Study of Antimicrobial Activity of New Zealand's Tea Tree Essential Oil , Grapefruit Seed Extract and its major Component.

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Abstract

Manuka oil sometime named New Zealand's tea tree oil is soluble in oil and come from nature. The α-pinene extracted from Manuka oil and R-limonene which is one of the component of extracted Citrex from Grapefruit were used to estimate the antimicrobial activity and to improve the capability of antiseptic. Disk diffusion and broth dilution methods were used to measure the antimicrobial activity. *Escherichia coli* which is gram-negative bacteria and *Staphylococcus aureus* which is gram-positive bacteria were used as strain. The antimicrobial activity of Manuka oil and α-pinene for *Escherichia coli, Staphylococcus aureus* is similar when the concentration of Manuka oil and α-pinene is 10$\mu\ell$. However, Antimicrobial activity of Manuka oil for *Escherichia coli, Staphylococcus aureus* is better than that of α-pinene when the concentration of Manuka oil and α-pinene is low. Antimicrobial activity of Citrex is superior to that of R-limonene. The proper ratio of Maunka

oil and Citrex can improve the antimicrobial activity. The proper ratio obtained from studies was 75% of Maunka oil and 25% Citrex for *Escherichia coli,* 25% of Maunka oil and 75% Citrex for *Staphylococcus aureus.*

Introduction

The antiseptic which is used in food, midicine and cosmetics to preserve the quality of production for a long time has brought about a revolution in human life.

The antiseptic protects food from oxidizing and being decayed by bacteria or microorganism when it is exposed in the air. There are generally used EDTA, Propionic acid calcium, Benzonic acid, Soldium, Sodium nitrite, Sorbic acid including Phenol derivatives BHA, BHT. BHA and BHT are generally used as antioxidant because of their Chelating antion but they are also used as antiseptic.[1-4] The safety of antiseptic in the medicine and cosmetic field should be more throughly examined so that the proper one can be selected.

Generally there are a little difference in the meaning of sterilization, disinfection and antiseptic. The sterilization means decreasing the vitality of pathogenic microorganism to prevent their propagation and infection and the disinfection means destroying all of the microorganism including spore. The antiseptic means preventing decay and destroying all of the microorganism not to propagate and grow. Generally the function of antiseptic is bacteriostatic action which restrains the propagation of microorganism and arrests the metabolism not to grow and the bactericidar action means destroying microorganism not to regenerate and has the function of physicochemistrical desinfection or antiseptic.

The purpose of this study is to get antiseptic which come from nature and can complement the defect irritation of parabens which has been widely used as antiseptic in cosmetics. And for that, the antiseptic effect of botanical antibiotic agent was examined and compared for the substance water soluble and soluble in oil, and that was used as basic information for getting superior antiseptic.

At present, there are known several kinds of natural antibiotic agent and New Zealand's tea tree oil is one of them. New Zealand's tea tree oil is soluble in oil and used as traditional folk remedy of New Zealand's natives. It is also used as partial antiseptic and antibiotic agent in Australia and composed of complex elements of kinds of terpene and kinds of triketone.[5-8)]

α-pinene and β-pinene of New Zealand's tea tree oil are bicyclic terpene of monoterpens and their molecular formula is $C_{10}H_{16}$ and they are safe as antiseptic. Also, the oil extracted form the leaves of Melaleuca alternilolia(tea tree) shich grows in Australia has been widely used for septicemia for about 80 years.[9)] According to the recent study, this oil has effect for propion bacterium pimple and gram-positive and gram-negative bacteria including *staphylococus aureus* which has tolerance to methicillin in the experimental condition.[10-12)]

Citrex extracted from Grapefruit Seed is known as water soluble substance having antibiotic effect and R-limonene, the one of its composition elements, is used as antibiotic agent.[13)]

Therefore, in this study, the antibiotic effect of botanical substance for gram-negative bacteria *"Eschericha coli"* ans gram-positive bacteria *"Staphylococcus aureus"* was measured to confirm if the natural botanical substance can be used as antiseptic.

As sample, New Zealand's tea tree oil and its main element, α-pinene and Citrex which is extracted from Grapefruit Seed and water-soluble and its main element, R-limonene are used. In this study, their antibiotic effect is measured and the value was compared with the efficiency of parabens, propyl para hydroxybenzoate(PPHB). Based upon the result, the method which can increase the antibiotic effect is suggested.

Material and Method

1. Strain and Medium

To examine the antibiotic effect parabens and natural botanical substance, the representative gram-negative bacteria, "Escherichia coli(ATCC 11105)" and gram-positive bacteria "Staphylococcus aureus(ATCC 6538)" are used as strain and nutrient broth was used to culture them. 250㎖ erlenmeyer flask was filled with nutrient broth, and the strains was inoculated in solid medium and cultured for 24hours at 30℃, 150rpm incubation. The culture fluid diluted O.D.(660mm) with disinfected medium was used as inoculum The strain was preserved in nutrient agar.

2. Reagent and Sample

Manuka oil is stock solution which is obtained from tea tree growing spontaneously in the high mountains in New Zealand and Table 1 shows the result of element analysis. The reagents used in the examination are Manuka oil(Tairawhiti, New Zealand), α-pinene(Fluka, Switzerland), propyl para hydroxybenzoate(Dan IL Chemical, Korea), Grapefruit Seed Extract Citrex(Quinabra, Brazil) and R-limonen(Fluka, Switzerland). All of them was the reagents for analysis and pure water was used.

