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Antibacterial activity of grapefruit seed extract and seven kinds of essential and blended essential oils

Young Sam Yuk
Professor, Department of Clinical Medical Science, Dankook University Graduate School of Health and Welfare

Grapefruit seed extract와 7종의 Essential oil 및 혼합 Essential oil의 항균 활성

육영삼
단국대학교 보건복지대학원 임상의과학과 교수

Abstract Objectives: Antibiotics help treat Vaginitis, and prolonged usage of antibiotics can lead to resistance. Methods: This study investigates the antimicrobial activity of two types of lactic acid bacteria using essential oils. After cultivation by adding grapefruit seed extract (GSE), eucalyptus, tea tree, clove bud, cinnamon, lemongrass, thyme, and ginger oils in a specific ratio, pathogenic microorganisms, namely *E. coli, C. albicans,* and lactic acid bacteria were released. The number of bacteria was measured using a medium suitable for the strains. Results: The essential oils and GSE inhibited pathogenic microorganisms, and the inhibitory concentration of GSE against pathogenic bacteria (*E. coli, C. albicans*) was confirmed. The non-inhibitory mixing ratio was also confirmed (50 μl of eucalyptus globulus (EG) oil and 50 μl of melaleuca alternifolia oil (tea tree oil, TTO) at 200 ppm GSE (pH 5.0, 5.5, 6.0)). Conclusion: Essential oils can be considered as an alternative to antibiotics because of their antibacterial properties. They are useful as auxiliary antibacterial agents for patients under long-term antibiotic treatment.

Key Words : Essential oil, Microbiome, Antibacterial, Lactic acid bacteria, Phytochemical

요 약 본 배경 : 질염은 항생제를 복용하는 방식으로 치료를 하고 있으며, 이러한 항생제의 지속적인 치료는 내성을 유발할 수 있다. 연구 방법 : Lactic acid bacteria 2종에 에센셜 오일을 이용한 항균 활성을 보고자 한다. SE(Grapefruit Seed Extract), eucalyptus, tea tree, clove bud oil, cinnamon oil, lemongrass oil, thyme oil, ginger oil을 일정 비율로 넣어 배양 후, 병원성 미생물- *E. coli, C. albicans*와 Lactic acid bacteria은 균주에 맞는 배지를 사용하여 균 수를 측정하였다. 결과 : Essential oil 7종과 GSE가 병원성 미생물에 억제 효과가 있으며, 병원성균(*E. coli, C. albicans*)에 대한 Grapefruit seed extract(GSE)의 억제농도를 확인하였다, 병원성균은 억제하고 Lactic acid bacteria는 억제하지 않는 배합비도 확인하였다(GSE 농도가 200ppm에서 *Eucalyptus globulus*(*EG*) oil 50㎕와 *Melaleuca alternifolia* oil(Tea tree oil, TTO) 50㎕(pH 5.0, 5.5, 6.0)). 결론 : 본 실험에서 Essential oil은 다양한 항균 활성 가지고 있어 항생제 대안으로도 생각할 수 있으며, 장기 항생제 치료환자에 대한 보조 항균제로서도 유용할 것으로 생각된다.

주제어 : 에센셜 오일, 항균활성, 마이크로바이옴, 유산균, 파이토케미칼

1. Introduction

1.1 Introduction

There are 100 trillion microorganisms in the human body; the number of genes in microorganisms is more than 100 times the number of human genes [1 -2]. Most microbiomes are present in the digestive system, including the intestine. They have also

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widely been found in the respiratory tract, genitals, oral cavity, and on the skin [3].

Vaginitis, also called “female cold,” includes bacterial vaginosis, vulvar candidiasis, trichomoniasis, and non-infectious vaginitis [4]. It is a type of change in the feminine microbiota characterized by Vaginitis can result from changes in the vaginal microbiota. [5-6]. Vaginal infections can lead to pain, discomfort, dissatisfaction in sexual relationships, and absence from school or work [7]. Currently, vaginitis is treated through prescribing antibiotics either orally or injecting them directly into the vagina. Continuous antibiotic treatment is usually recommended [4], but this can lead to antibiotic resistance [8-9]. Treatment for [10] vulvar candidiasis [11], and resistant infection [12] is weak. As an alternative to this, many phytochemical studies have recently been conducted on Vaginitis [13].

