Applied Chemistry Project

Project title Screening of Antimicrobial Thai Plants by Directed TLC-Bioautography

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Faculty of Science, Chulalongkorn University

Screening for Antimicrobial Thai Plants by Directed TLC-Bioautography

by
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Miss Thanita Leelakraisorn

In Partial Fulfillment for the Degree of Bachelor of Science
Program in Applied Chemistry (International Program)
Department of Chemistry, Faculty of Science

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Accepted by Department of Chemistry, Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Degree of Bachelor of Science Program in Applied Chemistry (International Program)

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Abstract

Medicinal plants have been discovered and used as traditional drugs since ancient years. At the present, modern drugs have killed a large number of people due to a drug of resistance, and the best solution to solve this problem was medicinal herbs. The goal of this project was to examine the antibiotic compounds from Thai herbs in our daily lives. However, Thai plants were only screened for antimicrobial activity as the time limited. In this study, 10 Thai plants were selected to screen the antimicrobial activity against methicillin-resistant *Staphylococcus aureus* by using directed TLC-bioautography and agar disc diffusion method. *S.aureus* was used to examine each crude extract as this bacteria can cause common severe illnesses. The methanol was used as the main solvent for plant extracts. However, there were 7 out of 10 crude extracts that represented the good separation of the metabolite spot on the TLC plate which were *Zingiber officinale*, *Piper nigrum*, *Andrographis paniculata*, *Centella asiatica, Curcuma longa*, *Garcinia mangostana*, and *Lycopersicon esculentum Mill*. For the other 3 plant extracts, they performed less separation on the TLC plate including *Allium sativum*, *Allium ascalonicum*, and *Cymbopogon citratus*. For the agar disc diffusion technique, *Centella asiatica* has been exhibited the highest antibacterial properties in the second trial with the amount of 700 ug crude extract. Furthermore, in the directed TLC-bioautography assay, the compounds of *Andrographis paniculata* and *Garcinia mangostana* have been performed to have clear zones on the TLC plate to represent antimicrobials.

Keyword: Medicinal plants, Antimicrobial, *Staphylococcus aureus*, Directed TLC-bioautography

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Chapter 1
Introduction

1.1 Introduction to the research problem and significance

Antimicrobial agents are significant to our human lives as they can inhibit the growth of microorganisms which are the major cause of infectious diseases. However, the main problem for today's pharmaceutical industry is the resistance of bacteria toward antimicrobial agents. This is also known as antimicrobial resistance (AMR) [1]. It occurs when the microorganisms, such as bacteria, fungi and viruses, are altered gradually over time which leads to not responding to antimicrobial medicines. Due to the World Health Organization, it has been reported that the drug resistance problem has killed almost 10 million people per year. The research also indicates that Thai people have been infected by bacteria due to drug resistance 88,000 people per year, and every 15 minutes, one Thai person dies. This will enlarge the risk of infectious diseases, serious illness and premature death. Therefore, it is important to search for new antimicrobial agents and natural products are one of the potent resources for antimicrobial agents, particularly plants which include herbs, vegetables, and fruits. At present, there are more than 1350 plants with antimicrobial activities and more than 30,000 antimicrobial components that have been extracted from plants [2]. Moreover, many studies have also been conducted on the antimicrobial potential of plant extracts.

One of the techniques to detect antimicrobial agents in plants is known as the directed TLC bioautography method. This method is speedy, useful, effective and easy to detect antimicrobial compounds as it can evaluate complex plant extracts. Thus, this is suitable for selective detection which combines with chromatography [3]. Therefore, this research aims to deal with the antimicrobial resistance problem by examining the antimicrobial activities of Thai plants with the directed TLC bioautography methods.

1.2 Main groups of Plant-derived antibacterial compounds

Class of natural compound	**Compound**	**Active against**
Phenolic compounds	Resveratrol	*S. aureus, E. coli, L. monocytogenes, C. jejuni*
	Baicalein	*M. smegmatis, MRSA, C. albicans*
	Catechin	*L. monocytogenes*
Alkaloids	Piperine	*S. aureus, E. coli, B. subtilis*
	Berberine	*E. coli, S. agalactiae*
	Reserpine	*Staphylococcus sp., Streptococcus sp., Micrococcus sp.*

Table 1: Example of plant-derived antibacterial compounds

1.2.1 Phenolic compounds

Phenolic compounds are mostly represented in plants that possess significant antimicrobial activities. Moreover, many studies have been reported that phenolic compounds provide various advantages such as antioxidant, anti-aging, antiproliferative, and antimicrobial activities.