Manuka oil, Citrex, α-pinene and R-limonene have strong viscosity, so it

is gard to get the determination of reagent. To solve this problem, the solution compounded with 95% Ehtanol in volume ratio 1:1 was used in all antibiotic experiments.

The sample in which 95% Ethanol is compounded in the ratio 1:1 was used in the amount of 2, 4, 8, 16, 20 ㎕/㎖ of broth and 2, 4, 8, 16, 20 ㎕/paper disk. And the sample in which Manuka oil and Citrex are compounded by 100:0, 75:25, 50:50, 0:100 are used by 2, 4, 8, 16, 20 ㎕/㎖ of broth and 2, 4, 8, 16, 20 ㎕/paper disk.

Table 1. Results of GC/MS Analysis of Manuka Oil Samples(%)

(A. L. Wilkins Confidential Report No. 94. 1. 1994)

a. usually including traces of α -thulene, γ -terpinene,terpineolene,terpincene-4-ol and α-terpineol

contents	sample A	sample B
α -pinene	1.08	1.54
β-pinene	0.14	0.11
myrcene	0.21	0.28
ρ-cymene	0.17	0.15
cineole+limonene	0.17	0.37
γ-terpinene	0.10	0.16
other monoterpene[a]	trace	0.31
ester(mainly C_{10} MWt 168-172)	0.55	0.50
α -cubebene	3.92	3.99
α -yanglene	0.32	0.32
α -copaene	6.13	5.59
β-elemene	0.53	0.57
α -gurjunene	0.98	1.06
β-caryophylene	2.47	2.63
aromadendrene	2.29	1.90
α -humulene	0.31	0.43
allo-aromadendrene	0.78	0.81
β-selinen	3.77	3.58
α -selinen[b]	4.16	4.53
α -farnesene	0.67	1.02
calamanene	16.98	11.85
δ-cadinene	3.94	6.10
cadina-1,4-diene	4.70	5.38
other sesquiterpene hydrocarbons[c]	12.35	16.35
spathulenol	0.40	0.63
caryophyllene epoxide	0.23	0.26
cubenol	0.86	1.12
other oxygenated sesquiterpene[d]	4.21	3.90
flavesone	5.18	4.65
isoleptospermone	4.47	4.76
leptospermone	15.93	15.15
total	100.00	100.00

b. may include a minor co-eluting viridiflorene contribution

c. mainly selinene,amorpene,muuroeiene and cadinene isomers and unknown sesquiterpene hydrocarbons of the type reported in Australian Tea Tree Oil (Brophy et al. J. Agric. food.1989. 37. 1330-1335)

d. usually including neroiidiol,viridiflorol and ledol

3. Minimum Inhibitory Concentraion got from Disk Diffusion Method

In the antibiotic experiment fulfilled by disk geffusion method, 50㎕ culture fluid diluted O.D(660mm) 0.5 with disinfected medium was patched in vutrient agar petri dish and paper disk(diameter 8mm) was put in the meddle of petri dish. The paper disk was moistened with the sample, in which Ethanol and other sample are compounded in 1:1, by 2, 4, 8, 16, 20 ㎕ and the petri dish was sealed by parafilm and cultured for 72 hours at 30℃. And them the size of round clear zone formed in 8㎜ paper disk was measured.

Manuka oil, α-pinene, Citrex and R-limonene used in antibiotic experiment were diluted Ethanol by 1:1 was filled by 2, 4, 8, 16, 20㎕/㎖ of broth and was cultured for 24 hours at 30℃.

After 25 hours, it was filled in culture tube which has disinfected medium and kiluted $10^2 \sim 10^3$ and patched in nutrient atar petri dish by 50 ㎕.

In case the compound sample of Manuka oil and Citrex was used, the three kinds, undiluted and diluted each 10^1 , 10^2 were patched in nutrient agar petri dish by 50㎕.

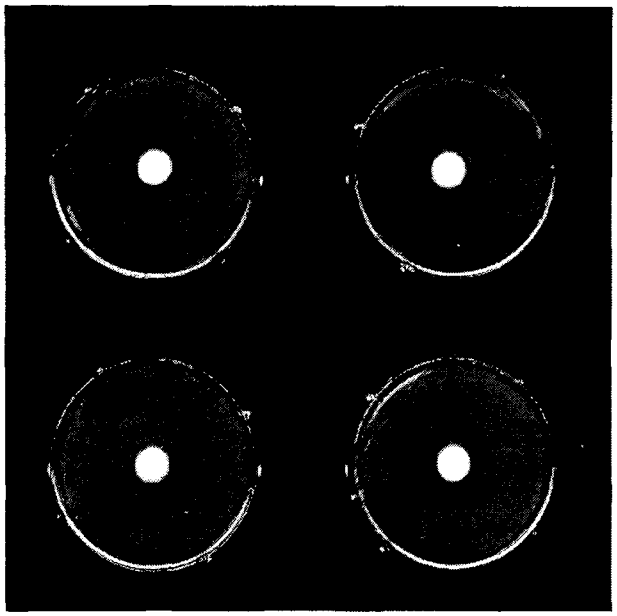
The patched petri dish was scaled with parafilm and cultured for 72 hours at 39℃ and the number of colony was counted.

