ANTIMICROBIAL ACTIVITY OF POTASSIUM ALUMINIUM SULFATE AND *PHYLLANTHUS EMBLICA* L. LEAVES EXTRACT AGAINST COMMON SKIN PATHOGENS, IN VITRO STUDY

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BY

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Author	PEERAPAS WEERAKIET
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As the use of antibiotics grows worldwide, the expansion of antibiotic-resistant bacteria becomes increasingly threatening to medical treatment. Many natural substances have been reported to have antimicrobial properties. The objectives of the study were to evaluate the antimicrobial property and the synergistic interaction between alum and *Phyllanthus emblica* leaves against common skin pathogens, including antibiotic-resistant *Cutibacterium acnes*, antibiotic-susceptible *Cutibacterium acnes*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Pseudomonas aeruginosa* and *Candida albicans*. Alum was diluted into four concentrations (2.5, 5, 10, 20%w/v). The *P. emblica* leaves were extracted with 95% ethanol by maceration method. The crude extracts were then collected and mixed into two concentrations (5, 25 mg/ml). The microorganisms were tested using the agar well diffusion method and the broth microdilution method to determine the minimum inhibitory zone (MIZ) and minimum inhibitory concentration (MIC) respectively. Finally, the synergistic interaction was evaluated by checkerboard assay to determine the fractional inhibitory concentration index (FIC index). The highest inhibition zone (21mm) was observed on *P. aeruginosa* with the alum concentration of 20%w/v. The lowest inhibition zone (6 mm) was noticed on *S. aureus* with 2.5% concentration of alum. *P. emblica* extract at a concentration of 5 mg/ml and 25 mg/ml demonstrated antimicrobial activity against *S. aureus*, *S. epidermidis, M. luteus* and *C. acnes*. The lowest MIC of alum solution (0.0195% w/v) was seen on *S. epidermidis*. The lowest MIC of *P. emblica* extract (0.097 mg/ml) was spotted on *C. acnes*. The highest FIC index (0.421) was observed on *M. luteus* and the lowest FIC index (0.046) was seen on *S. aureus*. In summary, both alum and *P. emblica* leaves exhibited substantial antimicrobial activity and can be combined for enhanced efficacy.

Keyword : Alum, *Phyllanthus emblica* leaves, Antibiotic-resistant Cutibacterium acnes, Antimicrobial activity, Synergistic interaction

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CHAPTER 1
Introduction

1.1 Background and significance of the research problem

There are millions of bacteria, fungi and viruses that form the skin microbiota. The physiology of the skin site, such as moist, dry and oily microenvironments, defines the composition of microbial communities. For instances, *Cutibacterium* species are mostly founded in sebaceous sites while *Staphylococcus* and *Corynebacterium* species are abundant in moist areas([1]).

Most of these microbes are non-pathogenic but, in some circumstances, where the skin is injured or when the equilibrium between the microflora is disarranged, skin diseases or even systemic diseases can occur([2]).

Acne vulgaris is a very common disease, affecting most adolescents and young adults aged 11-30 years ([3]). It is characterized by comedones, papules, pustules and nodules. Many complications can follow the disease, such as scarring which can lead to psychological problems on the affected person ([4]). The pathogenesis comprises of 4 main causes, abnormal follicular keratinization, *Cutibacterium acnes* (*C. acnes*) proliferation, sebaceous hyperplasia and inflammatory reactions ([5]).

Staphylococcus aureus (*S. aureus*) is a gram-positive bacteria and facultative anaerobe. It colonizes in the nares of nearly 20% of population. *S. aureus* is a major cause of skin and soft tissue infection. Furthermore, it associates with atopic dermatitis and complicates the treatment([6]).

Among all fungal species of the skin, *Malassezia* spp. are the most common. They consist around 80% of whole fungal flora. *Malassezia* spp. are thought to be the cause of several skin diseases, for instance, dandruff, seborrheic dermatitis and tinea vesicolor. Other fungal pathogens, namely *Trichophyton* spp. and *Candida* spp., are also found in normal skin microbiota but at lower number. However, these latter two species cause common diseases, like candidiasis and tinea corporis([2]).

Currently, the management of skin infections is depended on antibiotics. The judgment between topical and systemic therapy is normally determined by the severity of the disease([7]). While the uses of antibiotics are growing worldwide, the expansion of antibiotic-resistance bacteria becomes more threatening to human life and health([8]). In order to lower the antibiotics use, Complementary and alternative medicine (CAM) has been increasingly studied. CAM can reduce the antibiotic use by boosting the self-healing capacities and killing microbes by its own antimicrobial properties([9]).

Potassium aluminium sulfate or potash alum or alum is an odorless, colorless crystalline solids that occurs commonly in rocks and usually found in India, Egypt, Nepal and many parts of asian countries([9]). Potash alum has been use in various industries for long time , for instance food preservative, water purifier and deodorant ([10]). Recent studies showed that alum has antimicrobial properties against many bacteria and fungal species, for example *Enterococcus faecalis* (*E. Faecalis*), *Enterococcus faecium* (*E. Faecium*), *Escherichia coli (E. Coli*) , *Klebsiella pneumoniae* (*K. Pneumoniae*), *Staphylococcus aureus* (*S. aureus*), *Fusarium solani, Alternaria alternate and Botrytis cinerea* ([11, 12]). Also, there are many studies demonstrated that potash alum has synergistic effect when combine with some antibiotics or some plant extracts ([13, 14]).

Although there are many studies demonstrating antimicrobial properties of potash alum but still there has been neither research in efficacy of potash alum against *C. acnes* nor research considering the synergistic effect of potash alum and *Phyllanthus emblica* leaves extract in *C. acne*'s growth inhibition. Therefore, we investigated the efficacy of potash alum against *C. acne*s and other common skin pathogens, also we studied the synergistic effect of potash alum and *Phyllanthus emblica* leaves extract for bacteria's growth inhibition

1.2 Objectives

Primary objectives

1. To evaluate antimicrobial activity of potash alum against antibiotic-resistant *C. acne*s and antibiotic-susceptible *C. acnes*

2. To evaluate minimum inhibitory concentration of potash alum for inhibiting antibiotic-resistant *C. acne*s and antibiotic-susceptible *C. acnes*

Secondary objectives

1. To evaluate minimum inhibitory concentration of potash alum for inhibiting *Staphylococcus aureus*, *Staphylococcus epidermidis (S. Epidermidis)*, *Micrococcus luteus* (*M. Luteus*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Candida albicans* (*C. albicans*)

2. To evaluate minimum inhibitory concentration of *Phyllanthus emblica* leaves extract for inhibiting *S. aureus*, *S. epidermidis*, *M. Luteus*, *P. aeruginosa* and *C. albicans*

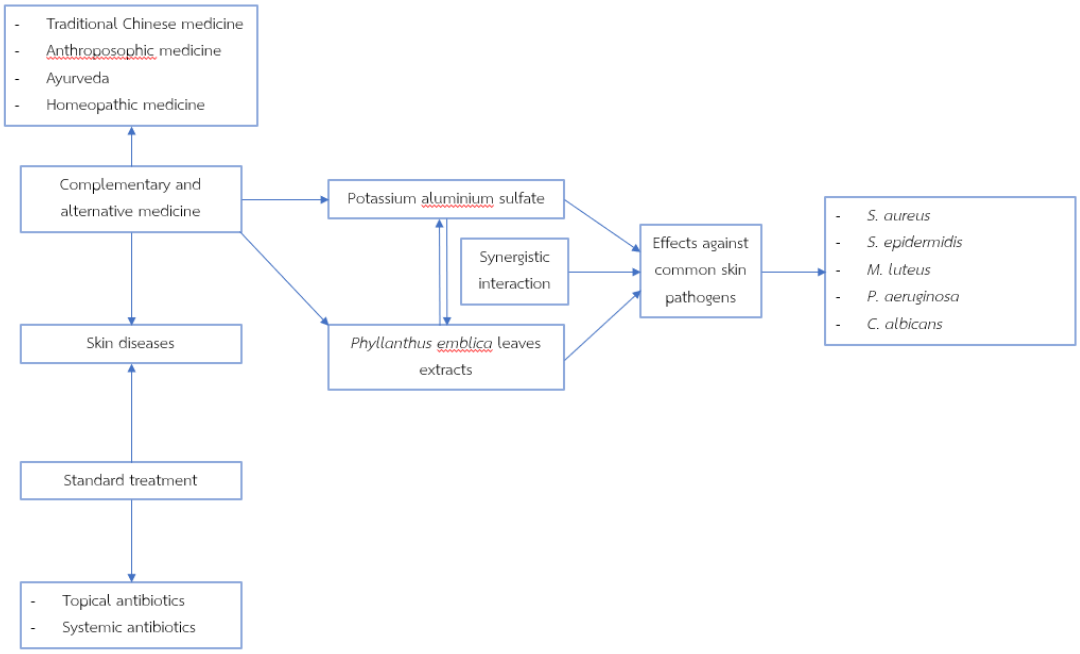
3. To evaluate synergistic interactions between potash alum and *Phyllanthus emblica* leaves extract

1.3 Hypothesis

1. Potash alum can inhibit *C. acnes*, *S. aureus*, *S. epidermidis*, *M. Luteus*, *P. aeruginosa* and *C. albicans* growth

2. Potash alum has synergistic effect with *Phyllanthus emblica* leaves extract

1.4 Conceptual framework



CHAPTER 2

Related concept, theories and literature review

2.1 Skin microbiota and skin diseases

Human skin is the habitat to millions of bacteria, fungi and viruses. These microbes provide protection against foreign pathogens, stimulate our immune system and produce vitamins([1]). The differences in skin temperature, humidity, sebaceous density and pH of each skin site create different microbial communities.