Resveratrol is one of the members of phenolic compounds that exist in over 100 medical and comestible plants such as Peanut, Chinese cinnamon, and Japanese knotweed, grapes, blueberries and mulberries. However, it is also represented as an active compound

in red wine. Moreover, resveratrol also provides health benefits and can inhibit the bacterial growth known as *S. aureus*, *E. coli*, *L. monocytogenes* and *C. jejuni* [4].

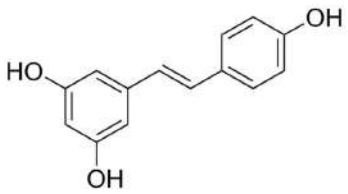


Figure 1: Structure of resveratrol

Baicalein is a type of flavone that is an active component found in traditional Chinese herb, *Scutella baicalensis*. It is also present in *Oroxylum indicum* and Thyme. This compound is significant for plants as it is the ingredient of flower pigmentation which can help to attract insects. It mainly has anti-cancer and anti-inflammatory properties. Moreover, it can inhibit the development of cell proliferation. From the previous research, the antimicrobial activity of baicalein has been reported that it can inhibit bacteria such as *M. smegmatis* and *MRSA*. However, MRSA is a dangerous bacterium because it can cause different parts of the body to become infected [5].

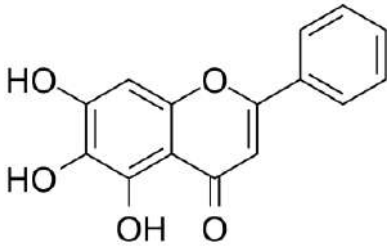


Figure 2: Structure of baicalein

Catechin is one type of phenolic compounds which are commonly presented in various herbs, fruits, vegetables and meats. However, the rich source of catechin is mostly found in green tea (*Camellia sinensis*). The tea has been reported to exhibit antimicrobial activities and consist of various catechin compounds. Moreover, it contains many benefits for human health such as anti-cancer, anti-inflammatory, anti-allergic and antimicrobial

properties. This compound has antimicrobial activity and can be active against *Listeria monocytogenes,* a gram-positive bacterium that exists in red meat [6].

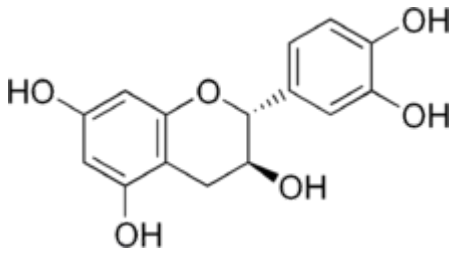


Figure 3: Structure of catechin

1.2.2 Alkaloids

Alkaloids are generally considered in both modern and traditional medicines with various pharmacological activities. They showed anti-cancer, anti-inflammatory, antimicrobial, and several other activities. Piperine is the main alkaloid present in *P. nigrum* (black pepper). It has many properties such as anti-inflammatory, anti-arthritic and antigenic. The piperine has been reported to exhibit antibacterial activity active against both gram-positive and gram-negative bacteria which were *Bacillus subtilis* and *E.coli*, respectively [7].

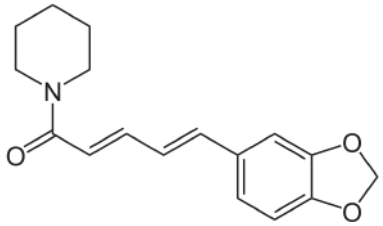


Figure 4: Structure of piperine

Berberine is an isoquinoline derivative alkaloid that is mostly found in barberry, tree turmeric and Amur cork tree. This compound usually exists in the roots, barks and rhizomes. Moreover, it affects antimicrobial, anti-inflammatory and antiviral activities. In

addition, berberine is effective as antimicrobials against *E. coli.* and *S. agalactiae,* the cause of invasive bacterial infection which lead to mortality [8].