Result

The antibiotic effect of each compounded sample, Manuka oil and α-pinene for *Escherichia coli*(ATCC 11105) and *Staphylococcus aureus*(ATCC 6538) is shown in Fig.1~5, In Fig.1~5, the clear zone in Ethanol and PPHB is not observed, but it is clearly appeared in all concetration of Manuka oil and α-pinene and the size gets bigger as the concentration increases.

Fig. 6 shows the clear zone of Ethanol, PPHB, Manuka oil, α-pinene, Citrx and R-limonene for *Escherichia coli*(ATCC 11105) by concentration. As being shown in Fig.6, 7, Ethanol formed clear zone 1㎜ for *Escherichia coli*(ATCC 11105) in all concentration and for *Staphylococcus aureus*(ATCC 6538), and it formed clear zone 1㎜ just in concentration 10 ㎖, so the test result was set up as blank. Also, the size of clear zone formed by α-pinene got bigger 5㎜ to 10㎜ as the concentration increases, but PPHB little formed the clear zone(0㎜ to 1㎜).

For *Escherichia coli*(ATCC 11105), Manuka oil especially in low concentration(1~2㎕) formed clear zone two times bigger than that α-pinene formed.



Left Right

S.aureus *E.coli*

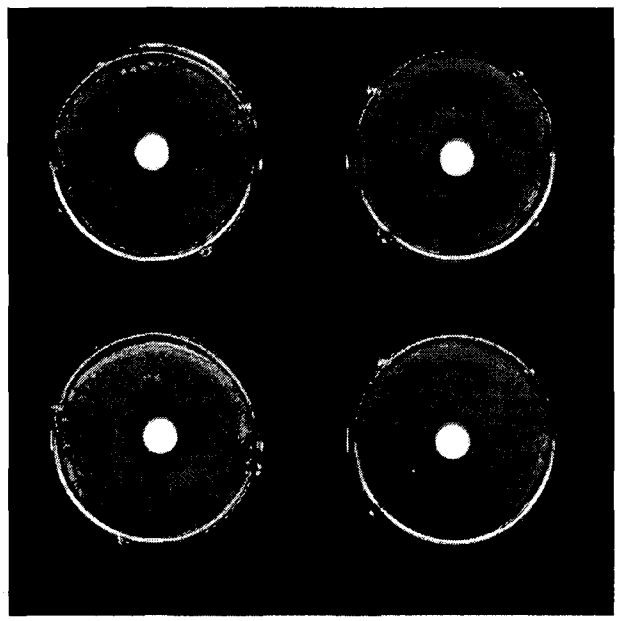
Ethanol

1 μl

S.aureus *E.coli*

PPHB

1 μl



S.aureus *E.coli*

Manuka

1 μl

S.aureus *E.coli*

α -pinene

1 μl

Fig. 1. The clear zone size of *Escherichia coli*(ATCC 11105) and *Staphyl- ococcus aureus*(ATCC 6538) for New Zealand's tea tree oil(Manuka), α-pinene and proryl para hydroxybenzoate(PPHB) at conc. of 1 μl.

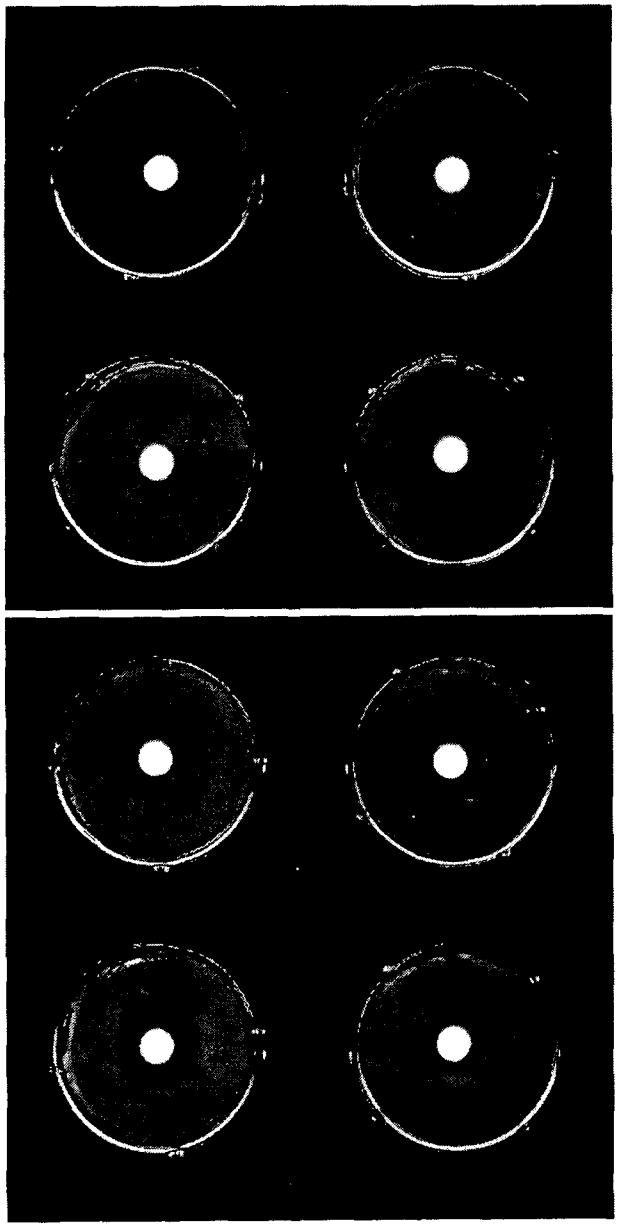


Fig. 2. The clear zone size of *Escherichia coli*(ATCC 11105) and *Staphyl- ococcus aureus*(ATCC 6538) for New Zealand's tea tree oil(Manuka), α-pinene and proryl para hydroxybenzoate(PPHB) at conc. of 2 μl.

