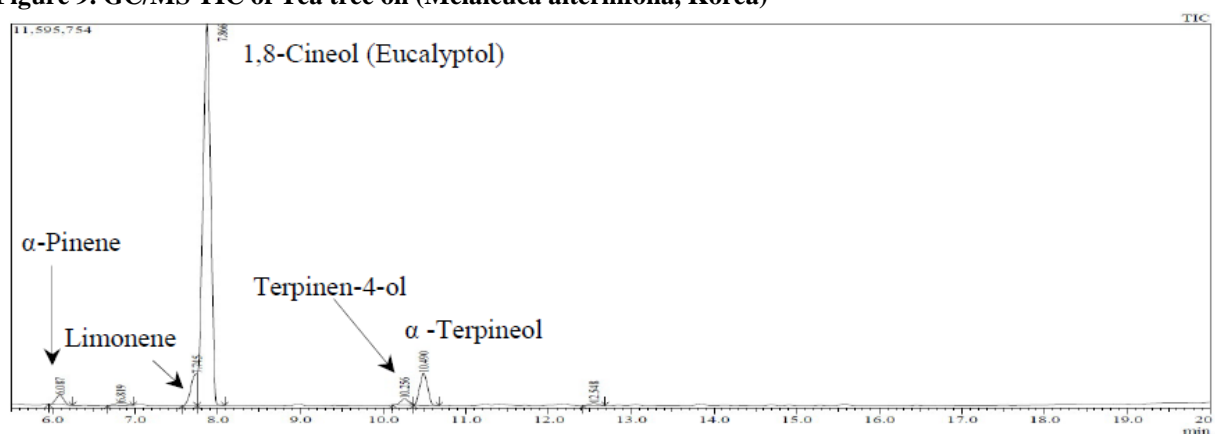


Figure 10. GC/MS TIC of Eucalyptus oil (*Eucalyptus radiata*, Germany)

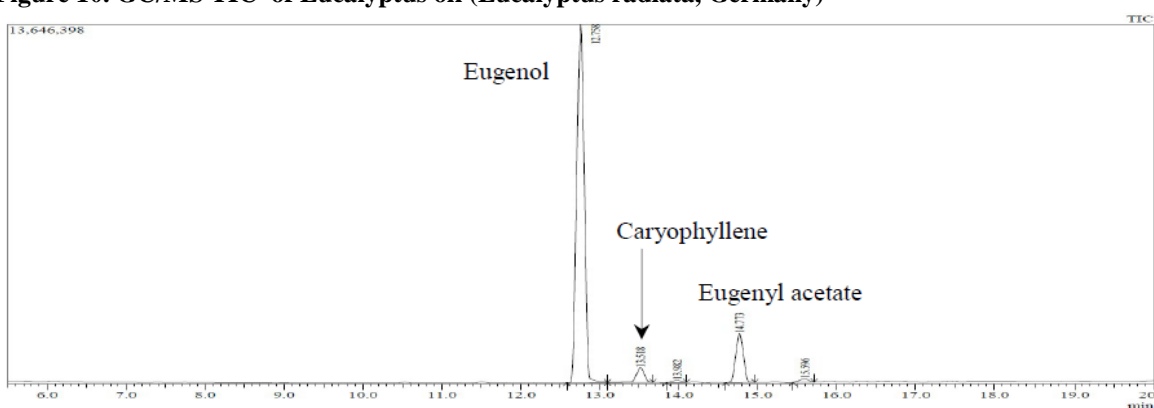


Figure 11. GC/MS TIC of Girofle(clove) oil (*Eugenia caryophyllus* (Clove), France)

3.4. Cytotoxicity on the agar overlay test of Lemongrass and Girofle oils

The cytotoxicity test using the agar middle layer method used in this experiment was performed by culturing the standardized cells and analyzing the cell changes for the test evaluation components, and it is highly reproducible, objective, relatively simple, fast and sensitive, and suitable for evaluating biological properties or toxicity of new components. The concentration of MBC (0.313%) of cinnamon bark oil measured in this experiment was higher than that recommended for use at concentrations below 0.1%.