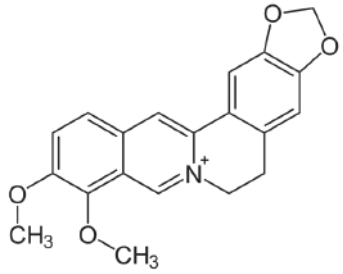


Figure 5: Structure of Berberine

1.3 Examples of plant products with antimicrobial activity

Common name	**Scientific name**	**Compound**	**Active against**
Ginger	*Zingiber officinale Roscoe*	Gingerol	*S. aureus*, *E. coli, S. pyogenes*
Black pepper	*Piper nigrum*	Piperine	*S. aureus*, *E. coli, B. cereus*, *S. faecalis*
Green chiretta	*Andrographis paniculata*	Andrographolide	*S. aureus*, *S. pyogenes*
Mangosteen	*Garcinia mangostana*	α-Mangostin	*S. aureus*, *E. coli,*
Turmeric	*Curcuma longa*	Curcuminoids	*S. aureus*, *E. coli, B. subtilis*

Table 2: The plant products with antimicrobial activity

1.3.1 *Zingiber officinale Roscoe* (Ginger)

Ginger (*Zingiber officinale Roscoe*) is a plant that has been commonly used as herbal drugs and spices since the ancient years which belongs to the Zingiberaceae family and Zingiber genus. The significant part of ginger that is generally used is known as rhizome as it can treat various diseases such as nausea, headaches, colds and digestive problems, due to the abundant bioactive compounds within the plant.

Based on phytochemical study, ginger is composed of more than 400 constituents. However, the significant bioactive compounds in Ginger are gingerol, shogaols and parasols. The 6-gingerol is responsible for its taste and aroma characteristics. It is determined as the most active component as compared to others due to the ability to resist the different pharmacological effects such as antioxidant, anti-inflammatory, and antipyretic activities in the ginger.

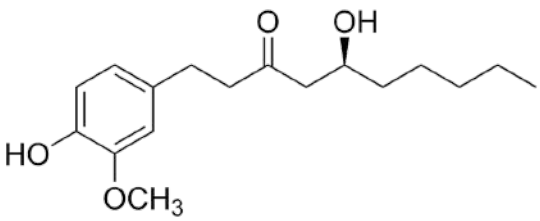


Figure 6: Structure of 6-gingerol

The antibacterial properties of ginger provide the ability to prevent infections. The ginger extracts can fight against various bacteria such as *Staphylococcus aureus* and *Streptococcus pyogenes*. Besides, from the research, the ginger extracts are effective against gram-positive bacterium more than gram-negative bacterium.

1.3.2 *Piper nigrum* (Pepper)

Piper species are a group of plants that have a unique aroma. *Piper nigrum* or black pepper is a member of the Piperaceae family originated in India. It is also known as "King of Spices" as people commonly used as a spice.

Besides, many compounds can be found in *P. nigrum* which is known as piperine, pellitorine, guineensine, and piperonal. However, the piperine in black pepper has been proven to be the main bioactive component that provides diverse pharmacological effects such as antibacterial, anti-inflammatory activities, etc.

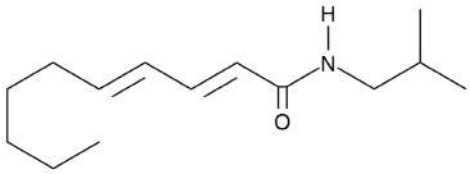


Figure 7: Structure of pellitorine

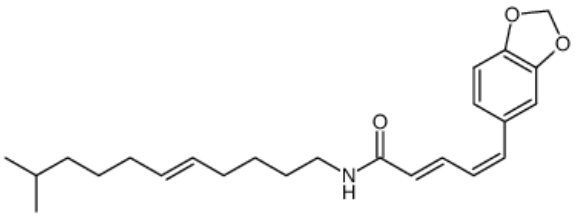


Figure 8: Structure of guineensine

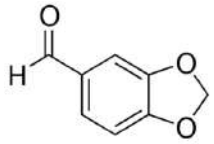


Figure 9: Structure of piperonal

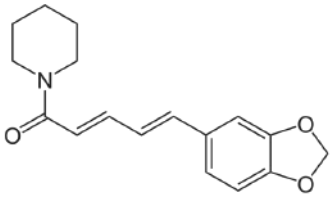


Figure 4: Structure of piperine

Black pepper can be considered as an antimicrobial agent as it can resist various pathogens. It was discovered to be the most effective with the gram-positive bacteria including *Staphylococcus aureus*, *Bacillus cereus*, and *Streptococcus faecalis* [9]. However, the gram-negative bacteria are less effective for black pepper.

The anti-cancer properties have been found for piperine, the main bioactive compound in *P. nigrum*. The alcohol extracts of black pepper displayed that the piperine is effective to resist lung cancer by changing lipid peroxidation. Piperine also provides anti-tumor activity due to the activation of cellular and immunomodulation characteristics. Furthermore, piperine can inhibit breast cancer cell-induced angiogenesis in our body by inhibiting the angiogenic process.