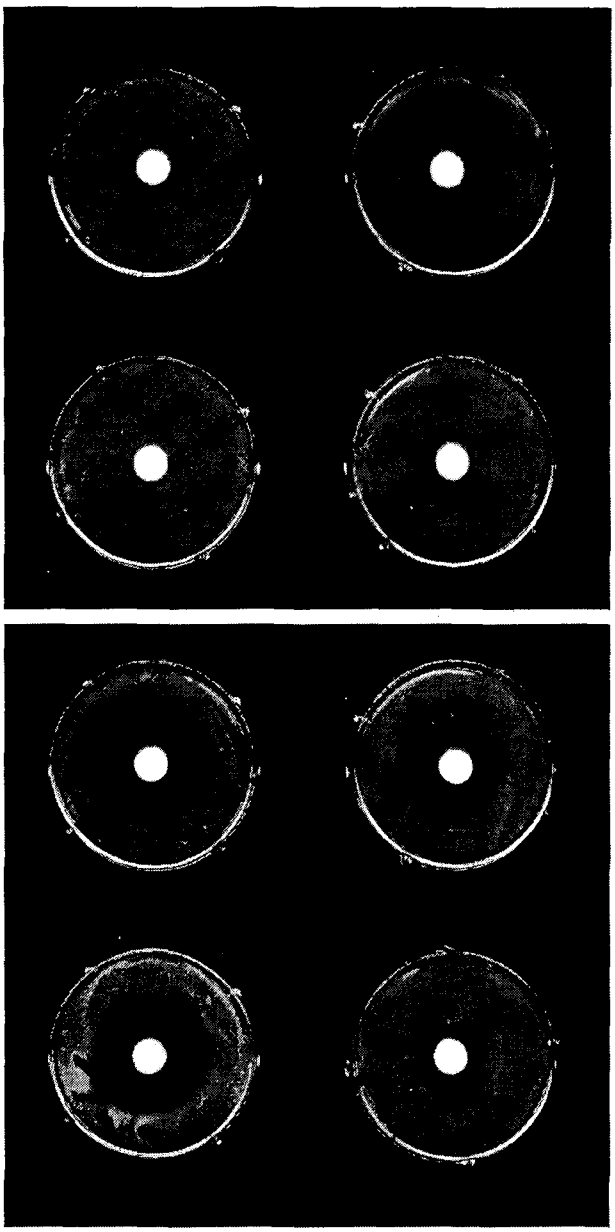


Fig. 3. The clear zone size of *Escherichia coli*(ATCC 11105) and *Staphyl- ococcus aureus*(ATCC 6538) for New Zealand's tea tree oil(Manuka), α-pinene and proryl para hydroxybenzoate(PPHB) at conc. of 4 μl.

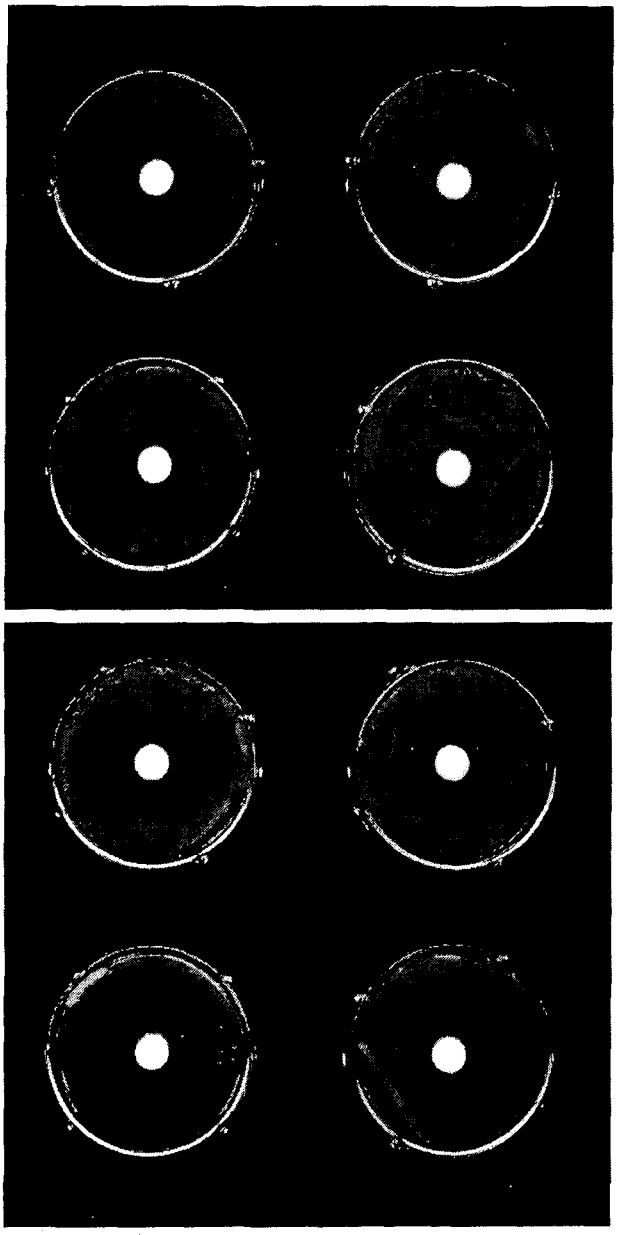


Fig. 4. The clear zone size of *Escherichia coli*(ATCC 11105) and *Staphyl- ococcus aureus*(ATCC 6538) for New Zealand's tea tree oil(Manuka), α-pinene and proryl para hydroxybenzoate(PPHB) at conc. of 8 μl.