Therefore, the toxicity test was carried out on an agar overlay test according to the concentrations of Lemongrass and Girofle (Clove) essential oils known to be non-toxic. Toxicity evaluation of Lemongrass and Girofle (clove) oils using agar middle layer method is shown magnified by microscope in Figure 12. The results exhibited that the concentration of the two oils was 3.13 mg / mL (0.313%), which is similar to that of living cells in unprocessed agar. The 0.313% concentration of Lemongrass oil in the two oils was consistent with the MIC for *S. mutans* and *C. albicans*, and was found to be antimicrobial and safe to use.

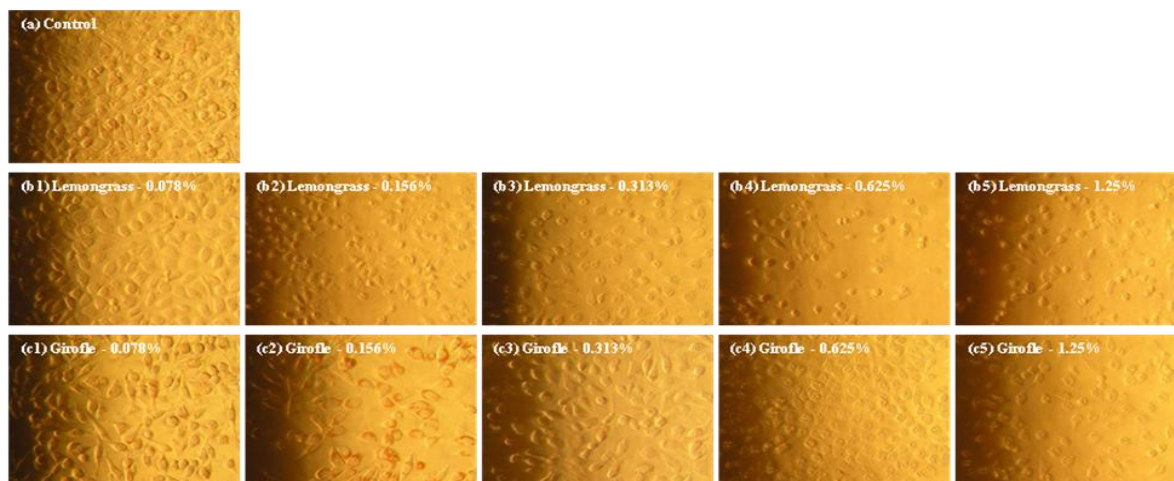


Figure 12. Optical microscopy of L929 mouse fibroblast cells in the agar overlay test with the samples: cells without any sample (a), Lemongrass (b1-b5), and Girofle (c1-c5) (with 200 × magnification).

IV. CONCLUSION

In this paper, 6 kinds of essential oils which have antibacterial effect and can be edible were selected to investigate the possibility of use as a natural antimicrobial active ingredient of oral mouthwash. First, the antimicrobial effect of *C. albicans* was measured among *S. mutans* and oral flora causing periodontal disease, and Cinnamon bark and Lemongrass oil showed the highest MBC of 3.13 mg/mL (0.313%), followed by Girofle (Clove) and Clove bud oil (MBC: 6.25 mg/mL (0.625%)) showing significant antimicrobial effects. The concentration of MBC (0.313%) of cinnamon bark oil measured in this experiment was higher than the concentration recommended to not be used at a concentration of 0.1% or more. Therefore, the toxicity test was carried out with an agar overlay test according to the concentrations of Lemongrass and Girofle (Clove) essential oils known to be non-toxic and it was found to be non-toxic at concentrations below 0.313%. The major components were identified by analyzing the components using GC/MS. Cinnamon bark was identified as cinnamaldehyde and eugenol, and Lemongrass was identified as a major ingredient of the antimicrobial effect through the analysis of constituents of essential oils such as citral components, neral and geranial, and girofle (Clove), eugenol. It was confirmed that information on the concentration of major components can be obtained according to the concentration used and the evaluation of the antimicrobial effect can be accurately performed. In addition, the optimum concentration could be determined through safety evaluation of the Lemongrass and Girofle (Clove) oils that represent low MIC and MBC among the evaluated essential oils. For these natural oils, in addition to antimicrobial effects against *C. albicans* in *S. mutans* and oral flora, further studies on other bacteria and component analysis and safety evaluation studies can be expected to be applied to the development of a safe and antimicrobial natural mouthwash.

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