Effects of Fatty Acid Salts against Trichophyton Violaceum

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Abstract. *Trichophyton violaceum* is an anthropophilic fungus. Dermatophytosis (Tinea) is fungal infection that can infect the scalp, glabrous skin, and nails. In general, Tinea can be spread by skin-to-skin contact or bathroom or floor materials. The treatments of Tinea need antifungal medication and good hygiene environment. The effective antifungal medication and infection prevention, and the creation of antifungal medication with high safety are required. In this study was focused on the antifungal effect of fatty acids potassium salts. The antifungal activity of nine fatty acid salts (butyrate, caproate, caprylate, caprate, laurate, myristate, oleate, linoleate, and linolenate) was tested on the spores of *Trichophyton violaceum* NBRC 31064. The results show that C6K, C8K, C10K, C12K, C18:2K, C18:3K was the most inhibit 4-log unit (99.99 %) of the fatty acids potassium incubated time for 10 min. It was observed that C12K and C18:3K was most high antifungal activity MIC. Commercially soap was lowest antifungal activity. This is because of the oleic acid is a major component of soap. Although further investigation is necessary to make clear antifungal mechanisms, our results suggest that fatty acid potassium will use to the development of a coating agent such as furniture.

1 Introduction

Dermatophytes are parasitic fungi that infect skin, hair and nails of both humans and animals [1]. David *et al.* suggest that dermatophytes infections are caused by 40 species of fungi which are grouped into three genera;*Trichophyton*, *Microsporum* and *Epidermophyton* [2]. *Tricophyton violaceum* is an anthropophilic dermatophyte. Dermatophyte infections are common worldwide, and infections of skin, hair, and nails caused by *Trichophyton* .spp [3], [4]. Antifungal agents such as itraconazole, ketoconazole, miconazole, clotrimazole, voriconazole, terbinafine, fluconazole is used as a therapeutic agent. However, although these antifungal therapy is usually well tolerated, the drug may cause gastrointestinal side effects and must be administered for a period of several weeks, making successful treatment of young children potentially difficult. We were searching for an alternative to the more secure and antifungal agents [3], [5], [6].

In this study, we focused on fatty acid salts, which are the main component of soap. Fatty acids, the raw material for the production of fatty acid salts, have been reported to show some antibacterial and antifungal activity. Fatty acids vary in length and degree of saturation, and naturally occurring fatty acids have a chain length of 4 to 28 carbons, which may be saturated or unsaturated [7]. Saturated fatty acids are straight chains and consist of a carbon chain with single bonds, while unsaturated fatty acids contain one or more carbon-carbon double bonds (C=C), which introduce fixed bends into the carbon chain [8]. Some report on the antimicrobial and antifungal effect of the fatty acid. For example, *Neisseria gonarrhoese* and *Helicobacter pylori*, *Aspergillus* spp. and *Penicillium* spp [9]-[11]. A previous study (Era *et. al*., 2015) indicated that the antifungal activity of nine fatty acid salts was tested on the spores of *Penicillium pinophilum* NBRC 6345 and *Penicillium digitatum* NBRC 9651. Potassium caprate showed the strongest antifungal activity at 4 log-units. At incubation times of 180 min, potassium caprylate and potassium laurate showed antifungal activities of 2 log-units against *P. pinophilum* NBRC 6345 [12]. The results confirm the effectiveness of the ability of C10K to inhibit fungal growth on orange rind. C10K effectively inhibited *P. pinophilum* NBRC 6345 growth on orange rind [12]. Thus, C10K shows promise as an antifungal agent. This result shows that fatty acid salts prevented fungal growth, and fatty acid salts can be forecasted from its effective antifungal agent for dermatophytes. Dermatophytes can be infected by towels and clothing, while Foot ringworm is infected through shoes, socks and the floor or bathroom floor mats. We believe that the fatty acid salt serve the prevention of dermatophytes infection.