Oily sites are dominated by *Cutibacterium* species. *Cutibacterium* species can use their lipases to break down triglyceride lipids in sebum which promote bacterial adherence([2]). While *Staphylococcus* and *Corynebacterium* species are the most abundant in moist areas. *Staphylococcus* species have developed many approaches to endure on the skin. They can use the urea which is present in sweat as a nitrogen source. Furthermore, they produce proteases to extract nutrients from the stratum corneum.

Fungal communities are different from the bacteria's. The composition of fungal communities is almost the same across core body. The largest fungal diversity is founded on the feet. *Malassezia* species are the most common, consisting of around 80% of the whole fungal flora. *Malassezia* genomes are enhanced with multiple lipase genes, therefore they can utilize the lipids of sebum to promote colonization.

Most of these microbes are beneficial but, in some circumstances, where the skin is injured or when the equilibrium between the microflora is disarranged, skin diseases or even systemic diseases can occur

Cutibacterium acnes is a gram-positive, oxygen-tolerant anaerobe. It is the most common microbe found in the microbiota of normal adults. *C. acnes* lives in hair follicles and sebaceous glands. It utilizes sebum triglycerides and consequently produce free fatty acid. Acne vulgaris is a common chronic inflammatory disease which affect more than 85% of adolescents and young adults. The disease is significantly associated with *C. acnes* proliferation. However, its pathogenesis also includes

abnormal follicular keratinization, increased sebum production and inflammatory reaction.

Staphylococcus aureus is a gram-positive bacteria and facultative anaerobe. *S. aureus* is the most common cause of skin and soft tissue infections([7]). The bacteria can produce multiple enzymes and toxins to infect the host. *S. aureus* also associates with atopic dermatitis, a common chronic skin disease affecting children and adults. Researchers founded that during flare stage, there was increased relative abundance of *S. aureus* in lesioned skin.

Furthermore, there are other well-known skin pathogens that cause many common dermatological diseases, for instance, *Trichophyton* causing onychomycosis and tinea corporis, *Candida* species causing candidiasis and *Pseudomonas aeruginosa* causing green nail syndrome.

2.2 Antibiotic and antifungal resistance

Antibiotics and antifungals have been used to fight microbes for more than 50 years([8]). Uncomplicated skin infections are usually treated by topical antibiotics and sometimes by oral antibiotics. However, they rarely need hospitalization. While complicated skin infections often require debridement, systemic antibiotics and hospitalization([7]). As a result of worldwide growing antibiotic uses, various types of antibiotic resistance genes are found in almost all regions of the world. Infections of antibiotic-resistant bacteria lead to higher risk of hospitalization and complications([15]).

In 2001, Poomsuwan P and Noppakun N studied antibiotic sensitivity of *C. acnes* in 70 Thai patients using E-test method. The results showed that 6.15% of *C. acnes* resisted to clindamycin, 6.15% resisted to erythromycin but no resistance to tetracyclines and doxycyclines were observed. Later, in 2017, Laochunsuwan studied antibiotic susceptibility of *C. acnes* isolated from 95 acne patients using E-test method([4]). The results demonstrated that 62.66% of *C. acnes* resisted to clindamycin, 64% resisted to erythromycin, 1.33% resisted to tetracyclines. However, no doxycycline-

resistant *C. acnes* was seen. As demonstrated, since 2001 the resistant *C. acnes* has been increasing significantly.

Antibiotic resistance is a process which is triggered by multiple factors such as inappropriate antibiotic prescribing in human medicine, Overprescription of broad-spectrum antibiotics and antibiotic overuse in agriculture([15]). Because of inventing new drugs is a very difficult and expensive task. Therefore, reduction of antibiotic usage is an important and helpful strategy to limit the increase of antibiotic resistance.

2.3 Complementary and alternative medicine

Complementary and alternative medicine (CAM) is a term used for describing various traditional medicines including ayurveda, homeopathy, anthroposophic medicine and traditional Chinese medicine. Each therapy has its theory and practice that have been developed throughout the years in different cultures. According to 12 Cochrane reviews, some CAM therapies are effective for respiratory tract infections, urinary tract infections and skin infections([9, 16]). Many CAM treatments have been researched in few clinical trials and demonstrated promising results for otitis media. Although some of the CAM focus on promoting self-healing capacity, there are several natural medicinal products that have antimicrobial effects. Multiple CAM treatments have not been studied in clinical trials but showed antimicrobial properties in vitro, namely, *Anemarrhena asphodeloides*, *Phyllanthus emblica* and *Asparagus racemosus*([9]).

Phyllanthus emblica Linn. or *Emblica officinalis* Gaertn. is commonly found throughout tropical southeast asia([17]). It has been used to produce many herbal drugs. In Thailand, *Phyllanthus emblica* is frequently used in a combination with *Terminalia belerica* and *Terminalia chebula* to form Triphala which is a renowned Thai traditional herbal drug([18]).

Phyllanthus emblica contains many bioactive compounds including apigenin, gallic acid, ellagic acid, chebulinic acid, quercetin, luteolin and so on. Chemical

compounds from each part of the plant is slightly different. However, the leaves contain gallic acid, chebulic acid, ellagic acid, chebulinic acid, chebulagic acid, amlic acid, alkaloids such as phyllantine and phyllantidine which have antimicrobial and anti-inflammatory properties([17-19]).

Many studies on antimicrobial properties of *Phyllanthus emblica* have been conducted. In 2007, Saeed S investigated on antimicrobial activities of *Emblica officinalis* and *Coriandrum sativum* against 10 different genera of Gram-positive bacteria and 2 isolates of *C. albicans* which were all isolated from urine specimens. The results showed that the *Emblica officinalis* exhibited zones of inhibition against all the tested isolates including *S. aureus*, *Micrococcus spp.*, *C. albicans* and *Bacillus subtilis*([20]). Niyomkam P also reported that both ethyl acetate and methanol extracts of *Phyllanthus emblica* leaves demonstrated inhibitory effect on *C. acnes*, *S. aureus* and *S. epidermidis*([21]).

2.4 General and safety information of potash alum

Potassium aluminium sulphate ([9]) is an inodorous, uncolored crystalline solids. Its chemical formula is $KAl(SO_4)_2$. It can be frequently seen in India, Egypt, Nepal, Italy, Philippines and many of Asian countries. PAS can be used in a variety of ways such as food preservative, water purifier, sundry applications and antimicrobial agents. The advantages of PAS are low-cost, availability, harmlessness and environment-friendliness([10]).

According to Food Safety Commission of Japan (FSCJ) ([22]), a tolerable weekly intake (TWI) for aluminium is 2.1mg/kg/week. The TWI was calculated from the lowest no-observed-adverse-effect level (NOAEL) of 30 mg/kg/day which obtained from studies in rats. No positive tests were acquired in Ames test on aluminium compounds including potassium aluminium suphate, so FSCJ concluded that aluminium in potassium aluminium sulphate has no human genotoxicity. The association between aluminium and the effects on bone or neurological diseases, such as Alzheimer's disease, were still controversial because of limited data.

Osuala F studied safety of potassium aluminium tetraoxosulphate in mice ([23]). Five grams of alum was dissolved in 750 ml distilled water then administered to each mouse at average amount of 0.01 ml. The administration was done every 8 hours for four days then the histological assessment was done on the last day of administration, 2 weeks after the administration and 5 weeks after the administration. There were no inflammation or any abnormal findings in histological sections of the kidney, liver and lungs but the brain showed a sign of cerebral edema which was non-specific finding. Also, no mouse died during the study. The authors concluded that alum is safe based on histological analysis.

Alzomor A.K. reported that potassium alum can not be absorbed through the cell membrane due to its negative ionic charge ([24]). Thus, the topical applications are safe and will not produce high level of aluminium.

2.5 Antibacterial effect of potash alum

Recent studies demonstrated various antibacterial properties of potash alum. Bacteria causing axillary malodor, *Micrococcus Luteus* (*M. luteus*), *Staphylococcus epidermidis* (*S. epidermidis*), *Corynebacterium xerosis* (*C. xerosis*), *Bacilus subtilis* (*B. subtilis*) were tested by different potash alum concentrations. The results showed excellent inhibitory effects on all tested bacteria. The lowest MIC was found in *Corynebacerium xerosis* at 1.875 mg/ml. While those for *S. epidermidis*, *B. subtilis*, *M. luteus* were 7.5, 5 and 3.75 mg/ml respectively([25]).

In another study, *Escherichia coli* O157:H7 were tested against potash alum in 0.25, 0.5, 1, 2 %v/v and the growth was monitored by a micro-plate reader every 30 min for 4 hr. the results demonstrated a dose-dependent bacterial's growth inhibition of alum solutions. 1% and 2% of alum solutions inhibited *E. coli*'s growth significantly, while 0.25% and 0.5% of alum solutions had no significant effect on *E. coli*'s growth. Also, the study showed time-dependent effect on *E. coli*'s growth as *E. coli* was reduced significantly at 3, 2.5 hour with 1% and 2% alum solution respectively([26]).

L. Amadi and N. Ngerebara([27]) studied antimicrobial properties of alum against bacteria isolated from oyster, *Proteus* spp., *Bacilus subtilis*, *Staphylococcus aureus* , *Escherichia coli* , *Klebsiella* spp., *Pseudomonas aeruginosa* and *Vibrio* spp. The results showed dose-dependent inhibitory effect of alum disregarding the type of bacteria. The highest sensitivity was observed in *proteus* spp., *E. coli*, *B. subtilis* by using 3% concentration of alum. The least inhibitory effect was seen in *Klebsiella* spp. By using 3% concentration of alum. The study suggested that alum displayed broad spectrum antibacterial effect especially in Gram negative bacteria.