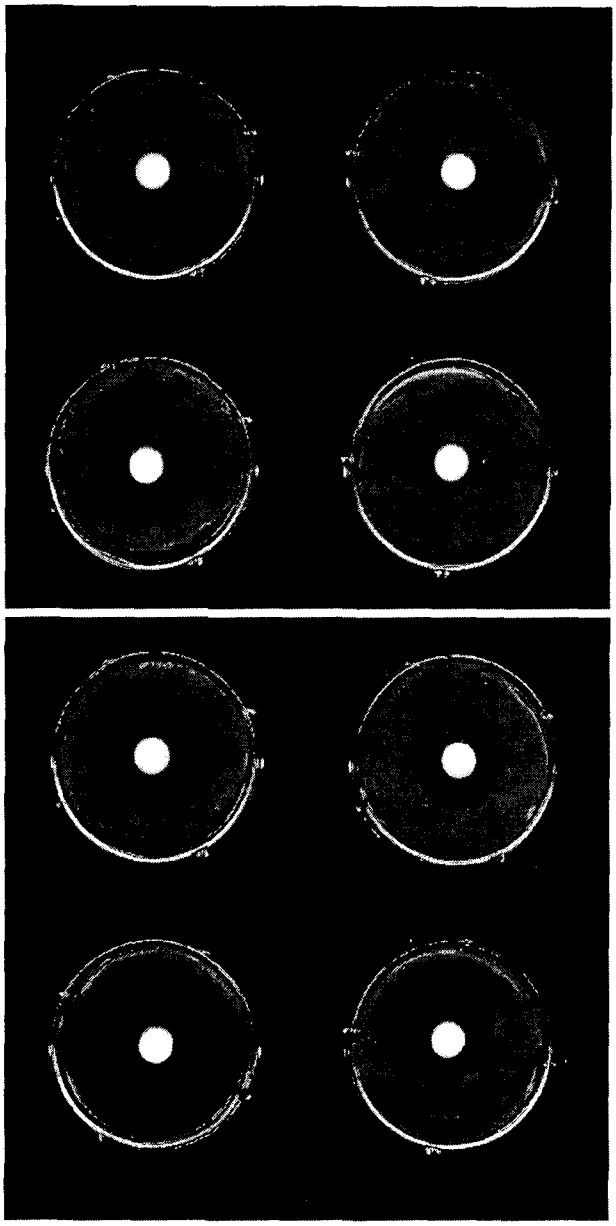


Fig. 5. The clear zone size of *Escherichia coli*(ATCC 11105) and *Staphyl- ococcus aureus*(ATCC 6538) for New Zealand's tea tree oil(Manuka), α-pinene and proryl para hydroxybenzoate(PPHB) at conc. of 10 μl.

Table 2. The colony form unit(cfu) of *Escherichia coli*(ATCC 11105) produced by proryl para hydroxybenzoate(PPHB), New Zealand's tea tree oil (Manuka) and α-pinene using broth dilution method

unit : cfu/ml

Conc. / Material	1 μl/ml	2 μl/ml	4 μl/ml	8 μl/ml	10 μl/ml
Ethanol	$9.56x10^4$	$8.98x10^4$	$9.77x10^4$	$9.66x10^4$	$1.08x10^4$
PPHB	$1.32x10^3$	$5.42x10^2$	$1.13x10^2$	2.4x10	0
Manuka	$6.76x10^2$	$1.72x10^2$	3.6x10	0	0
α-pinene	$5.84x10^2$	$3.23x10^2$	7.8x10	0	0

Table 3. The colony form unit(cfu) of *Staphylococcus aureus* (ATCC 6538) produced by proryl para hydroxybenzoate(PPHB), New Zealand's tea tree oil(Manuka) and α-pinene using broth dilution method

unit: cfu/ml

Conc. / Material	1 μl/ml	2 μl/ml	4 μl/ml	8 μl/ml	10 μl/ml
Ethanol	$1.46x10^4$	$1.27x10^4$	$1.34x10^4$	$1.55x10^4$	$1.22x10^4$
PPHB	$1.07x10^3$	$3.32x10^2$	9.6x10	2.1x10	0
Manuka	$4.33x10^2$	$1.03x10^2$	1.6x10	0	0
α-pinene	$4.75x10^2$	$1.46x10^2$	3.8x10	0	0