2 Materials and Methods

2.1 Fungus stains

The spore suspension of *T. violaceum* NBRC 31064 was obtained from the NBRC (Biological Resource Center, NITE, Tokyo, Japan). The fungus was initially grown on Sabouraud dextrose agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) in the dark and the culture stocks were stored at 4°C. Plant-pathogenic fungi were routinely sub-cultured on Sabouraud dextrose agar in slants in the dark at 27°C so that the fresh cultures were available for later use.

2.2 Preparation of spore suspensions

Fungus was grown on Sabouraud dextrose agar slants at 27°C until well sporulated, approximately 10-14 d. Spores were harvested in sterile distilled water using a sterile inoculation loop and gentle agitation; 0.9% NaCl were added to aid wetting of the spores. The spore concentration was determined by counting using a hemacytometer (Thoma, Sunlead Glass Corp., Saitama, Japan). The initial spore concentration was adjusted to 3.0×10^4 spores/mL.

2.3 Source of fatty acids

Nine fatty acids, butyric acid (C4:0), caproic acid (C6:0), and Iinoleic acid (C18:2) were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Caprylic acis (C8:0), capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), oleic acid (C18:1), and Linolenic acid (C18:3) were obtained from Tokyo Chemical Industry Co., Ltd. Potassium salts of fatty acids were also prepared by mixing fatty acid salt with the KOH: potassium hydroxide pellets (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and water. Samples were prepared at 350 mM concentrations. The samples were then stirred for 2h at 75°C. KOH aq was added to yield theoretical neutralization at pH 10.5 of fatty acid salts. Potassium butyrate (C4K), potassium caproate (C6K), potassium caprylate (C8K), potassium caprate (C10K), potassium laurate (C12K), potassium myristate (C14K), potassium oleate (C18:1K), potassium linoleate (C18:2K), potassium linolenate (C18:3K), and the blank were all adjusted using a KOH pH-adjusted solution (pH 10.5) [12]. All fatty acid salts and the KOH pH- adjusted solution were filter-sterilized at low temperature (4-6°C) using a 0.20-μm Millipore filter (Toyo Roshi Kaisya, Ltd., Tokyo, Japan).

2.4 Effect of fatty acid salts on fungal spores

Solutions of 400 μL of fatty acid salts (final concentration of 175 mM in the tubes) and 400 μL of the spore suspension (3.0×10^4 spores/mL) were prepared in 1.5 mL plastic tubes. Spores mixed with the KOH pH-adjusted solution were used as controls [12]. Final pH of all samples were a range of pH 9.2-10.8. The mixtures were incubated at 25°C. Samples were counted at 0, 10, 60, and 180 min by plating (100 μL) on Sabouraud dextrose agar. Fungal colonies were counted after incubation for 3 d or 7 d at 27°C. Viable counts (log10 CFU) of spore was subtracted from the viable count of the control (log10 CFU), and the difference was used as a measure of the antifungal activity. All experiments were performed at least thrice.

2.5 Determination of minimum inhibitory concentrations (MICs)

The MIC is defined as the lowest concentration of drug sufficient for inhibiting visible growth of spores after 10 min of incubation. MICs against fungi were determined using the two-fold dilution method [13], [14]. Each fatty acid salt was separately inoculated with 400 μL of *T. violaceum* NBRC 31064 at 3.0×10^4 spores/mL. 1.5 mL plastic tubes containing 400μL of each of fatty acid salts were inoculated separately with 400 μL of the fungi. The tubes, each containing a total volume of 800 μL, were incubated at 25°C for each organism for 10 min. After incubation, samples were plating on Sabouraud dextrose agar, incubated at 27°C for 7 d, and then examined for the growth of spores. Following incubation, the end point was visually assessed and expressed in mM. The lowest concentration of the antifungal treatment that inhibited visible growth of the fingi after incubation was taken as the MIC of the treatment [14].

2.6 Effect of C12K combined with other fatty acid salts

C12K was mixed with short-chain fatty acid salts (C4K, C6K), medium-chain fatty acid salts (C8K, C10K) or long-chain fatty acid salts (C14K, C18:1K, C18:2K, C18:3K); final concentrations of each fatty acid salt of 0.175, 1.75, 17.5, 35, 87.5, 130 mM. Final concentration of C12K was 5.5 mM. These samples were used to measure the antifungal activity of C12K mixed with other fatty acid salts [12].