Similarly, a study using potash alum solutions against *Neisseria gonorrhea* and *Candida albicans* (*C. albicans*) was conducted([28]). The results showed that *C. albicans'* growth was inhibited when using at least 10% w/v of alum concentration. While *N. gonorrhea* can be inhibited by using 2.5% w/v of alum concentration.

2.6 Antifungal effect of potash alum

Irshad M studied antifungal activity of potash alum against *Candida albicans*([29]). The results showed that potash alum 10 mg/ml were effective in eliminating the fungus from the acrylic resin. Another study on antifungal property of potash alum against *C. albicans* was done by Mohad and Fatalla. They evaluated the colonization of *C. albicans* in soft lining dental materials. The results showed significant decline in the amount of *C. albicans* on the soft dental lining materials immersed in alum solution.

Amadi L evaluated antifungal effect of 1% w/v potash alum solution on postharvest tomatoes([30]). The fungi included *Aspergillus niger* (*A. niger*), *Aspergillus flavus* (*A. flavus*), *Fusarium* spp., *Penicillium* spp., *Rhizhopus stolonifera (R. stolonifera*), *Geotrichum candidum* (*G. candidum*) and *Saccharomyces cerevisiae* (*S. cerevisiae*). *G. candidum* had 9 mm of DIZ (diameter inhibition zone), which was the highest. *A. niger* had 8 mm of DIZ value. While both *Penicillium* and *Fusarium* spp. had 7 mm of DIZ. However, 1% w/v alum solution failed to inhibit *R. stolonifer*.

2.7 Synergism with plant extracts

Antibacterial activity of *Gongronema latifolium* (*G. latifolium*) combined with potassium aluminium sulphate was tested against four Gram-negative bacteria, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi (S. typhi*), *Pseudomonas aeruginosa* and two Gram-positive bacteria, *Bacillus subtilis* (*B. subtilis*), *Staphylococcus aureus*([31]). Methanolic leaf extract, one of three *G. latifolium* extracts, showed the largest mean of inhibition zone on *E. coli, S. typhi*. Enhancement of efficacy was seen in most of extracts which combined with alum. However, *P. aeruginosa*, *S. typhi*, *K. pneumoniae* appeared to be resistant to aqueous leaf extract with alum. In another study([14]), combination of turmeric rhizomes extracts (*Curcuma longa*) and alum were used against four bacteria, *Stapylococcus aureus*, *Escherichia coli*, *Bacillus cereus (B. cereus*), *Pseudomonas fluorescens* (*P. fluorescens*), and five fungi, *Saccharomyces cerevisae* (*S. cerevisae*), *Candida albicans* (*C. albicans*), *Aspergillus flavus* (*A. flavus), Aspergillus tereus* (*A. tereus*), *Penicillium crystallium* (*P. crystallium*). The largest inhibition zone was spotted in ethanolic turmeric extract and alum at concentration of 0.3g on Gram negative bacteria, *E. coli* (16.6 ±0.8 mm) and *P. fluorescens* (15.3 ± 1.1 mm), and Gram positive, *S. aureus* (15 ± 0 mm). Also, ethanolic turmeric extract with alum showed highest antifungal activity on *A. tereus* (35 ± 1mm). the authors concluded that combination of turmeric rhizomes extracts, and alum demonstrated the synergistic effect which enhanced antimicrobial activity of the extracts.

2.8 Synergism with inorganic compound

Potash alum and hydrogen peroxide (H_2O_2) were studied for antibacterial activity([12]). Comparison and combination between Potash alum and hydrogen peroxide were evaluated against five bacteria, *Enterococcus faecalis (E. faecalis), Enterococcus faecium (E. faecium), Escherichia coli (E. coli), Staphylococcus aureus (S. aureus) and Klebsiella pneumoniae (K. pneumoniae)*. H_2O_2 bacterial susceptibility trend was *E. coli > K. pneumoniae > S. aureus > E. faecium >E. faecalis*. Potash alum bacterial susceptibility trend was *K. pneumoniae > S. aureus > E. faecalis > E. coli >*

E. faecium. In comparison, H_2O_2 showed more inhibition zones than potash alum in all tested concentrations. However, both H_2O_2 and potash alum at 35 mg/ml showed more zone of inhibition than azithromycin against *E. faecalis, E. faecium* and *S. aureus* but less zone of inhibition than azithromycin against *E. coli* and *K. pneumoniae*. Combination of H_2O_2 and potash alum in 1:1 ratio demonstrated more zone of inhibition than H_2O_2 alone against *E. faecalis* and *E. faecium*, also more than potash alum alone in all tested bacteria.

2.9 Synergism with antibiotics

The synergistic interaction of antibacterial activity between alum and antibiotics, cefotaxime and tetracycline([13]), was evaluated against multi-drug resistant microbes, *Staphylcoccus aureus (S. aureus), Klebsiella pneumoniae (K. pneumoniae), Proteus vilagaris (P. vilagaris), Pseudomonas aeruginosa (Ps. Aeruginosa), Pseudomonas fluorescence (Ps. Fluorescence), Escherichia coli (E. coli)* and *Candida albicans (C. albicans)*. Alum solutions were prepared in 4, 8, 16, 20% w/v concentrations. While Cefotaxime was prepared in 500mg/5ml concentration and Tetracycline was prepared in 250 mg/5ml. The results showed that 20% alum solution was the MIC in all isolates, except C. albicans which had MIC of 10% alum concentration. Enhancement was observed in every bacterium tested by combination of cefotaxime-20% alum and tetracycline-20% alum, for instance, in *E. coli* 20% alum solution alone had 38 mm zine of inhibition, cefotaxime alone had no zone of inhibition and combination of 20% alum with cefotaxime had 46 mm zone of inhibition.

2.10 Mechanism of action

Although many studies demonstrated antibacterial and antifungal activities of alum, the mechanism remained unclear ([10]). It was proposed that alum reduced acidity of the bacterial cell walls or deleted the bacterial cell walls ([13]). Similarly, Amadi L suggested that fungal cell walls were deleted or lowered the acidity by alum ([30]). Further investigations regarding antimicrobial mechanism of alum are required.

2.11 Antimicrobial susceptibility testing

Many laboratory methods can be used to assess the in vitro antimicrobial property of an extract or a pure compound. The most widely used and basic methods are the disk diffusion and broth or agar dilution methods.

Diffusion methods([32, 33])

1. Agar disk diffusion method

Agar disk diffusion method has been widely used for routine antimicrobial susceptibility testing. Mueller-Hinton agar plates are inoculated with a standardized inoculum of the test microorganism. Then, up to 12 commercially prepared paper disks, containing the test compound at a desired concentration, are set on the inoculated agar surface. The agar plates are subsequently incubated in appropriate conditions. Later, the diameters of the inhibition zones are measured in millimeters (Fig.1). The agar disk diffusion method provides qualitative results by classifying the test microorganism as susceptible, intermediate or resistant. The results are interpreted by the criteria from the Clinical and Laboratory Standards Institute (CLSI). The advantages of this method are simplicity and low cost.

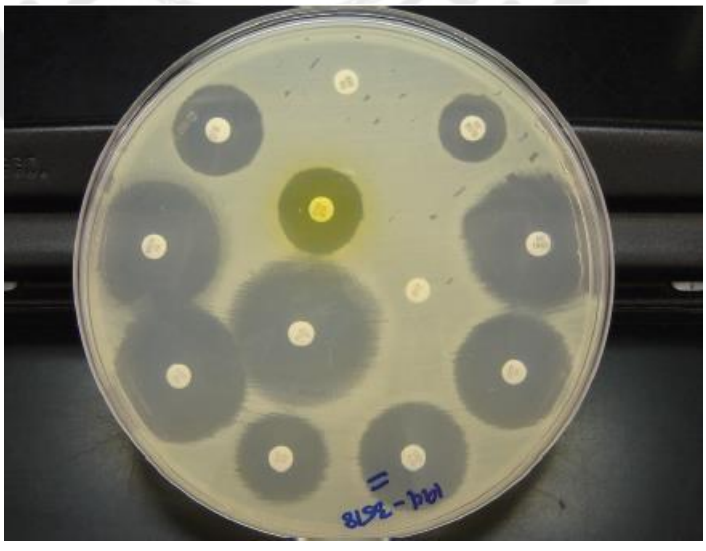


Figure 1 Agar disk diffusion method

2. Antimicrobial gradient method

Test strips, filled with an increasing concentration gradient of the antibiotic, are placed on the inoculated agar surface. After overnight incubation, the minimum inhibitory concentration (MIC) are interpreted. The MIC are determined at the intersection of the strip and the growth inhibition ellipse (Fig.2). The antimicrobial gradient method is rapid and easy to use. However, this method can be expensive if more than a few drugs are tested.

Figure 2 Antimicrobial gradient method

3. Agar well diffusion method

Agar well diffusion method is similar to the agar diffusion method. The agar plates are inoculated with a volume of the tested microbe. Instead of using antibiotic-filled paper disks, the agar well diffusion method use a sterile cork borer to create holes. Then, a small volume of antimicrobial agent or extract solution is introduced into each hole. The agar plates are subsequently incubated, and the inhibition zone of each hole is measured (Fig.3).