1.3.3 *Andrographis paniculata* (Kariyat)

Andrographis paniculata is considered a medicinal plant that is commonly used all over the world. It has a significantly bitter taste or also known as “King of bitter” which is used as a herbal drug to cure different diseases such as common fever, diabetes, liver disorders, and respiratory tract infection [10]. The aerial part of *A. paniculata* is the common part that is being used. Their extracts contain several compounds such as flavonoids, diterpenoids, lactones, etc. *A. paniculata* has been found to have a wide range

of pharmacological properties including antimicrobial, anti-inflammatory, anticancer, and anti-hepatitis.

Various active compounds have been found after the ethanol and methanol extracts from *A. paniculata* which include more than 20 diterpenoids and over 10 flavonoids [11]. From the several studies, andrographolide ($C_{20}H_{30}O_5$) is a diterpenoid, the major bioactive compound in *A. paniculata* which has several pharmacological properties, particularly active against various cancer cells and is known as a chemotherapeutic agent.

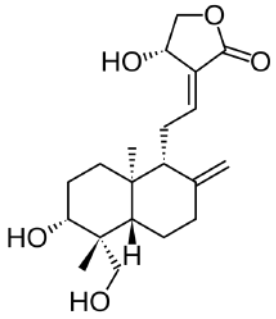


Figure 10: Structure of andrographolide

The various types of extracts from *A. paniculata* have been reported to show antibacterial properties against both pathogenic and nonpathogenic bacteria. According to the experiment, the methanolic extract from the *A. paniculata* leaves provided the highest antibacterial activity against *E. faecalis*. However, dichloromethane extract provided the lowest antibacterial activity. From the studies, the methanol extracts can also resist *S. aureus* and *S. pyogenes* which are human pathogenic bacteria.

1.3.4 *Curcuma longa* (Turmeric)

In general, *Curcuma longa is also* known as turmeric which belongs to the Zingiberaceae family. It has been reported to provide various pharmacological effects such as anti-inflammatory effects, anti-diabetic, anti-carcinogenic, etc.

C. longa is composed of various compounds such as alkaloid, essential oil, turmeric oil, yellow matter curcumin, starch grains, etc. The turmeric mainly contains essential oil known as zingiberene. However, curcuminoids and sesquiterpenoids are determined as the main bioactive components in *C. Longa.* that provide different bioactivities [12].

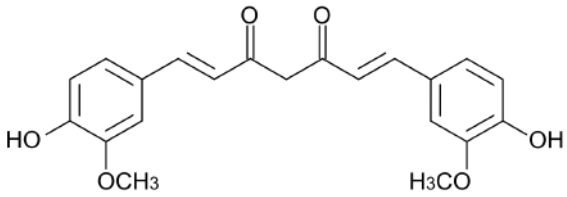


Figure 11: Structure of curcuminoids

C. longa has been reported to have antimicrobial activity. It can against the growth of bacteria such as *E. coli, B. subtilis*, and *S. aureus* due to the curcuminoids in turmeric extracts [13].

It can also inhibit *Enterococcus faecalis* which is a gram-positive bacterium that reside in the human and animal gastrointestinal tracts. Furthermore, fungals that *C. Longa* can against are *Aspergillus parasiticus*, *Penicillium digitatum*, and *A. flavus* [14].

1.3.5 *Garcinia mangostana (Mangosteen)*

Garcinia mangostana or mangosteen belongs to the Clusiaceae family which is commonly known as “Queen of Fruits” as it has pleasant aroma, juicy and unique sweet taste [15]. The peel of mangosteen is normally used to cure various ailments such as fever, diarrhea, skin infections and inflammation. Moreover, the *G. mangostana* extracts have been reported as antibacterial, antioxidant and anti-inflammatory agents [16].

G. mangostana contains several bioactive constituents such as xanthones, phenolic acids, anthocyanins, and flavonoids [17]. Some of the main xanthones can be isolated from mangosteen extracts which are known as α-mangostin, β-mangostin, garcinone, gartanin, etc. However, α-mangostin is the major compound in mangosteen that has antioxidant properties.

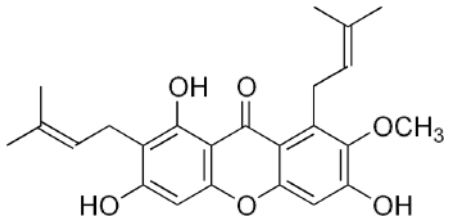


Figure 12: Structure of α-mangostin

From the several studies, the mangosteen pericarp showed antimicrobial activity which is effective against various bacteria such as *S. aureus*, *E. coli,* and *Salmonella typhi* [18]. Moreover, the α-mangostin has been reported to be a potent antibacterial agent against various bacterial strains [19].