3 Results and discussion

3.1 Antifungal activity of saturated fatty acid salts

Antifungal effects of saturated fatty acid potassium salts against *T. violaceum* NBRC 31064 are shown for Figure 1. The average initial population of fungi at 0 min in all samples was approximately 3.0×10^4 spores/mL. Fungus was incubated for 3 d. Final concentration of fatty acid salts were 175 mM. In the saturated fatty acid C6K, C8K, C10K, C12K produced a 4 log-units reduction in the growth of *T. violaceum* NBRC 31064 after incubation for 10 min. Thus, C6K, C8K, C10K, C12K suppressed 99.99% of fungal growth. C4K produced a 1 log-units reduction in the growth of *T. violaceum* NBRC 31064 after incubation for 180 min.These results show that the compound with the 6 to 12-C chain produced combination the highest antifungal effect. Saturated fatty acid salts exerted an antifungal effect, and no effect was produced by the pH-adjusted solution alone.

No growth samples were cultured up to 10 d. Than this, the high antifungal effect samples was confirmed that there is a persistent.

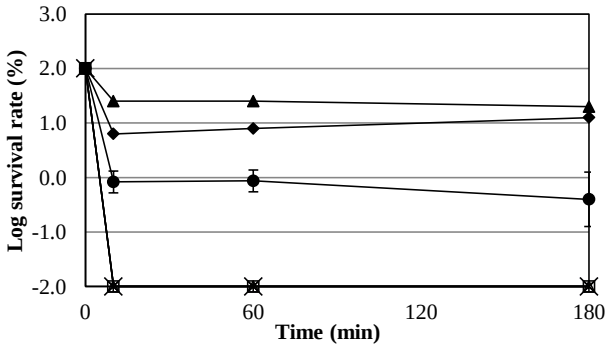


Figure 1. Antifungal activity of 175 mM saturated fatty acid salts against *T. violaceum* NBRC 31064. Spores were counted at the time of inoculation (0 min) and after 10, 60, 180 min of incubation by means of plating 100 µL portions of the same samples. Log survival rate (expressed log CFU/mL) of the samples stored at 27℃ for 3 d were enumerated at the specified time points on Sabouraud dextrose agar. Symbols: ◆, C4K; □, C6K; △, C8K; ×, C10K; ✻, C12K; ●, C14K; ▲, Control (KOH pH-adjusted). Experiments were performed in triplicate, and the error bars represent the standard deviation.

In previously reported, Against *P. pinophilum* NBRC 6345, C8K and C12K produced an antifungal effect of 2 log-units (suppressing 99% of growth) following incubation for 180 min. Further, C6K was ineffective after 180 min [12]. Desbois *et al*. has been reported for bacteria, it remains unclear exactly how free fatty acids exert their antibacterial activities but the prime target seems to be the bacterial cell membrane [15]. Further the various essential processes that occur within and at the membrane and this allows them to interact with the cell membrane to create transient or permanent pores of variable size [15]. Believes that the effect is also similar in *T. violaceum*.

3.2 Antifungal activity of unsaturated fatty acid salts

Antifungal effects of unsaturated fatty acid potassium salts against *T. violaceum* NBRC 31064 are shown for Figure 2. The average initial population of fungi at 0 min in all samples was approximately 3.0×10^4 spores/mL. Fungus was incubated for 3 d. Final concentration of fatty acid salts were 175 mM. In the unsaturated fatty acid C18 :2K, C18 :3K produced a 4 log-units reduction in the growth of *T. violaceum* NBRC 31064 after incubation for 10 min. Thus, C18:2K, C18:3K suppressed 99.99% of fungal growth. C18 :1K produced a 1 log-units reduction in the growth of *T. violaceum* NBRC 31064 after incubation for 180 min.These results show that the compound with the 18-C cahin has more than 2 double bonds produced combination the highest antifungal effect. Unsaturated fatty acid salts exerted an antifungal effect, and no effect was produced by the pH-adjusted solution alone.