This experiment used grapefruit seed extract (GSE) [14-16], which is an effective substitute to antibiotics against bacteria and viruses, such as Escherichia coli (E. coli), Cand*ida species*, such as *Candida albicans* (*C. albicans*) and *Candida tropicalis*, etc.; eucalyptus globulus (EG) oil [17-18], which is effective against bacteria, such as *Escherichia coli* (*E. coli*); and melaleuca alternifolia oil (tea tree oil, TTO), which is effective against viruses, such as Candida species and bacteria that cause dermatitis [19-21]; clove bud oil (syzygium aromaticum, CBO) [22], cinnamon oil (cinnamomum zeylanicum, CO) [23], lemongrass oil (cymbopogon citratus to report the antibacterial activity using essential oils of LO) [24], thyme oil (thymus vulgaris, TO) [25], and ginger oil (zingiber officinale, GO) [26].

2. Materials and Methods

2.1 Test strain

The strains used for the antibacterial activity test of essential oils included two types of lactic acid bacteria, a yeast infection-related fungus, and two types of bacteria that cause food poisoning. Table 1 shows.

Table 1. Strain and media used in this study

Strain	Medium
Lactobacillus plantarum (KCTC 3104)	MRS agar
Streptococcus thermophilus (KCTC 37119)	MRS agar
Candida albicans (KCTC 7752)	PDA
Escherichia coli 0157:H7 (ATCC 43895)	TSA

2.2 Essential oil

Among the essential oils used in the experiment, GSE and eucalyptus oil were Biochemica products, tea tree oil (leaf) was AEOC, clove bud oil, cinnamon oil, lemongrass oil, thyme oil, ginger oil were Euro aroma products. Table 2 summarizes the botanical names.

Table 2. Essential oils used in this study

Essential Oil	Botanical Name	Brand
Grapefruit Seed Extract		Chemiebuero
Eucalyptus Oil	*Eucalyptus globulus*	Biochemica
Tea Tree Oil(Leaf)	*Melaleuca alternifolia*	AEOC
Clove Bud Oil	*Syzygium aromaticum*	Euroaroma
Cinnamon Oil	*Cinnamomum zeylanicum*	Euroaroma
Lemongrass Oil	*Cymbopogon citratus*	Euroaroma
Thyme Oil	*Thymus vulgaris*	Euroaroma
Ginger Oil	*Zingiber officinale*	Euroaroma

2.3 Antibacterial Effects of GSE and Seven Essential Oils in a Mixed Culture of Lactic Acid Bacteria and Pathogenic Microorganisms

The antibacterial test of essential oils in a mixed culture of lactic acid bacteria and pathogenic microorganisms followed the test method of Denkova et al. [27] and Cunha et al. [28]. Each pathogenic microorganism was used after confirming the presence or absence of homogeneous colony formation in trypticase soy agar (Difco, MI, USA), adjusting the bacterial concentration to 0.5 MacFarland standard (1.5 x 108 CFU/mL), and diluting in 10 times. Lactic acid bacteria were re-inoculated with MRS broth (Difco) after confirming the presence or absence of homogeneous colony formation in MRS agar (Difco, MI, USA), incubating in microaerobic conditions (10% CO2) at 35°C for 24 hours, and cultivating the bacterial solution at 3000 rpm. After centrifugation for 10 minutes, the supernatant was discarded, the bacterial mass was washed a second time with sterile physiological saline, and the concentration of the bacteria was adjusted to the 0.5 MacFarland standard (1.5 x 108 CFU/mL), followed by dilution in 10 steps. To mix and cultivate pathogenic microorganisms and lactic acid bacteria, the probiotics medium MRS broth (Difco) and tryptic soy broth (Difco) were mixed; a culture medium for pathogenic microorganisms (on the other hand, MRS broth (Difco)) was used for lactic acid bacteria cultivation. After inoculation, 10 μl of lactic acid bacteria strain and 10 μl CFU/mL each, and 10 μl of pathogenic strains were inoculated with 10 μl CFU/mL and GSE, eucalyptus, tea tree, clove bud, cinnamon, lemongrass, and thyme oils. After adding ginger oil in a certain ratio, the culture was kept under microaerobic (10% CO2) conditions at 35°C for 72 hours (however, *C. albicans* was kept under aerobic conditions). Pathogenic microorganisms (tryptic soy broth (Difco)-*E. coli*), potato dextrose agar (Difco, MI, USA)-*C. albicans*), and lactic acid bacteria (silver MRS agar (Difco)) were measured using a suitable medium.