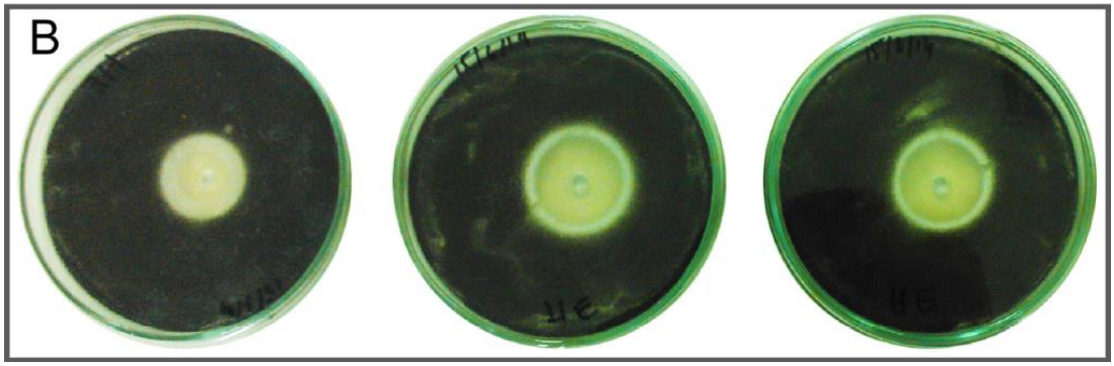


Figure 3 Agar well diffusion method

Dilution methods([32-34])

Dilution methods are reference methods for determining the MIC values of the antimicrobial agents. Dilution methods have been used not only for susceptibility testing but also for the comparative testing of various antimicrobial testing.

1. Broth macrodilution method

Serial two-fold dilutions of antimicrobial agent are prepared. Then, each dilution is added in to test tubes (minimum volume of 2 ml), containing liquid growth medium and standardized microbial suspension. The test tubes are incubated under suitable conditions. The MIC value is determined as the lowest concentration of antimicrobial agent that completely inhibits growth of the organism which represent as no turbidity is observed in the MIC test tube (Fig.4). Disadvantages of macrodilution method are time-consuming, errors during the preparation of each antimicrobial solution and relatively large amount of space and reagents required.

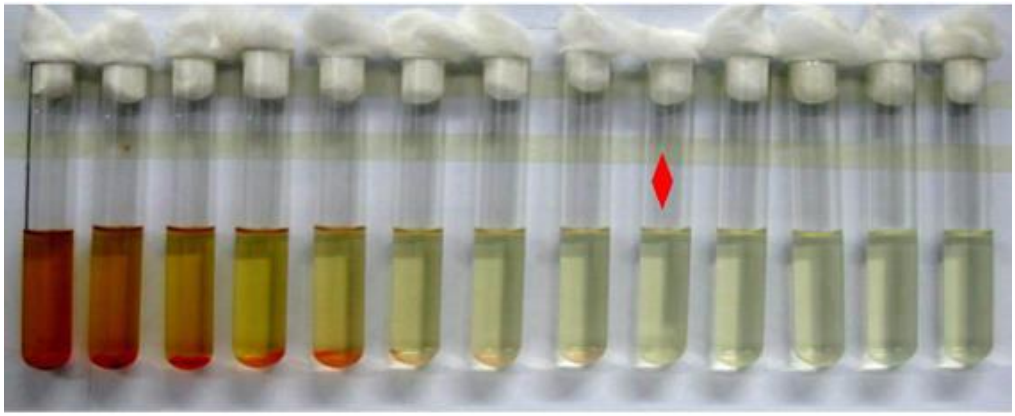


Figure 4 Broth macrodiltuion method

2. Broth microdilution method

The microdilution method uses small sterile microtitration plates, usually containing 98 wells. Each well has a volume of 0.1-0.2 ml. The procedure is similar to the macrodilution method. The wells are filled with serial two-fold dilutions of antimicrobial agent and standardized microbial suspensions. The wells are mixed and incubated in appropriate conditions. Later, the MIC is determined visually (Fig.5).

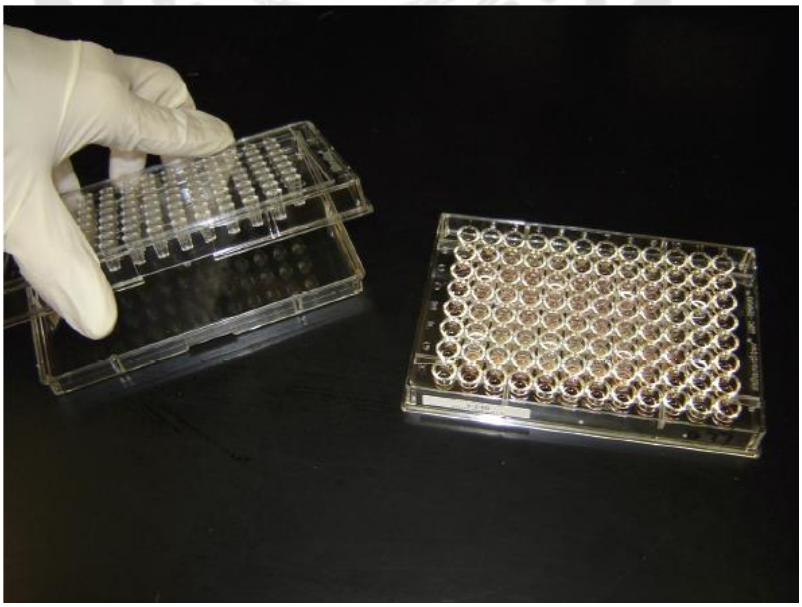


Figure 5 Broth microdilution method

2.12 Antibacterial synergy testing

Several different methods are frequently used for synergy testing. However, they do not always provide the same results. The reference method for synergy testing has not been established.

1. Checkerboard assay([35-37])

The checkerboard assay is a modification of broth microdilution method. The procedure is produced in 96-well microtiter plate. Each well contains a volume of bacterial inoculum and specific concentrations of antibiotics. A checkerboard assay layout is demonstrated in Figure 6.

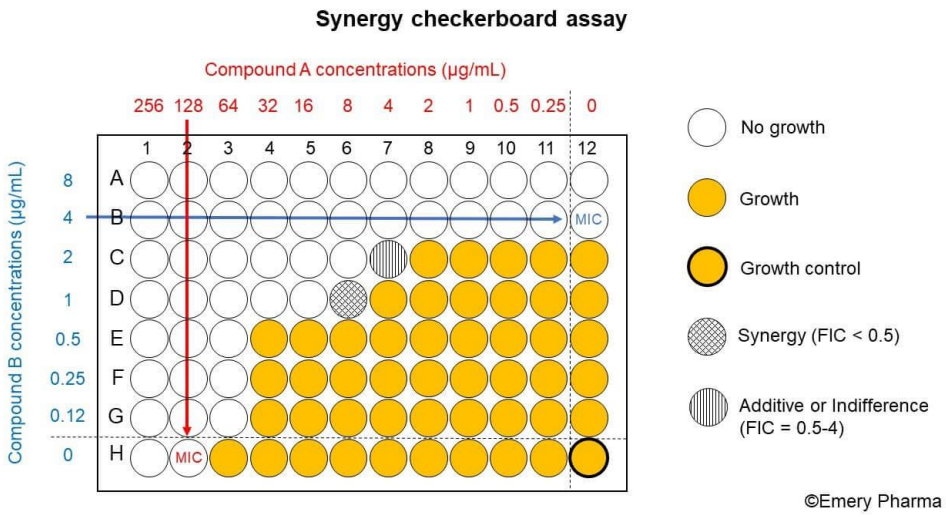


Figure 6 Checkerboard assay

Synergistic activity is evaluated by calculating the fractional inhibitory concentration index (FIC index). The FIC index is the sum of the FIC values of two drugs in a well. FIC value of each drug is calculated by dividing the MIC of each antibiotic in combination by the MIC of that drug alone. An FIC index of $\leq$ 0.5 is considered synergistic, while an FIC index of > 4.0 is antagonistic and the FIC index between 0.5-4.0 is considered indifference.

2.Time-kill assay([35, 36, 38])

Time-kill assay (TKA) compares the colony count at a given period of time, usually 24 hours, between the combination of antimicrobial agents and the most active agent alone. The process starts with adding standard inoculum of organism in broths. The first broth contains a combination of antimicrobial agents. The second and third broth are filled with single antimicrobial agent at the same concentration. The fourth broth is an antibiotic-free growth control. Then a small portion of the mixture is taken from each broth for colony count at the beginning of the test, at interval time points and at 24 hours. Synergistic interaction is established when the colony count at 24 hours from the combination broth is ≥2 $\log_{10}$ less than the count from the broth containing the most active single antimicrobial agent. If the colony count at 24 hours from the combination broth is ≥3 $\log_{10}$ less than the initial colony count, the combination is considered bactericidal. TKA is more labor-intensive and slower than the checkerboard assay (Fig.7).