No growth samples were cultured up to 10 d. Than this, the high antifungal effect samples was confirmed that there is a persistent.

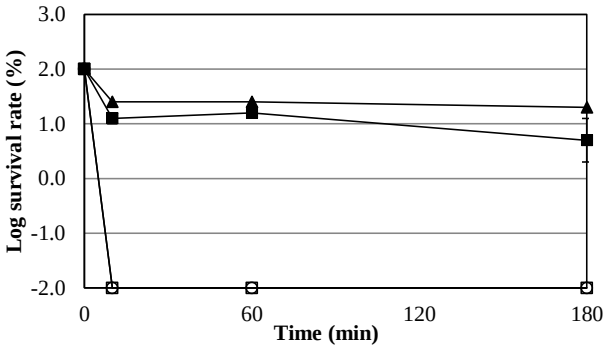


Figure 2. Antifungal activity of 175 mM unsaturated fatty acid salts against *T. violaceum* NBRC 31064. Spores were counted at the time of inoculation (0 min) and after 10, 60, 180 min of incubation by means of plating 100 µL portions of the same samples. Log survival rate (expressed log CFU/mL) of the samples stored at 27℃ for 3 d were enumerated at the specified time points on Sabouraud dextrose agar. Symbols:■, C18:1K; ○, C18:2K; ◇ , C18:3K;▲, Control (KOH pH-adjusted). Experiments were performed in triplicate, and the error bars represent the standard deviation.

In previously reported, Against *P. pinophilum* NBRC 6345 and *P. digitatum* NBRC 9651, C18:2K and C18:3K were ineffective after 180 min [12].

3.3 Antifungal effect of short contact time in the case of saturated fatty acid salts

Figure 3. showed the antifungal effect against *T. violaceum* NBRC 31064 of fatty acid salts to shorten the incubation. The results of C4K was similar to the results of Figure 1. However, C14K produced an antifungal effect of 1log-units (suppressing 90% of growth) following incubation for 60 sec. These results suggest that compound with the 6 to 12-C chain produced and the highest antifungal effect. These results raise the possibility that fatty acid salts was affecting the surface of the cell.

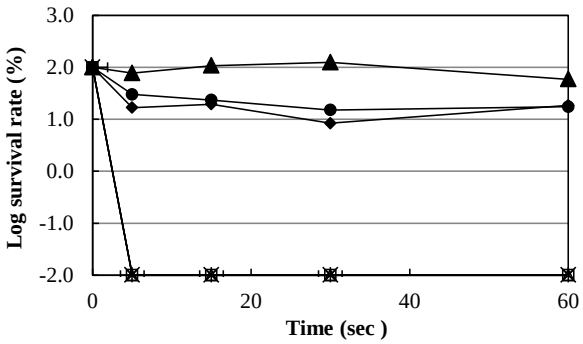


Figure 3. Antifungal activity of 175 mM saturated fatty acid salts against *T. violaceum* NBRC 31064. Spores were counted at the time of inoculation (0 min) and after 5, 15, 30, 60 sec of incubation by means of plating 100 µL portions of the same samples. Log survival rate (expressed log CFU/mL) of the samples stored at 27℃ for 3 d were enumerated at the specified time points on Sabouraud dextrose agar. Symbols: ◆, C4K; □, C6K; △, C8K; ×, C10K; ✻, C12K; ●, C14K; ▲, Control (KOH pH-adjusted).

3.4 Antifungal effect of short contact time in the case of unsaturated fatty acid salts

Figure 4. showed the antifungal effect against *T. violaceum* NBRC 31064 of unsaturated safatty acid salts to shorten the incubation. The results of C18 :1K were similar to the results of Figure 1.