3. Results

3.1 Antibacterial Effects of GSE and Seven Essential Oils in a Mixed Culture of Lactic Acid Bacteria and Pathogenic Microorganisms

Table 3 shows the antibacterial effect of GSE in the mixed culture of lactic acid bacteria and pathogenic microorganisms. At a culture pH of 5.0, *C. albicans* had a GSE concentration of 500 ppm or higher, *E. coli* had 100 ppm or higher, *L. plantarum* had 500 ppm or higher, and *S. thermophilus* had 1000 ppm or higher. The inhibitory effect was observed at the GSE concentration of 2000ppm or more, *E. coli* of 150ppm or more, *L. plantarum* of 1000ppm or more, and *S. thermophilus* of 500ppm or more. It was tested that it did not appear.

Table 3. Antimicrobial activity of grapefruit seed extract by concentration according to pH [*4]

pH	GSE con.[*1] (ppm)	*C. albicans*	*E. coli* 0157:H7	*L.plantarum*	*S. thermophilus*
5.0	100	+[*2]	+	+	+
	200	+	−	+	+
	300	+[*3]	−	+	+
	400	+	−	+	+
	500	−	−	−	+
	1000	−	−	−	−
	1500	−	−	−	−
	2000	−	−	−	−
	2500	−	−	−	−

[*1] The concentration of GSE is an appropriate concentration in 10 ml of a medium prepared in a 1:1 ratio of MRS broth and PDA broth.
[*2] "+" means that the bacterial concentration is set to around 10^6, and the bacteria are alive after 72 hours of incubation.
[*3] "−" means that the bacteria concentration is set to about 10^6,

and the bacteria have not survived after 72 hours of incubation.
4) "pH" is the pH of the medium adjusted with apple cider vinegar.

Table 4 shows the results of the antibacterial effect of each combination of GSE and essential oils in the mixed culture. When the concentration of GSE was 100ppm, 50μl of ginger oil showed inhibitory effect only on *E. coli*. At 200 ppm GSE, clove bud, cinnamon, lemongrass, thyme, ginger oils inhibited only *E. coli*. At 300 ppm GSE, lemongrass, thyme, and ginger oils inhibited *C. albicans*. Clove bud, cinnamon, lemongrass, thyme, and ginger oils inhibited *E. coli* at 200 ppm of GSE. *L. plantarum* was inhibited by thyme and ginger oils, and *S. thermophilus* only by lemongrass oil.

Table 4. Antibacterial activity of GSE and essential oils at pH 5.0

GSE con. (ppm)	Essential oil Name	con. (μl)	*C. albicans*	*E. coli* 0157:H7	*L. plantarum*	*S. thermophilus*
100	Clove Bud Oil	each	+	+	+	+
	Cinnamon Oil	50	+	+	+	+
	Lemongrass Oil		+	+	+	+
	Thyme Oil		+	+	+	+
	Ginger Oil		+	−	+	+
200	Clove Bud Oil	each	+	−	+	+
	Cinnamon Oil	50	+	−	+	+
	Lemongrass Oil		+	−	+	+
	Thyme Oil		+	−	+	+
	Ginger Oil		+	−	+	+
300	Clove Bud Oil	each	+	−	+	+
	Cinnamon Oil	50	+	−	+	+
	Lemongrass Oil		−	−	+	−
	Thyme Oil		−	−	−	+
	Ginger Oil		−	−	−	+

Table 5 shows the antibacterial effect of the solution of GSE and two types of essential oils at pH 5.0 in the mixed culture. When the GSE concentration was 100 ppm, the mixed solution of lemongrass and thyme oils suppressed *E. coli* and *S. thermophilus,* the mixed solution of cinnamon and ginger oils suppressed *C. albicans* and *L. plantarum.* When the GSE concentration was 200 ppm, the mixed solution of lemongrass and thyme oils did not inhibit only *L. plantarum,* but suppressed the remaining 3 types of bacteria; the mixed solution of cinnamon and ginger oils suppressed all 4 types of bacteria. A 300 ppm GSE concentration showed the same result as the 200ppm concentration.