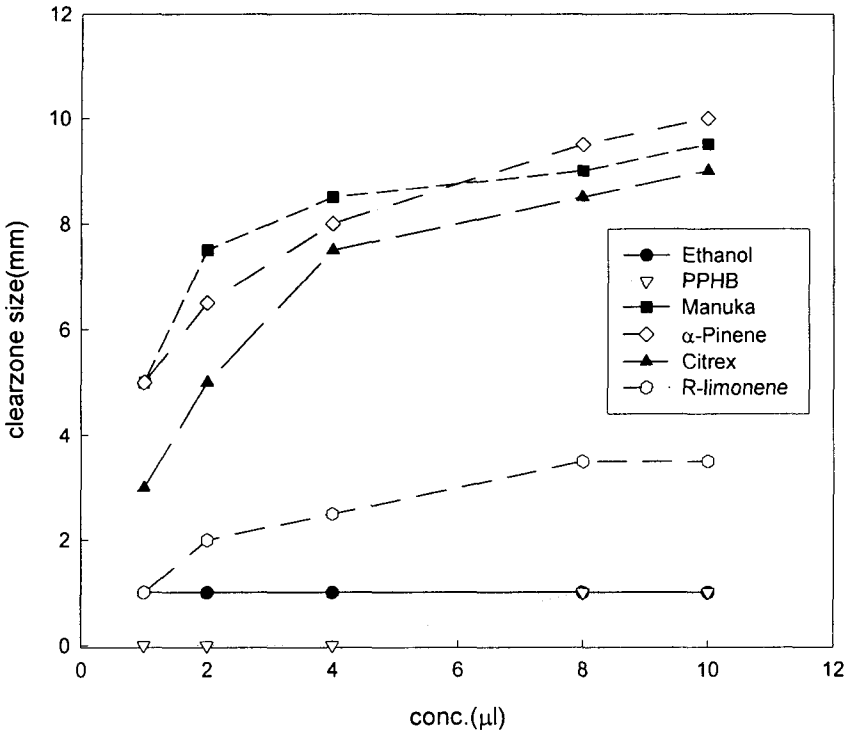


Fig. 6. The clear zone size of *Escherichia coli* (ATCC 11105) for proryl para hydroxybenzoate(PPHB), New Zealand's tea tree oil(Manuka), Grapefruit seed extract(Citrex) and R -limonene.

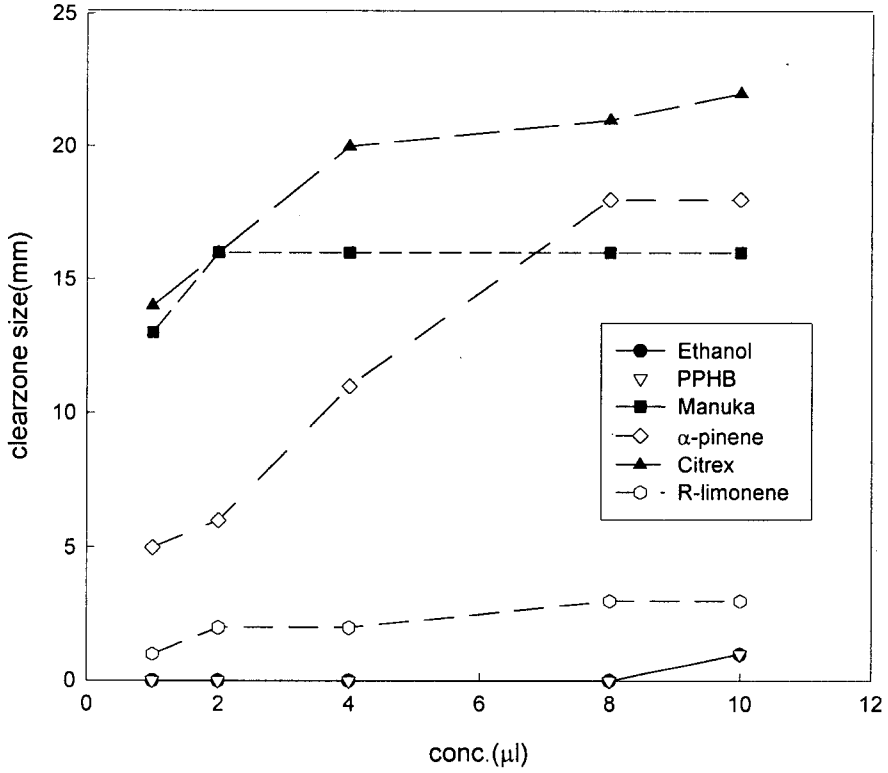


Fig. 7. The clear zone size of *Staphylococcus aureus*(ATCC 6538) for proryl para hydroxybenzoate(PPHB), New Zealand's tea tree oil(Manuka), α-pinene, Grapefruit seed extract(Citrex) and R-limonene.