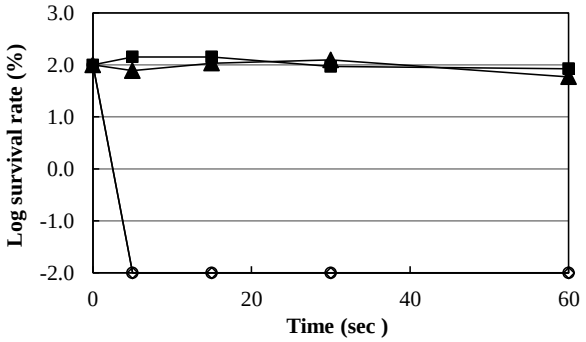


Figure 4. Antifungal activity of 175 mM unsaturated fatty acid salts against *T. violaceum* NBRC 31064. Spores were counted at the time of inoculation (0 min) and after 5, 15, 30, 60 sec of incubation by means of plating 100 µL portions of the same samples. Log survival rate (expressed log CFU/mL) of the samples stored at 27℃ for 3 d were enumerated at the specified time points on Sabouraud dextrose agar. Symbols:■, C18:1K; ○, C18:2K; ◇, C18:3K;▲, Control (KOH pH-adjusted).

3.5 MICs of fatty acid salts and other reagents tested

Two-fold dilution samples of the 175 mM solution inoculated with fungi were incubated for 10 min, and then applied to the agar medium, and MIC were determined after 7 d of culture. The experimental results showed that, form the nine fatty acid potassium salts tested, C12K and C18 :3K had the best antifungal effect against *T. violaceum* NBRC 31064 (Table 1). The MIC of C12K and C18 :3K were 5.5 mM (Table 1). The peak of antifungal effect of saturated fatty acid salts was 12 carbon chain and one more thing the peak of antifungal effect of unsaturated fatty acid salts was 3 double bonds in 18 carbon chain. Similarly, Isaacs *et al*. reported that fatty acids and monoglycerides containing 8-12 carbons showed stronger antiviral and antibacterial activities than their long-chain counterparts [16]. Desbois *et al*. investigated the C12 has the best balance between hydrophobic and hydrophilic groups among saturated fatty acids [17]. Moreover, Zheng *et al*. suggest that the anti bacterial effect of long-chain unsaturated fatty acids was due to their inhibition of fatty acid biosynthesis [10].

Table 1. MICs of fatty acid salts and other reagents tested against *T. violaceum* NBRC 31064.

T. violaceum NBRC 31064 MIC (mM)	
Potassium butyrate (C4K)	>175
Potassium caprote (C6K)	175
Potassium caprylate (C8K)	43.8
Potassium caprate (C10K)	21.8
Potassium laurate (C12K)	5.5
Potassium myristate (C14K)	>175
Potassium oleate (C18:1K)	>175
Potassium linoleate (C18:2K)	21.8
Potassium linonate (C18 :3K)	5.5

The MIC is the lowest concentration of a drug inhibiting visible growth of spores after 10 min of incubation. MIC, minimum inhibitory concentration.