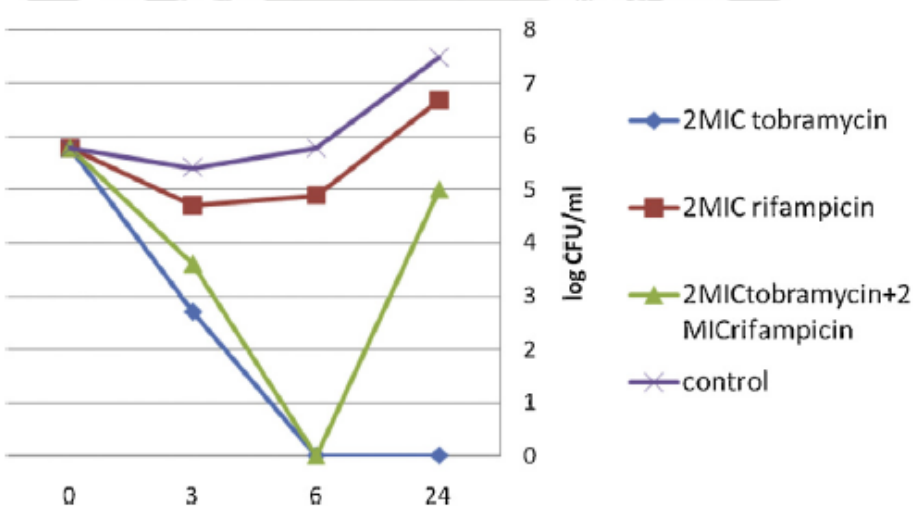


Figure 7 Time-kill results

3. Double disc synergy test

The concept of double disc synergy test is similar to the disc diffusion method. Discs containing tested antimicrobial agents are placed 20 mm (or sum of the radii of the zone of inhibition of each drug) apart over an inoculated agar medium. Synergy is recognized when there is bridging of the zone of inhibition or an increase in the zone of inhibition ≥ 2 mm compared to the zone of inhibition of single agent. Antagonism is shown by the loss of the zone of inhibition at the intersection of tested agents (Fig.8). Although this method is simple and inexpensive, it has not been routinely used nowadays because of its qualitative and subjective results.

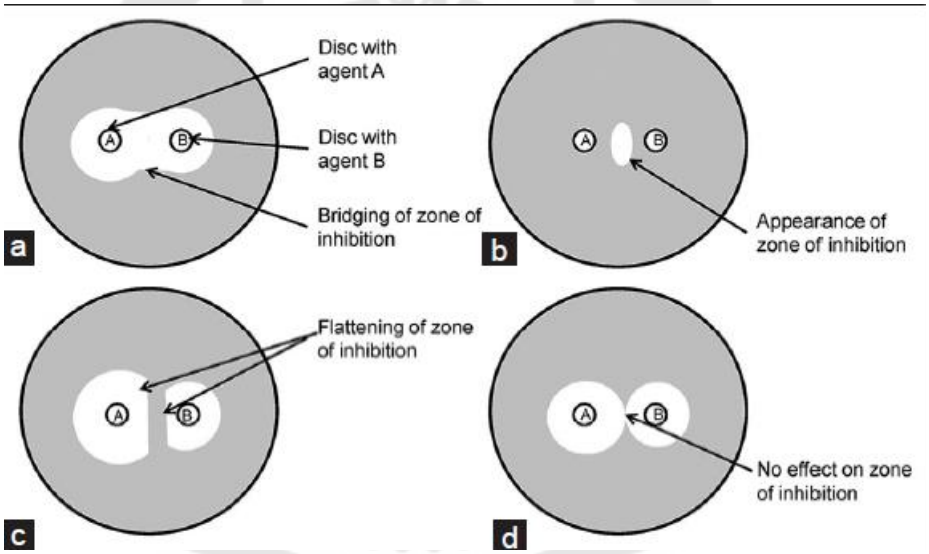


Figure 8 Double disk synergy test

2.13 Literature review

Table 1 Literature review

Title	Authors	Methods	Conclusions
Potassium aluminium sulphate(alum) inhibits growth of human axillary malodor-producing skin flora in vitro([25])	Al-Talib H, Yaziz H, Zulkafli NF et al.	Antimicrobial activity of alum in different concentrations (0.937-20 mg/ml) against axillary skin flora (*Micrococcus luteus, Staphylococcus epidermidis, Corynebcterium xerosis* and *Bacilus subtilis*)	Alum has excellent antimicrobial activity against axillary malodor-producing skin flora
In vitro study of concentration-effect and time-course pattern of white alum on *Escherichia coli* O157:H7 growth([26])	Shahriari R, Salari S, Shahriari S	Alum in 0.25, 0.5, 1, 2% concentrations were tested against *Escherichia coli* O157:H7. Optical density was recorded for four hours to acquire growth pattern data	White alum showed antibacterial effect against *Escherichia coli* O157:H7. The study exhibited dose and time-dependent effect of alum.

Table 1 Literature review (continue)

Title	Authors	Methods	Conclusions
Susceptibility profiles of alum on bacteria isolated from shellfish bivalve oyster([27])	Amadi L and Ngerebara N	Alum in 1.5, 2, 2.5, 3% concentrations were tested against *Proteus* spp., *Bacilus subtilis, Staphylococcus aureus, Escherichia coli, Klebsiella* spp., *Pseudomonas aeruginosa and Vibrio* spp. Later, the results were compared with standard treatment, ofloxacin.	Alum showed broad spectrum antibacterial activity against tested bacteria. However, ofloxacin had the highest antibacterial profiles among the tested solutions.
Preliminary studies on antifungal and antibacterial activity of alum as medicine preparation for vaginal discharge([28])	Shalli FG, Taufikurohmah T, Suyatno	Alum in 2.5, 5, 10, 15, 20 and 25% concentrations were tested against *Candida albicans* and *Neisseria gonorrhoeae*	Alum had antimicrobial activity against *Candida albicans* and *Neisseria gonorrhoeae*. Alum had the possibility to be used as a treatment for vaginal discharge.

Table 1 Literature review (continue)

Title	Authors	Methods	Conclusions
Antimycotic potential of alum on postharvest deterioration of tomato([30])	Amadi L, Udoh EJ, Thompson RU et al.	Antifungal activity of 1% alum concentration against *Aspergillus niger, Aspergillus flavus, Fusarium* spp., *Penicillium* spp., *Rhizopus stolonifera, Geotrichum candidum and Saccharomyces cerevisiae*.	1% concentration of alum was effective antimycotic agent against broad spectrum of fungal genera, except *Rhizopus stolonifera*.
In vitro activity of potash alum against *Candida albicans* on acrylic resin([39])	Irshad M, Younas M, Shabir H et al.	30 acrylic discs contaminated with *Candida albicans* were kept in 10 mg/ml alum solution for 2 hours. Later, colony counts was done to identify antifungal effect of alum. Positive and negative controls were 5.25% sodium hypochlorite and phosphate buffered saline respectively.	Both 5.25% sodium hypochlorite and 10 mg/ml alum solution demonstrated significant ($p<0.0001$) antifungal activity compared with the negative control.

Table 1 Literature review (continue)

Title	Authors	Methods	Conclusions
Antimicrobial properties of hydrogen peroxide and potash alum alone and in combination against clinical bacterial isolates([12])	Khurshid H, Rafiq M, Nazir F	Antibacterial activity of alum compare with hydrogen peroxide (4.4, 8.7, 17.5 and 35 mg/ml for both solutions) and in combination against	Antibacterial effects of alum and hydrogen peroxide were improved when the concentrations of the solutions were increased.
		Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Staphylococcus aureus and Klebsiella pneumoniae.	Hydrogen peroxide and alum in combination had more zone of inhibition than alum alone in all tested bacteria and more than hydrogen peroxide in *Enterococcus faecalis and Enterococcus faecium*.

Table 1 Literature review (continue)

Title	Authors	Methods	Conclusions
Synergistic antibacterial interaction between alum and antibiotics on some microorganisms([13])	Ali ZM	Alum in 4, 8, 16, 20% concentrations were tested against *Staphylococcus aureus, Klebsiella pneumoniae, Proteus vilgaris, Pseudomonas aeruginosa, Pseudomonas flourecence, Escherichia coli, Candida albicans*. Later, the inhibition zones of 20% alum plus cefotaxime and 20% alum plus tetracycline were compared with various antibiotic combinations	20% alum were effective against all tested bacteria including *Candida albicans*. Combinations alum plus cefotaxime and alum plus tetracycline had more inhibition zone than all tested antibiotic combinations, except clarithromycin plus gentamycin.
Antibacterial activity of aluminium potassium sulfate and *Syzygium aromaticum* extract against pathogenic microorganisms ([40])	Bnyan IA, Alta'ee AH and Kadhum NH	Antibacterial activity of alum in 10, 20, 30, 40 and 50% w/v concentrations together with *Syzygium aromaticum* (clove) extract in the same concentrations as	The minimum inhibitory zone was 20% concentration for each solution. Thus, alum and clove can be used as natural antimicrobial agents.

Table 1 Literature review (continue)

Title	Authors	Methods	Conclusions
		alum. Test organisms were *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli and Klebsiella pneumoniae*	
Profilistic study of bioactivities of extracts of Gongronema latifolium incorporated with alum on some clinical bacteria([31])	Amadi L, Ngerevara N and Okafor C.	Antibacterial activity of aqeous, ethanolic and methanolic leaf extracts (0.1-0.3g)c of *Gongronema latifolium*, with or without 1% concentration of alum, were tested against *Klebsiella pneumoniae, Salmonella typhi*, *Pseudomonas aeruginosa, Bacilus subtilis and Staphylococcus aureus*.	Synergism of *Gongronema latifolium* and alum was obviously seen. However, *Gongronema latifolium* can be effectively used on it's own.

CHAPTER 3

Materials and methods

3.1 Tested bacteria

All bacteria were acquired from Microbiology department, Faculty of medicine, Srinakharinwirot university. Bacteria used in this study included clinical isolates of *Cutibacterium acnes* (*C. acnes*), standard strains of *Staphylococcus aureus* (ATCC 25923) (*S. aureus*), *Staphylococcus epidermidis* (ATCC 12228) (*S. epidermidis*), *Micrococcus lutues* (ATCC 10240) (*M. luteus*), *Pseudomonas aeruginosa* (ATCC 27853) (*P. aeruginosa*) *and Candida albicans* (ATCC 10231) (*C. albicans*).