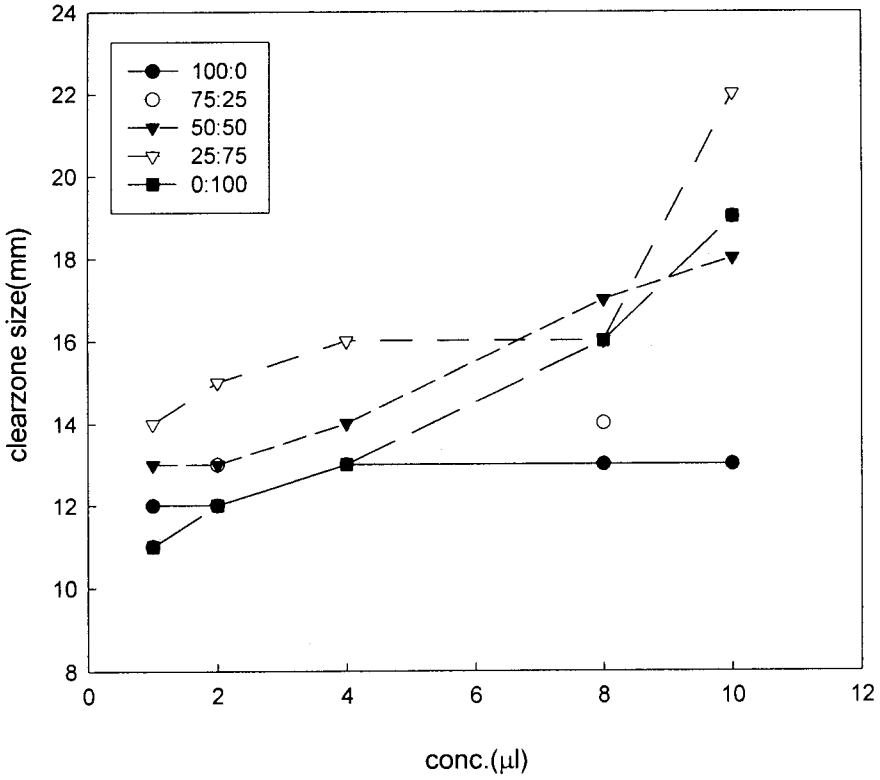


Fig. 8. The clear zone size of *Escherichia coli*(ATCC 11105) for ratio of concentration between New Zealand's tea tree oil (Manuka) and Graphfruit seed extract(Citrex).

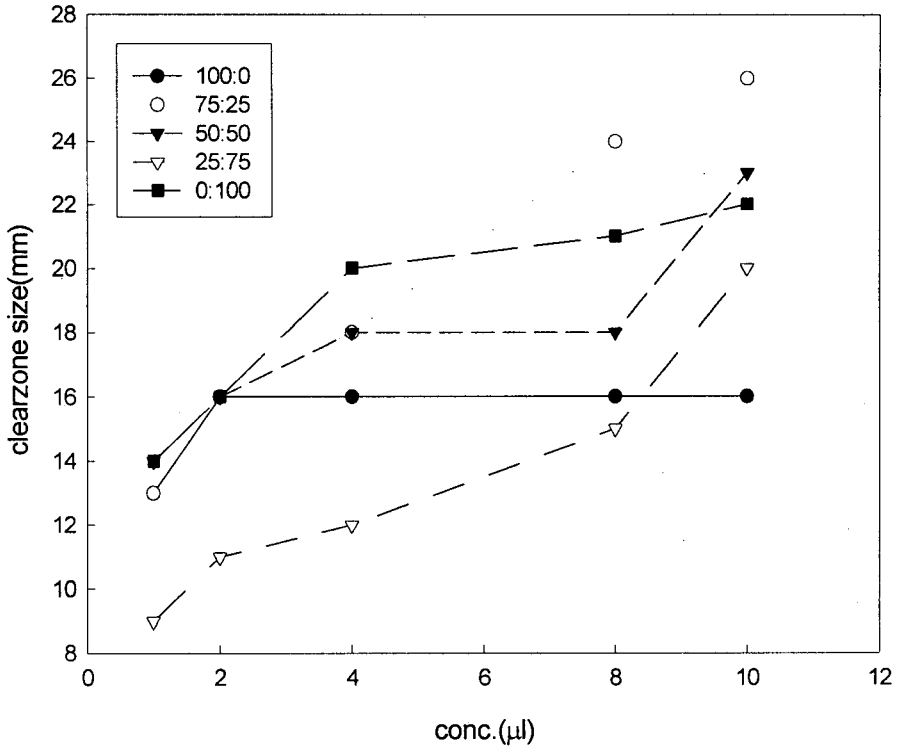


Fig. 9. The clear zone size of *Staphylococcus aureus*(ATCC 6538) for ratio of concentration between New Zealand's tea tree oil(Manuka) and Grapefruit seed extract(Citrex)

It is because Manuka oil is complexed substance of terpenes(α -pinene) and triketones so in the low concentration, triketones substance having lower volatility than α -pinene increases the inhibitory effect and several ingredients included in Manuka oil act together and increase the effect.
It has more powerful antibiotic effect than single ingredient, α -pinene, but further investigation is required.
The inhibitory effect of Manuka oil and α -pinene was stronger for *Staphyloccus aureus*(ATCC 6538) than for *Escherichia coli*(ATCC 11105) and it gets stronger as the concentration increases. The antibiotic effect of Manuka oil and α -pinene is stronger for gram-positive bacteria "*Staphyloccus aureus*(ATCC 6538)" than for gram-negative bacteria, "*Escherichia coli*(ATCC 11105)". The number of colony was counted by broth dilution method according to the change of concentration of Ethanol, PPHB, Manuka oil, α -pinene, Citrex and R-limonene and the Table 2 and 3 shows the value. The number of colony was little decreased (10^4cfu) in the concentration of Ethanol 10$\mu\ell$, and it decreases 10^3cfu to 10 cfu in PPHB 8$\mu\ell$/mℓ.

On the other hand, it is not much decreased in Manuka oil and α -pinene. This result verifies that the antibiotic effect of Manuka oil and α -pinene is stronger than that of PPHB. The number of colony of Manuka oil and α -pinene for strain is each 4.33×10^2cfu ~1.6×10cfu and 4.75cfu ~3.8×10cfu. Namely the number of colony of Manuka oil is 1.0~ 2.3 times higher than that of α -pinene and the number of colony for Staphyloccus aureus(ATCC 6538)is lower than that for Escherichia coli(ATCC 11105).
The MIC of Manuka oil, α -pinene and PPHB for strain is each 8$\mu\ell$, 8$\mu\ell$, 10$\mu\ell$. Namely, the MIC value of Manuka oil and α -pinene is lower than that of PPHB. As a result, the antibiotic effect of Manuka is the strongest and α -pinene and PPHB each follows after that. And the effect

was stronger for gram-negative bacteria "*Escherichia coli*(ATCC 11105)" than for gram-positive bacteria "*Staphyloccus aureus*(ATCC 6538)".