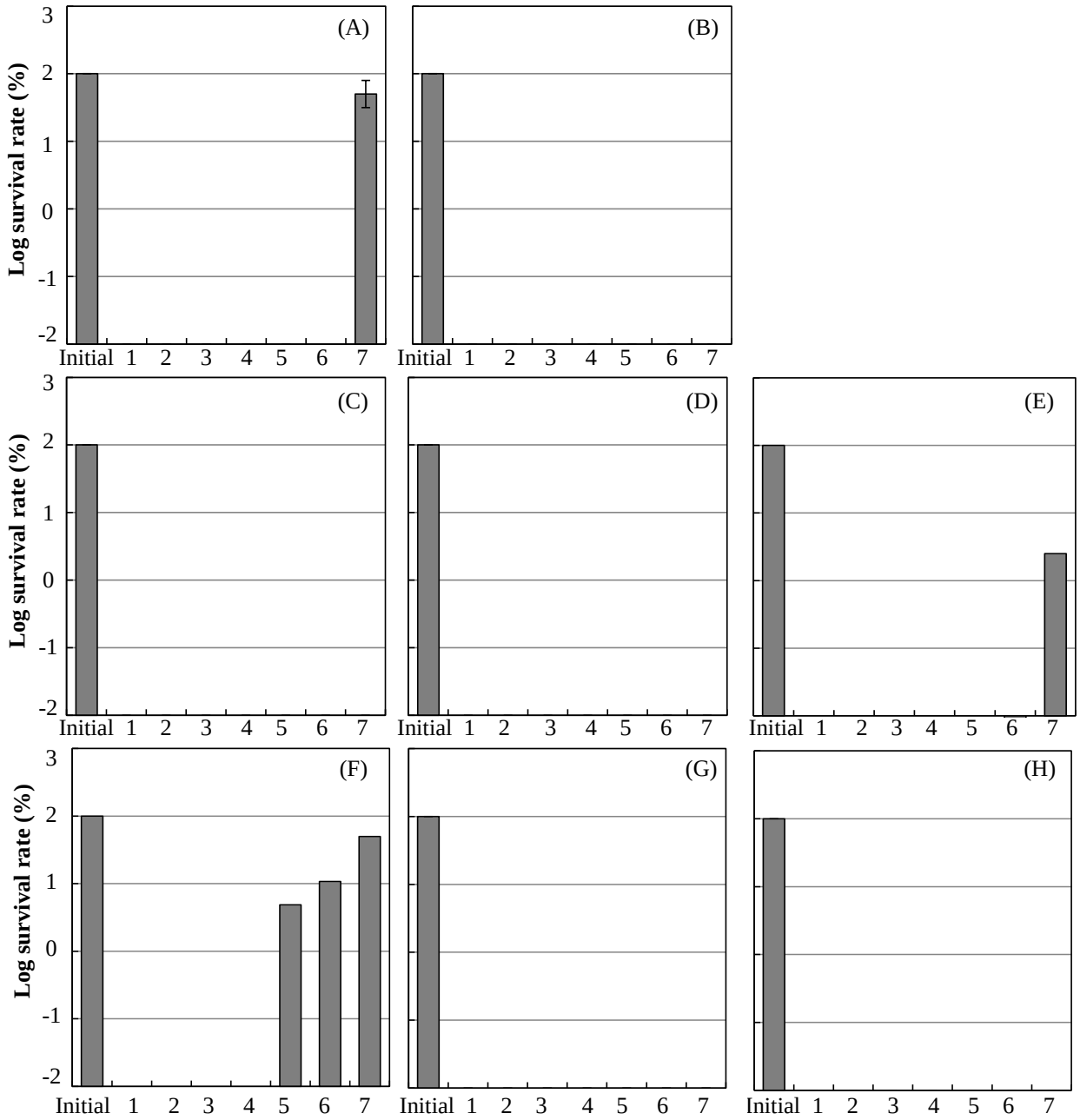
Suggest that cis double bond increased the activity of a straight-chain fatty acid and, the addition of a second double bond further increased the toxicity of the compounds to grampositive bacteria [18]. Unsaturated fatty acid was potassium linonate (C18 :3K) was effective. Kodicek reported consistent with us, the toxicity in increasing order: oleic acid < linoleic acid < linolenic acid [19]. However, this does not agree with Kabara *et al.* Bayliss and Fuller *et al*. reported that the MIC of linoleic acid was somewhat lower than that of linolenic acid[18], [20], [21]. Thus, these results were suggested that similar to previous results. In the results *P. pinophilum* NBRC 6345 MIC was 175 mM [12]. MIC was better of *T. violaceum* NBRC 31064 that comparison of *T. violaceum* NBRC 31064 with *P. pinophilum* NBRC 6345. The difference of the effect of the fungi strain has not been clarified. Antifungal effect was not know the different reasons by the fungi strain.

3.6 Effect of C12K mixed with other fatty acid salts

Soap can be produced using coconut, palm, or olive oils, various fatty acids. Carbon number of fatty acids are generally used the range of 8 to 22 [10]. The main component of soap is fatty acid salts bearing the carbon number and the salts formed from them. The fatty acid salts are characterized by micelle formation at high concentration. The micelle formation depends on the concentration of them, the critical micelle concentration (CMC) of long-chain fatty acid salt is low. Thus, we investigated the effect on the antifungal activity of C12K further by studying the antifungal effect of C12K, which showed the highest antifungal activity, against *T. violaceum* NBRC 31064 when mixed with other salts. Figure. 5 (A)~(H) shows the effect of the antifungal activity with mixing C12K and other fatty acid salts (C4K, C6K, C8K, C10K, C14K, C18:1K, C18:2K, or C18:3K).