C. acnes strains used in this study were clinically isolated by Laochusuwan A. (SWUEC/E-272/2559). 75 strains of clinically isolated C. acnes were collected in the study. 48 out of 75 strains are antibiotic-resistant, which resist either clindamycin, erythromycin or both. The other 27 strains are antibiotic-susceptible.

The present study used three antibiotic-resistant strains, which were *C. acnes* no. 8 (clindamycin and erythromycin resistant), *C. acnes* no.23 (clindamycin and erythromycin resistant) and *C. acnes* no. 41 (erythromycin resistant), and three antibiotic-susceptible strains, including *C. acnes* no. 50, 69 and 74.

3.2 Preparation of alum solution

Potash alum was purchased from Merck, Darmstadt, Germany. Fifty grams of alum were dissolved in 100 ml distilled water to obtain 50% concentration. Later, a 50% alum solution was diluted into four concentrations 2.5, 5, 10, 20% w/v slightly modification from previous studies ([13, 28])

3.3 Preparation of *Phyllantus emblica* leaf extract

Phyllanthus emblica leaf powder was acquired from Microbiology department, Faculty of medicine, Srinakharinwirot university. The plant was extracted with 95% ethanol, by maceration method. Then, the *Phyllanthus emblica* leaf extract was dissolved in dimethyl sulfoxide (DMSO) (Amresco, Ohio, USA) to create two concentrations: 5 mg/ml and 25 mg/ml.

3.4 In vitro antibacterial activity testing of alum and *Phyllantus emblica* extract

Antimicrobial activity of alum and *Phyllanthus emblica* extract was evaluated using the agar well diffusion method . *C. acnes* was cultured in Brain-Heart infusion medium supplemented with 10% horse serum. The other tested bacteria were cultured in Mueller-Hinton medium. *C. acnes* was adjusted to $1x10^8$ CFU/ml. While other tested bacteria were adjusted to $1x10^7$ CFU/ml. *C. albicans* was cultured on Sabouraud Dextrose Agar (SDA) and adjusted to $1x10^8$ CFU/ml suspensions compared to 0.5 McFarland standard.

Bacteria were spread on the surface of the appropriate medium. Subsequently, 4-mm diameter wells were created in the agar using a sterile cork borer. Different concentrations of alum solutions and *Phyllanthus emblica* extracts were filled in each well (50 µl each). Brain Heart infusion and Mueller-Hinton broth were used as negative controls. *C. acnes* was incubated at 37°C for 72 hours in anaerobic condition. Other tested bacteria were incubated at 37°C for 24 hours. *C. albicans* was incubated at 30°C for 72 hours. Later, the diameter of inhibition zones around the well were measured and recorded in millimeters (mm).

Broth microdilution method was used to determine the minimum inhibitory concentration (MIC) ([41]), a serial two-fold dilution of potash alum solutions and *Phyllanthus emblica* extracts were produced in 96-well microtiter plates in an initial concentration of the minimum inhibitory concentration acquired from agar well diffusion method (50 µl). 50 µl of suspension for each bacterium and fungus ($1x10^6$ CFU/ml) was added in each well. 30 µl of 0.02% resazurin was added to each well to evaluate the growth of microorganisms. If the color of resazurin does not turn from blue to red, then

the growth of microorganisms was inhibited. The MIC was defined as the lowest concentration of solutions that inhibited visible growth of tested bacteria ([42]).

3.5 Synergy test

Synergistic interaction between alum and *Phyllanthus emblica* extract was evaluated by checkerboard analysis ([37]). 96-well microtiter plates were used in the method. Eight concentrations of *Phyllanthus emblica* extract and twelve concentrations of alum were added to each well (Fig.9). The concentrations were calculated from the MIC of each tested solution ([35]). The concentrations were prepared in 2-fold serial dilutions, the initial concentration was twice the MIC of *Phyllanthus emblica* extract and twice that of the alum solution.

The fractional inhibitory concentration index (FIC index) is the sum of the FIC values of the tested compound. The fractional inhibitory concentration (FIC) of each tested solution is calculated by dividing the concentration of the substance in that well by the MIC of that substance alone ([36]).

$$\text{FIC of agent A} = \frac{\text{MIC of agent A in combination}}{\text{MIC of agent A alone}}$$

$$\text{FIC of agent B} = \frac{\text{MIC of agent B in combination}}{\text{MIC of agent B alone}}$$

$$\text{FIC index} = \text{FIC of agent A} + \text{FIC of agent B}$$

Synergy is identified when FIC index is less than or equal to 0.5. No interaction is determined when FIC index is between 0.5 and 4. Antagonism is interpreted when FIC index is more than 4 ([35]).

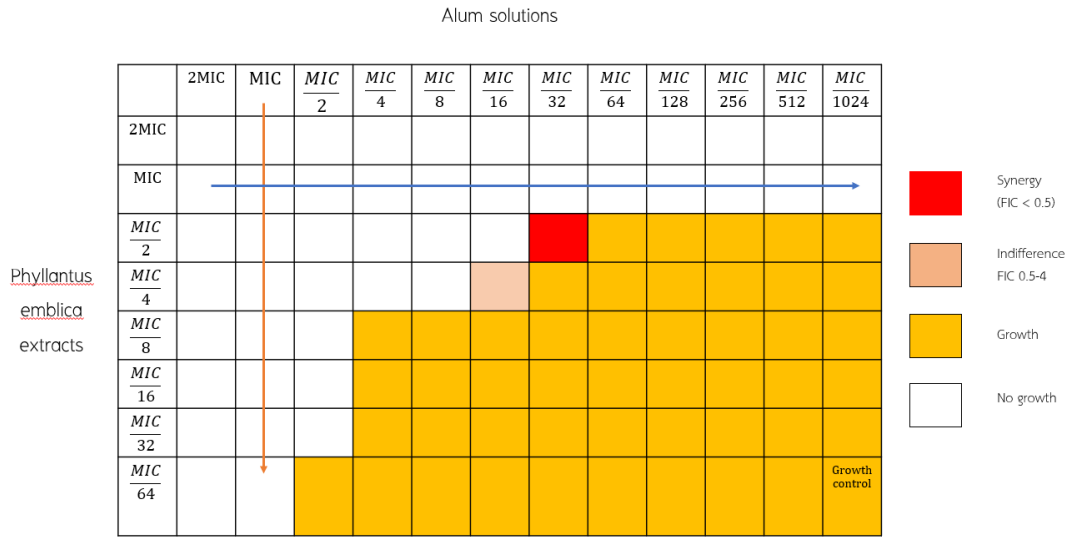


Figure 9 Checkerboard assay

3.6 Statistic analysis

Statistic/Data analysis (Stata) version 16 will be used to perform statistical analysis in this study. Descriptive statistic will be mainly used in the study. Minimum inhibitory concentration (MIC) and minimum inhibition zone (MIZ) will be reported as mean and standard deviation (SD). Wilcoxon-Ranksum test were used to evaluate the difference of quantitative data.

CHAPTER 4

Results

Antimicrobial activities of alum solutions and *Phyllanthus emblica* extracts were evaluated by agar well diffusion method. Four different alum solutions (2.5%, 5%, 10%, 20%) and two concentrations of (5 mg/ml, 25 mg/ml) *P. emblica* extracts were tested on six bacteria and one fungus.

In accordance with Davis and Stout's classification, the zones of inhibition can be graded into four groups which include weak response (diameter < 5 mm), moderate response (diameter 5-10 mm), strong response (diameter 10-20 mm) and very strong response (diameter > 20 mm).

As demonstrated in table 2, alum solutions of 10% and 20% demonstrated strong inhibitory effects on antibiotic-susceptible *Cutibacterium acnes* (*C. acnes*). However, alum solutions failed to inhibit antibiotic-resistant *C. acnes*. Furthermore, the 20% alum showed a very strong inhibitory effect on *Staphylococcus aureus* (*S. aureus*), *Staphylococcus epidermidis* (*S. epidermidis*), *Micrococcus luteus* (*M. luteus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*). In contrast, 10% and 5% alum solutions exhibited strong inhibitory effects on the same organisms. 2.5% alum solutions also exhibited inhibitory effects on mentioned organisms, however only moderate responses were observed. The largest inhibition zone (26 mm) was seen on *M. luteus*. As shown, higher alum concentrations demonstrated a greater inhibitory effect on tested organisms. Nevertheless, alum exhibited no antifungal activity against *Candida albicans* (*C. albicans*).

Table 2 Minimum inhibitory zones (MIZ) in millimeter (mm) of alum solutions by agar well diffusion method.

Tested organisms		Alum solutions			
		2.5%	5%	10%	20%
		MIZ (mm)			
Antibiotic-resistant strains	*C. acnes* (no. 8)	-	-	-	-
	C. acnes (no. 23)	-	-	-	-
	C. acnes (no. 41)	-	-	-	-
Antibiotic-susceptible strains	*C. acnes* (no. 50)	-	-	10	12
	C. acnes (no. 69)	-	-	-	-
	C. acnes (no. 74)	-	-	11	15
S. aureus		6	11	13.5	23
S. epidermidis		9	12	15.5	24.5
M. luteus		8.5	13	19	26
P. aeruginosa		7.5	12	14.5	21.5
C. albicans		-	-	-	-

P. emblica extracts (25 mg/ml) exhibited very strong antibacterial effects on most of *C. acnes* including both antibiotic-resistant and antibiotic-susceptible strains. Only *C. acnes* no.8 and no.74 were moderately inhibited by the extracts. The means of the minimum inhibitory zones of *P. emblica* extracts on antibiotic-resistant and antibiotic-susceptble *C. acnes* were 18.7 ± 7.6 and 18 ± 9 mm, respectively. The mean MIZ value

on antibiotic-resistant *C. acnes* was a little higher than on antibiotic-susceptble *C. acnes.* However, there were no statistical differences between the two groups. *P. emblica* extracts also showed strong inhibitory effects against *S. aureus*, *S. epidermidis* and *M. luteus* with only 5 mg/ml concentration. Nevertheless, *P. emblica* extracts were ineffective in inhibiting the growth of *P. aeruginosa* and *C. albicans*. (Table 3,4)

Table 3 Minimum inhibitory zones (MIZ) in millimeter of *P. emblica* extracts by agar well diffusion method.