Table 5. Antimicrobial activity of GSE and two mixed essential oils at pH 5.0

GSE con. (ppm)	Essential oil Name	con. (μl)	*C. albicans*	*E. coli* 0157:H7	*L. plantarum*	*S. thermophilus*
100	Lemongrass Oil + Thyme Oil Cinnamon Oil + Ginger Oil	each 50	+	−	+	−
		each 50	−	−	+	−
200	Lemongrass Oil + Thyme Oil Cinnamon Oil + Ginger Oil	each 50	−	−	+	−
		each 50	−	−	−	−
300	Lemongrass Oil + Thyme Oil Cinnamon Oil + Ginger Oil	each 50	−	−	+	−
		each 50	−	−	−	−

Table 6 shows the antibacterial effect of the solution mixed with GSE and three kinds of essential oils in the mixed culture under 5.0 pH

level. The blending ratio that inhibits pathogenic bacteria and does not inhibit lactic acid bacteria was not observed. However, at 300ppm GSE concentration, a mix of 25μl clove bud, 25μl cinnamon, 25μl lemongrass oils (A); a mix of 25μl lemongrass, 25μl thyme, 25μl ginger oils (B); and a mix of 25μl clove bud, 25μl cinnamon, 25μl lemongrass, 25μl thyme oils (C) was added to *C. albicans* and *E. coli.* A, B, and C inhibited *L. plantarum, S. thermophilus,* and *L. plantarum*, respectively.

Table 6. Antibacterial activity of GSE and 3 blended essential oils at pH 5.0

GSE con. (ppm)	Blending Essential oil	*C. albicans*	*E. coli* 0157:H7	*L. plantarum*	*S. thermophilus*
100	A [1)]	−	+	+	+
	B [2)]	−	+	+	+
	C [3)]	−	+	+	+
200	A	−	+	+	+
	B	−	+	+	−
	C	−	+	+	−
300	A	−	−	−	+
	B	−	−	+	−
	C	−	−	−	+

1) "A" is a blend of 25μl of clove bud oil, 25μl of cinnamon oil, and 25μl of lemongrass oil.
2) "B" is a blend of 25μl of thyme oil, 25μl of ginger oil, and 25μl of lemongrass oil.
3) "C" is a blend of 25μl of clove bud oil, 25μl of cinnamon oil, 25μl of lemongrass oil, and 25μl of thyme oil.

Table 7 shows the antimicrobial activity of the eucalyptus and tea tree oils mixed solution at 200ppm GSE concentration, 5.0 and 5.5 pH in the mixed culture wherein eucalyptus oil and tea tree oil mixed solution inhibited *C. albicans* and *E. coli,* respectively. It did not inhibit *L. plantarum* and *S. thermophilus*, which are beneficial bacteria, and lactic acid bacteria, respectively.

Table 7. Antibacterial activity of mixed eucalyptus oil and tea tree oil by pH [1)] when the concentration of GSE is 200ppm

pH	GSE con. (ppm)	Blending oil Name	Essential Con.	*C. albicans*	*E. coli* 0157:H7	*L. plantarum*	*S. thermophilus*
5.0	200	Eucalyptus oil + Tea tree oil	each 50μl	−	−	+	+
5.5	200	Eucalyptus oil + Tea tree oil	each 50μl	−	−	+	+
6.0	200	Eucalyptus oil + Tea tree oil	each 50μl	−	−	+	+

1)"pH" is the pH of the medium adjusted with apple cider vinegar.

Table 8 shows the antimicrobial activities of the 7 mixed solutions at pH 5.0 in the mixed culture.

Table 8. Antibacterial activity of GSE and 7 kinds of essential oils at pH 5.0

GSE con. (ppm)	Concentration of 7 blended essential oils [1)]	*C. albicans*	*E. coli* 0157:H7	*L. plantarum*	*S. thermophilus*
100	Each 10μl	−	−	+	−
200	Each 10μl	−	−	+	−
300	Each 10μl	−	−	+	−

1) "7 blended essential oils" include eucalyptus oil, tea tree oil, clove bud oil, cinnamon oil, lemongrass oil, thyme oil, and ginger oil.