2. The antibiotic effect of Citrex and R-limonene

The size of clear zone in the concentration 1㎕ to 10㎕ was measured by disk diffusion method to measure the antibiotic effect of Citrex and R-limonene for Escherichia coli(ATCC 11105) and Staphyloccus aureus(ATCC 6538). As a result, the size of clear zone of Citrex and R-limonene got bigger respectively 11㎜ to 19㎜ and 1.0㎜ to 3.5㎜ as the concentration of sample increases 1㎕ to 10㎕. (Fig. 6)

For Staphyloccus aureus(ATCC 6538), the clear zone of Citrex and R-limonene increased each 14.0㎜ to 22㎜ and 1.0㎜ to 3.0㎜ as the concentration increases.

It verifies that the antibiotic effect of Citrex much stronger than that of R-limonene.

The antibiotic effect of Citrex is a little stronger for Staphyloccus aureus(ATCC 6538) than for *Escherichia coli*(ATCC 11105) and R-limonene is stronger for *Escherichia coli*(ATCC 11105) than for *Staphyloccus aureus*(ATCC 6538). In this experiment, it is resulted that the antibiotic effect of Citrex for *Escherichia coli*(ATCC 11105) and *Staphyloccus aureus*(ATCC 6538) is stronger than that of R-limonene as a simple. That is because the composition elements of Citrex, ascorbic acid, citric acid, bioflavonoids, peptidese have the antioxidant effect and resisting power and they act together.

3. The antibiotic effect by the compound ratio of Manuka oil and Citrex . Manuka oil and Citrex have very strong antibiotic effect in which they forms the clear zone 12㎜ to 14㎜ for *Escherichia coli*(ATCC 11105) and *Staphyloccus aureus*(ATCC 6538). It results from the strong

antibiotic effect of Manuka oil. And Manuka oil which is soluble in oil spreads slowly, so the size of clear zone dosen't increases in a certain degree even though the concentration of Manuka oil increases. On the other hand, the antibiotic effect of Citrex gets stronger as the concentration increases because the spread speed of water soluble Citrex is so fast. Namely, from the strong antibiotic effect of Manuka oil and the fast spread speed and good antibiotic effect of Citrex, we can obtain their proper compound ratio in which the antibiotic effect become stronger.

For that, the antibiotic effect was experimented by the compound ratio of Manuka oil and Citrex. Fig 8 and 9 show the changing of clear zone measured by diffusion method in the concentration 1㎕ to 10㎕. The compound ratio of Manuka oil and Citrex was each 100:0, 75:25, 50:50, 25:75, 0:100. As being shown in Fig 8 and 9, the size of clear zone in all ratio got bigger as the concentration increases 1㎕ to 10㎕, and the compound sample also have strong antibiotic effect for gram-negative bacteria *Escherichia coli*(ATCC 11105)as Manuka oil and Citrex did. The sample in which 25% Manuka oil and 75% Citrex were compounded formed 13㎜ clear zone in the concentration 1㎕ and this size is near to that formed in other ratio but in 8㎕/㎖, the clear zone was 24㎜ and in 10㎕/㎖, it was 26㎜ and this value is higher than that obtained from other ratio. As a result, the antibiotic effect depends on the compound ratio of Manuka oil.

4. The compound of 75% Manuka oil and 25% Citrex was most effective for *Escherichia coli*(ATCC 11105) and the compound of 25% Manuka oil and 75% Citrex was effective for *Staphyloccus aureus*(ATCC 6538). The proper compound of Manuka oil and Citrex can be applied in both water soluble and oil soluble material and the antibiotic effect

depends on the compound ratio (M/C) of Manuka oil and Citrex, so proper formula is required for each strain

Conclusion

To get antibiotic agent which came from nature, the inhibitory effect of New Zealand's tea tree oil and α-pinene, Citrex(Grapefruit Seed Extract) and R-limonene for *Escherichia coli*(ATCC 11105) and *Staphyloccus aureus*(ATCC 6538) was measured by disk diffusion and broth dilution method and the result is as follows;

1. The antibiotic effect of Manuka oil, α-pinene, Citrex, R-limonene which came from mature was much stronger than that of PPHB.

2. In the concentraion 10$\mu\ell/m\ell$, the antibiotic effect of Manuka oil and α-pinene for *Escherichia coli*(ATCC 11105) and *Staphylococcus aureus*(ATCC 6538) doesn't have difference but in the concentration less than 2$\mu\ell/m\ell$, Manuka oil is much stronger than α-pinene.

3. The antibiotic effect of Citrex for *Escherichia coli*(ATCC 11105) and Staphylococcus aureus(ATCC 6538) was much stronger than R-limonene in all concentration.

4. The compound of 75% Manuka oil and 25% Citrex was most effective for Escherichia coli(ATCC 11105) and the compound of 25% Manuka oil and 75% Citrex was effective for Staphylococcus aureus(ATCC 6538).
The proper compound of Manuka oil and Citrex can be applied in both water soluble and oil soluble material and the antibiotic effect depends on the compound ratio (M/C) of Manuka oil and Citrex, so proper formula is

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