Tested organisms		*Phyllanthus emblica extract* (5mg/ml)	*Phyllanthus emblica extract* (25mg/ml)
		MIZ (mm)	
Antibiotic-resistant strains	*C. acnes* (no. 8)	-	10
	C. acnes (no. 23)	-	24
	C. acnes (no. 41)	-	22
Antibiotic-susceptible strains	*C. acnes* (no. 50)	-	20
	C. acnes (no. 69)	-	26
	C. acnes (no. 74)	-	8
S. aureus		10	(Not done)
S. epidermidis		17	(Not done)
M. luteus		11.3	(Not done)
P. aeruginosa		-	-
C. albicans		-	-

Table 4 Mean MIZ of *P. emblica extracts* on each *C. acnes* group

Tested organisms	$MIZ_{P. emblica}$ (mean ± SD)	*p*-value*
Antibiotic-resistant *C. acnes* (n = 3)	18.7 ± 7.6	0.9273
Antibiotic-susceptible *C. acnes* (n = 3)	18 ± 9	

Minimum inhibitory concentrations (MIC) were evaluated by broth microdilution method. Fifty microliters suspension of each bacterium ($1x10^6$ CFU/ml) was added into each well with fifty microliters of prearranged alum solution or P. emblica extract. The MIC represented the lowest concentration of solutions that inhibited visible growth of tested bacteria.

The lowest MIC of alum solution, found on S. epidermidis, was 0.0195 mg/ml. while the highest MIC of alum solution, which was 0.156 mg/ml, was found in S. aureus and P. aeruginosa. The lower MIC means the organism is more susceptible to alum solutions than the higher one. Thus, alum solution had the greatest inhibitory effect on S. epidermidis, followed by M. luteus, antibiotic-susceptible C. acnes, S. aureus and P. aeruginosa.

The lowest MIC of P. emblica extract , observed in antibiotic-susceptible C. acnes, was 0.097 mg/ml, whereas 0.78 mg/ml was the highest MIC P. emblica, detected in S. aureus. The mean MIC on antibiotic-resistant C.acnes was 0.325 ± 0.113 mg/ml which was higher than 0.130 ± 0.057 mg/ml observed for antibiotic-susceptible C. acnes. However, there was no statistical differences. (Table 5, 6)

Tested organisms which had both MIC of alum solution and MIC of P. emblica extract will be further investigated by checkerboard assay.

Table 5 Minimum inhibitory concentrations (MIC) of alum solutions and *P. emblica* extracts by broth microdilution method

Tested organisms		MIC_{Alum} (%w/v)	$MIC_{P. emblica}$ (mg/ml)
Antibiotic-resistant strains	*C. acnes* (no. 8)	-	0.39
	C. acnes (no. 23)	-	0.39
	C. acnes (no. 41)	-	0.195
Antibiotic-susceptible strains	*C. acnes* (no. 50)	0.078	0.097
	C. acnes (no. 69)	-	0.097
	C. acnes (no. 74)	0.078	0.195
S. aureus		0.156	0.78
S. epidermidis		0.0195	0.39
M. luteus		0.029	0.29
P. aeruginosa		0.156	-
C. albicans		-	-

Table 6 Mean MIC of *P. emblica* extracts on each *C. acnes* group

Tested organisms	$MIC_{P.\ emblica}$ (mean ± SD)	*p*-value*
Antibiotic-resistant *C. acnes* (n = 3)	0.325 ± 0.113	0.0679
Antibiotic-susceptible *C. acnes* (n = 3)	0.130 ± 0.057	

Table 7, 8 and figure 12 demonstrated the FIC of alum solution and *P. emblica* extracts on each microbe, respectively. Table 9 showed the FIC index of tested bacteria. FIC index was the sum of FIC_{Alum} and $FIC_{P.\ emblica}$ on the same organism. FIC index determines the interaction between two tested substances whether they are synergistic, antagonistic or noninteractive. The results of table 9 showed that every FIC index of tested bacteria was below 0.5, thus there was synergistic interaction between alum solution and *P. emblica* extracts on every microbe. The highest FIC index, observed in *M. luteus*, was 0.421, while the lowest FIC index, seen in *S. aureus* was 0.046.

Table 7 Fractional inhibitory concentration of alum solutions by checkerboard assay

Tested organisms		MIC_{Alum} (%w/v)	$MIC_{Alum\ in\ combination}$ (%w/v)	FIC_{Alum}
Antibiotic-susceptible strains	*C. acnes* (no. 50)	0.078	0.0024	0.031
	C. acnes (no. 74)	0.078	0.0006	0.007
S. aureus		0.156	0.0024	0.015
S. epidermidis		0.0195	0.0024	0.123
M. luteus		0.029	0.0098	0.338

Table 8 Fractional inhibitory concentration of *P. emblica* extracts by checkerboard assay

Tested organisms		$MIC_{P. emblica}$ (mg/ml)	$MIC_{P. emblica}$ in combination mg/ml)	$FIC_{P. emblica}$
Antibiotic-susceptible strains	*C. acnes* (no. 50)	0.0975	0.012	0.123
	C. acnes (no. 74)	0.195	0.024	0.123
S. aureus		0.78	0.024	0.031
S. epidermidis		0.39	0.024	0.062
M. luteus		0.29	0.024	0.083

Table 9 Fractional inhibitory concentration index (FIC index) of alum solutions and *P. emblica* extracts by checkerboard assay

Tested organisms		FIC_{Alum}(%w/v)	$FIC_{P. emblica}$(mg/ml)	FIC index
Antibiotic-susceptible strains	*C. acnes* (no. 50)	0.031	0.123	0.154
	C. acnes (no. 74)	0.007	0.123	0.130
S. aureus		0.015	0.031	0.046
S. epidermidis		0.123	0.062	0.185
M. luteus		0.338	0.083	0.421

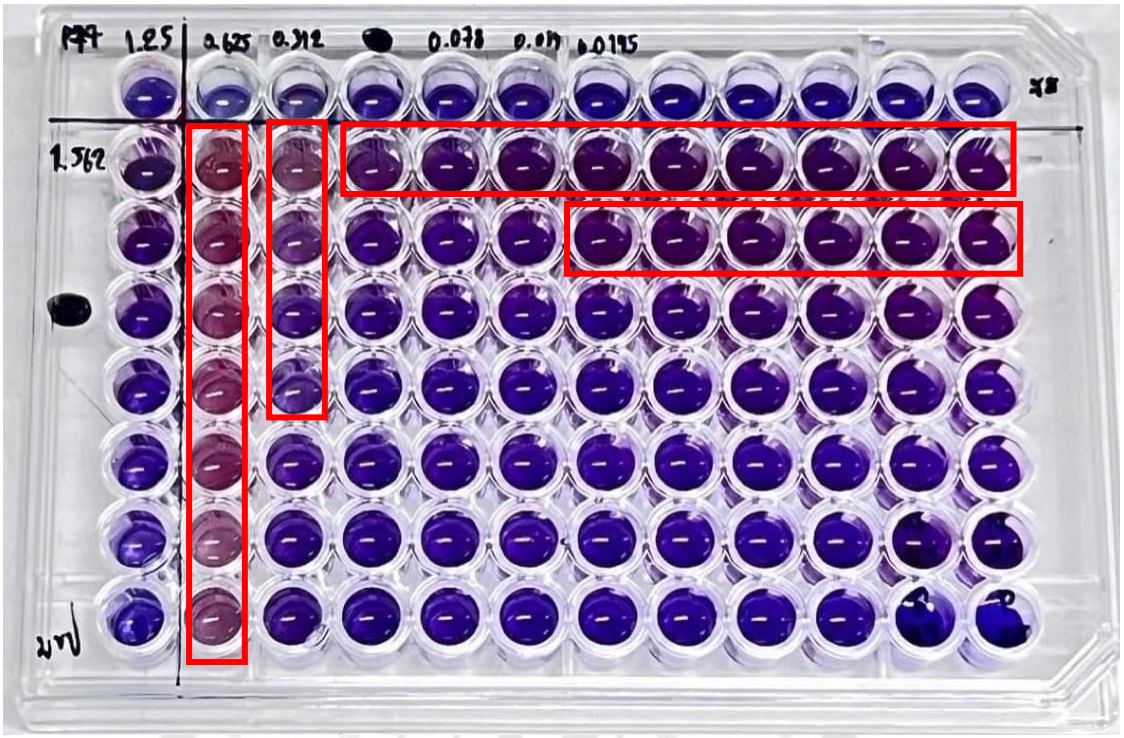


Figure 10 Abnormal incidents of checkerboard assay

CHAPTER 5

Discussion

This study found that alum showed stronger inhibitory effect on antibiotic-susceptible *Cutibacterium acnes* than antibiotic-resistant *C. acnes*. Although the exact mechanism of alum resistance is not fully understood, several antibiotic resistance mechanisms of *C. acnes* had been reported. Energy-dependent drug efflux was found to be the most common mechanism([43]). Other mechanisms include protection of ribosome by resistance proteins, which reduce ribosome's affinity for antibiotics, or the bacteria could produce enzymes capable of deactivating the antibiotics([44]). Therefore, alum resistance mechanism could be one of previously mentioned mechanisms. Because no previous studies have been conducted to evaluate the antimicrobial effects of alum on clinically isolated *C. acnes* , no comparison could be made. However, the results of this study could lead to further investigations into how alum interacts with *C. acnes*. Alum showed antibacterial activity against two out of three antibiotic-susceptible *C. acnes* strains with a mean minimum inhibitory concentration of 0.078% w/v. However, it failed to inhibit antibiotic-resistant *C. acnes* with concentrations up to 20% w/v. Since no previous study has been conducted to evaluate the antimicrobial effect of alum on *C. acnes*, more studies should be carried out to compare the results among them.