C12K mixed with C4K showed the same antifungal activity (4 log-units) as C12K alone (Figure. 5A). Thus, addition of C4K did not affect the activity of C12K. In addition, the effect of mixing C12K with C4K did not change, regardless of concentration. Similar results were obtained when C12K was mixed with short-chain fatty (C6K) and medium-chain fatty acid salts (C8K or C10K) and log-chain fatty acid saits (C14K, C18 :2K or C18 :3K). However addition of long-chain fatty acid salts (C18:1K) inhibited the antifungal activity of C12K (Figure. 5F). The antifungal activity of C12K decreased when mixed with long-chain fatty acid salts, and decreased more strongly as the concentration of long-chain fatty acids increased. However, addition of 3.5 mM of long-chain fatty acid salts did not affect the antifungal activity of C12K. This may have been due to the low

concentration; addition of 3.5 mM of any compound did not inhibit C12K.



Figures 5. The effect of mixing with other fatty acid salts (C4K, C6K, C8K, C10K, C14K, C18:1K, C18:2K or C18:3K) on the antifungal activity of C12K. A C12K with C4K, B C12K with C6K, C C12K with C8K, D C12K with C10K, E C12K with 14K, F C12K with 18:1K, G C12K with 18:2K, H C12K with 18:3K. Samples: initial, fungal suspension; sample 1, 5.5 mM C12K and 0.175 mM other fatty acid salts; sample 2, 5.5 mM C12K and 1.75 mM other fatty acid salts; sample 3, 5.5 mM C12K and 17.5 mM other fatty acid salts; sample 4, 5.5 mM C12K and 35.0 mM other fatty acid salts; sample 5, 5.5 mM C12K and 87.5 mM other fatty acid salts; sample 6, 5.5 mM C12K and 130 mM other fatty acid salts ; sample 7, 175 mM other fatty acid salts alone. Spores were counted at the time of inoculation (0 min) and after 10 min of incubation by means of plating 100 μL portions of the same samples. Log survival rate (expressed log CFU/mL) of the samples stored at 27 ℃ for 3 d were enumerated at the specified time points on Sabouraud dextrose agar.

4. Conclusions

Of the nine fatty acid salts, saturated fatty acid salts tested, potassium lauric (C12K) had the most antifungal effect on *T. violaceum* NBRC 31064, and MIC was 5.5 mM. This is in agreement with the results of several other investigators [18], [20], [22]. C6K, C8K, C10K, C12K and C18:2K, C18:3K can inhibit the ability of *T. violaceum* to take up nutrients, such as amino acids, thereby effectively starving the *T. violaceum* of the nutrients it requires to remain viable [23], [24]. A control solution at the same pH as the fatty acid salt solutions did not affect fungal growth, and we concluded that the antifungal activity was due to the fatty acid salts themselves, not pH.

The type of fatty acid salts that act is different it has been confirmed by the species of fungi [12]. Also, it has antifungal effect was revealed that the carbon is from 10 to 12 saturated fatty acid salts.

C4K mixed with C12K, was shown to increase the antifungal effect of C4K (Figure 5A). However, C18 :1K showed a reduction in antifungal effect in the mixture of 35 mM (Figure 5F). The kind of oil used for soap making are beef fas, palm oil, coconut oil, olive oil. It is known that the soap contained many of medium-chain fatty acids and long chain fatty acids [10]. From this study, the soap comprising oleic acid has been shown that low antifungal effect. The experimental results showed the effectiveness of lauric acid salts was prevention for dermatophytes infections. We believe that the fatty acid salt serve the prevention of dermatophytes infection. However, further experiments are required to determine their precise antifungal mechanism.

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