4. Discussion

Grapefruit seed extract (GSE) [14-16],

eucalyptus globulus oil (EG) [17-18], melaleuca alternifolia oil (tea tree oil, TTO) [19-21], clove bud oil (syzygium aromaticum, CBO) [22], which has antibacterial and antifungal properties; cinnamon oil (cinnamomum zeylanicum, CO) [23], lemongrass oil (cymbopogon citratus, LO) [24], thyme oil (thymus vulgaris, TO) [25], and ginger oil were used to develop a blending ratio that makes lactic acid bacteria [29] effective against pathogens survive and inhibits pathogens by mixing essential oils, such as (Zingiber officinale, GO) [26]. Thyme, ginger, eucalyptus, and tea tree oils were mixed with GSE. GSE [14-17], which is effective against *E. coli* at a pH level of 5.0 and a GSE concentration of more than 200 ppm were observed in the experiment. It was confirmed that *E. coli* was inhibited at 5.5 to 150 or higher, and *C. albican*s was also inhibited at pH 5.0, with GSE concentration at 500 ppm or higher, and at 5.5 to 1000 ppm or higher, *L. plantarum, S. thermophilus* was also confirmed to be inhibited. As a result of the antibacterial activity test of the mixed solution of clove bud [26], cinnamon [27], lemongrass [28], thyme [29], and ginger oils [30], *C. albicans, E. Coli* inhibition and lactic acid bacteria were not observed. When GSE was 300ppm, with a mix of clove bud, cinnamon, lemongrass oils (25μl each); a mix of lemongrass, thyme, ginger oils (25 μl each); and a mix of clove bud, cinnamon, lemongrass, and thyme oils (25 μl each) showed inhibitory effects on *C. albicans and E. coli*, but not on lactic acid bacteria.

EG oil is known to have inhibitory power against *C. albicans, E. coli* and Ent faecium (18-20), and TTO is known to have antimicrobial activity against *C. albicans* and MRSA (21-25). In this experiment, it was confirmed that 50 μl of EG oil and 50 μl of TTO were inhibited, but not lactic acid bacteria at pH 5.0, 5.5, 6.0, and 200 ppm GSE concentration. Clove Bud Oil, Cinnamon Oil, Lemongrass Oil, Thyme Oil Ginger Oil, Eucalyptus oil, and Tea tree oil each 10 μl of mixed solution did not inhibit only *L. plantarum* regardless of the concentration of GSE.

Essential oils, such as EG oil and TTO are not stable when taken in large amounts or for a prolonged period. Hence, there are concerns about the side effects with regard to women's vaginal management. Thus, a lot of time should be invested in stability experiments. Additionally, since the management of domestic essential oils has not been established, a study on essential oil management guidelines is considered necessary.

Blending of essential oils that do not inhibit beneficial bacteria but only harmful bacteria has a GSE concentration of 200 ppm at pH levels of 5.0 and 5.5. Further, the antibacterial effect of essential oils found in a mixed solution of eucalyptus and tea tree oils is an alternative to antibiotics. It is believed that this will be possible, and the product development research for the purpose of prevention is considered to be necessary for feminine quality management products that use essential oils as raw material.

5. Conclusion

This study investigated the blending ratio of seven kinds of essential oils and GSE that inhibit pathogenic microorganisms and do not inhibit lactic acid bacteria. A mixed culture of lactic acid bacteria (LAB) and pathogenic microorganisms (*E. coli, C. albicans*) was prepared. The inhibitory concentration of grapefruit seed extract (GSE) was confirmed (*C. albicans*: pH 5.0-500ppm or higher, 5.5-1000ppm or higher, *E-coli*: pH 5.0-200ppm or higher, 5.5-150ppm or higher). The mixing ratio that inhibits pathogenic bacteria and does

not inhibit lactic acid bacteria (50 μl of eucalyptus globulus (EG) oil and 50 μl of melaleuca alternifolia oil (Tea tree oil, TTO) at 200 ppm GSE (pH 5.0, 5.5, 6.0)) was confirmed. In conclusion, essential oils have various antibacterial activities and can be considered as an alternative to antibiotics. These are considered to be useful as auxiliary antimicrobial agents for patients under long-term antibiotic treatment. Vaginitis of candida genus, such as vulvar candidiasis is more about prevention than treatment. Further research regarding the same is needed.

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육 영 삼(Young-Sam Yuk) **[정회원]**

· 1994년 8월 : 단국대학교 일반대학원 미생물학(이학석사)
· 2016년 8월 : 단국대학교 보건대학원 보건학박사
· 2017 ~ 현재 : 단국대학교 보건복지대학원 교수

· 관심분야 : 에센셜오일 피부 항노화
· E-Mail : y60320@gmail.com