Alum also demonstrated a very strong inhibitory effect on both Gram-positive and Gram-negative bacteria. The results concurred with a previous study([27]) that evaluated the susceptibility of alum to multiple bacteria, including *S. aureus*, *P. aeruginosa* and others. The study concluded that alum exhibited broad-spectrum antibacterial potency, particularly against Gram-negative bacteria. Regarding Gram-positive bacteria, Al-Talib et al. studied the antimicrobial effect of alum against axillary bacterial flora, including *M. luteus* and *S. epidermidis*([25]). They reported that the minimum inhibitory concentration of *M. luteus* was 3.75 mg/ml (0.375% w/v) and of *S. epidermidis* was 7.5 mg/ml (0.75% w/v). They used the broth dilution method,

followed by subculture was done to determine colony-forming units (CFUs) and the minimum inhibitory concentrations. Thus, the MIC values in their study differed from ours. However, they concluded that alum had substantial inhibitory effects against all tested bacteria.

According to the results, none of the tested alum concentrations exhibited antifungal activity against *C. albicans*. Thus, this contrasts with Udemezue's findings([45]), which reported inhibition zones of 13.50 ± 0.71 mm starting to be seen at an alum concentration of 25 mg/ml or 2.5%w/v. The differences between our results and Udemezue's may be attributed to two factors. First, the volume of test solutions added to each well differs: Udemezue filled 0.5 ml (500 µl) of alum solution into 6-mm diameter wells, whereas this study used only 50 µl of alum solution for each 4-mm diameter well. Second, the strains of *C. albicans* were different. Udemezue collected the isolates from vulvo-vaginal candidiasis patients. While our study used a standard strain of *C. albicans*.

The results indicated that alum has antibacterial effects which increase with higher alum concentrations and concurred with Ali([13]) who described that the inhibition zones of six bacterial isolates (*Staphylococcus aureus*, Klebsiella pneumoniae, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Escherichia coli*) and one fungus (*Candida albicans*) increased with the incremental concentration of alum solutions.

Phyllanthus emblica extracts demonstrated strong inhibitory effects on both antibiotic-susceptible and antibiotic-resistant *C. acnes.* this agrees with previous study([46]) which reported that the mean inhibition zones of *P. emblica* extracts on antibiotic-susceptible and antibiotic-resistant *C. acnes* are 17.48 and 13.76 mm, respectively. Furthermore, Deewongkij reported that the mean of the MIC of *P. emblica* on antibiotic-susceptible *C. acnes* was found to be 0.5567 ± 0.348 mg/ml([47]). Whereas, the MIC of *P. emblica* on antibiotic-resistant *C. acnes* was 0.56 ± 0.85 mg/ml. The differences in MIC values between the latter study and ours may be due to two factors. First, the sample size of *C. acnes* in the previous study, which contained 44 specimens

of antibiotic-resistant *C. acnes* and 31 specimens of antibiotic-resistant *C. acnes*. Second, the variation of the main active ingredients acquired from extraction method may be higher in this study([46]).

As the results indicate*, P. emblica* extracts also exhibited stronger inhibitory effect against Gram-positive bacteria than Gram-negative bacteria and fungi. Similar results were reported by Gandhi et al. who evaluated antimicrobial activity of *P. emblica* leaf extracts against *S. aureus*, *E. coli* and *C. albicans*([48]). The study showed that inhibition zones were observed at a concentration of 25 mg/ml for both *S. aureus* and *E. coli* with mean diameters of 19 mm and 9 mm, respectively. However, the inhibition zones of *C. albicans* began to appear clearly at a concentration of 75 mg/ml with a mean diameter of 7 ± 0.49 mm. They concluded that *P. emblica* leaf extract was more effective against Gram-positive bacteria than Gram-negative bacteria and showed very limited efficacy against fungi. Moreover, past study([21]) showed similar results, indicating that ethyl acetate extracts of *P. emblica* were capable of inhibiting the growth of Gram-positve bacteria, including *C. acnes*, *S. epidermidis* and *S. aureus*. The MIC values were found to be 2.5, 0.312 and 0.312 mg/ml, respectively.

Generally, the main differences between Gram-positive and Gram-negative bacteria are that Gram-negative bacteria are surrounded by the outer membrane containing lipopolysaccharide and a thin peptidoglycan cell wall, while Gram-positive bacteria lack an outer membrane but have multiple layers of peptidoglycan cell wall. The outer membrane is well-known for its protective function. It also contains a few self-survival enzymes, including phospholipase and protease. Moreover, Gram-negative bacteria contain the periplasm, which is an aqueous compartment densely packed with proteins. The periplasm helps Gram-negative bacteria isolate harmful degradative enzymes, such as RNAse or alkaline phosphatase. Thus, Gram-negative bacteria are normally more resistant to antibiotics than Gram-positive bacteria([49]).

Checkerboard assay was used to evaluate fractional inhibitory concentration index (FIC index) of alum solutions and *P. emblica* extracts on tested bacteria in order to assess the interaction between the two suspensions.

As shown in table 9, all of the FIC indexes were below 0.5 which indicated synergistic interaction between alum solutions and *P. emblica* extracts on every studied organism. The highest FIC index (0.421) was reported on *M. luteus* and the lowest FIC index was seen on *S. aureus*.

There was no preceding study that investigated the interaction between alum solution and *P. emblica* extract. Nonetheless, numerous research demonstrated synergistic interaction between alum solutions and natural substances. For instance, crude *Turmeric rhizome* extracts and alum were tested against four bacterial (*S. aureus*, *E. coli*, *B. cereus*, *P. fluorescens*) and five fungal isolates (*Saccharomyces cerevisiae*, *C. albicans*, *Aspergillus flavus*, *A. terreus*, *Penicillium crystallium*)([14]). The results showed that there was an enhancement of antimicrobial effect when *Turmeric rhizome* extracts were incorporated with alum.

The antimicrobial mechanism of alum solution was not fully understood. However, some studies suggested that it damaged or reduced the acidity of bacterial cell walls. While *P. emblica* extract has tannin as the main active ingredient for antibacterial action([46]). Tannin or tannic acid is natural polyphenol which can strongly bind to proteins or metal ions and consequently forms complexes([50]). Hence, it can inhibit bacteria's proteins transport, microbial adhesion, and extracellular microbial enzymes such as lipase, protease, or hyaluronidase([51]). This study found that there was a synergistic interaction between alum solution and *P. emblica* extract. Thus, the mechanism of synergy might be explained as the bacteria's cell walls were weakened by alum solution. Consequently, *P. emblica* extract, containing tannin, can react more easily to the bacterial proteins.

As shown in Figure 12, the combinations of alum solutions and *P. emblica* extracts at high concentration exhibited abnormal incidents. In particular, the concentrations, which were higher than the MIC, of both alum solution (0.078%) and *P. emblica* extracts (0.195 mg/ml) failed to inhibit *C. acnes* growth. On the contrary, the combinations of low concentration alum and *P. emblica* extracts tended to inhibit bacteria's growth substantially. This phenomenon could be explained as tannin-metal

complex formation. Slabbert([50]) reported that aluminium (Al) can form colorless complexes with tannins. Moreover, Tangarfa and Hassani([52]) studied tannic acid adsorption on fluorite surface and concluded that the formation of tannin-metal complexes was enhanced by increasing initial tannic acid concentration. Dönmez and Aksu([53]) also described that the initial concentrations provide an important driving force to overcome all mass transfer resistance of the metal ion. In summary, high concentration of alum solutions and *P. emblica* extracts reacted to each other and formed complexes more readily than at low concentrations. Therefore, they lost their antibacterial property more than at low concentrations.

For further studies, higher concentrations of alum solutions and *P. Emblica* extracts should be tested against *C. albicans*. Additionally, more microorganisms such as *Kytococcus sedentarius*, dermatophytes and *Malassezia furfur* should be evaluated. Lastly, clinical trials should be conducted to assess the therapeutic effects of alum and *P. emblica* against cutaneous infections.

Conclusion

Potash alum and *P. emblica* are well-known natural products which have been utilized throughout human history, especially in the treatment of diseases. Numerous studies reported that they have antibacterial and antifungal effects.

In summary, this study showed that alum solutions have an antibacterial effect against only antibiotic-susceptible *C. acnes* and no inhibitory effect on antibiotic-resistant *C. acnes*. Moreover, while alum solutions exhibited substantial inhibitory effect on both Gram-positive and Gram-negative bacteria, they failed to inhibit *C. albicans*. Alternatively, *P. emblica* extracts demonstrated a strong inhibitory effect on Gram-positive bacteria, including antibiotic-resistant *C. acnes*. However, they showed no effect on *P. aeruginosa* and *C. albicans*. Synergistic interaction between alum solutions and *P. emblica* extracts were observed. Nonetheless, when high concentrations of alum solutions and *P. emblica* extracts were used, tannin-metal complexes form extensively, causing them to lose their antimicrobial properties.

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