1.4 Detection of antimicrobial agents by bioautography

Bioautography by thin-layer chromatography (TLC) is the most important method to use for the detection of antimicrobial compounds. In general, paper chromatography (PC) and thin-layer chromatography (TLC) are powerful tools for screening antimicrobial properties by the way of bioautography. The bioautography method is generally based on the disk diffusion technique wherein the antibacterial compound is transferred from the chromatography to the agar plate and the bacteria allow it to grow on the agar media and then observe the amount of space around every antibiotic disk as shown in the Figure 14. The bioautographic methods can be classified into three methods, including contact bioautography or agar diffusion, direct thin-layer chromatography bioautography, and agar overlay or immersion bioautography. These methods are used to detect antimicrobial agents in a compound mixture [20].

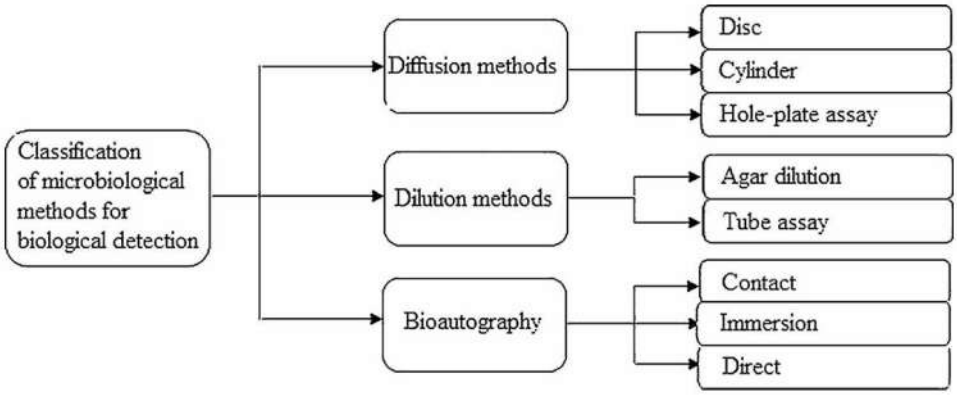


Figure 13: Classification of microbiological methods for biological detection

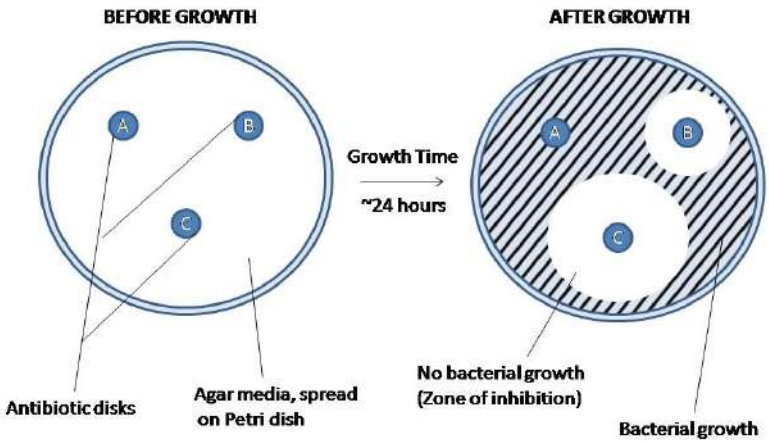


Figure 14: Schematic overview of the disk diffusion technique

1.4.1 Contact bioautography or agar diffusion

In agar diffusion or contact bioautography, the antimicrobial agent is spread from the TLC plate or developed paper to the inoculated agar. The TLC plate was placed face down on the vaccinated agar layer over time to activate the diffusion. Then, the chromatogram was removed and incubated in the agar layer. The zone of inhibition on the agar surface at the point in the plate chromatography is indicative of antimicrobial substances. According to Figure 15, the inoculation with microbial strains and chromatogram was placed down for allowing diffusion into agar media with incubation and then the result will perform the zone of inhibition to identify the antimicrobial. The period of incubation is estimated to be 24 hours or 1 day but depending on each of the substances [21]. The disadvantages of biological exposure are the difficulties in obtaining complete contact between agar and plate and adherence of the sorbent to the substrate. Another problem may arise due to the different diffusion of components, especially the insoluble components, from the chromatogram to the agar plate. This is a technique similar to the microbiologist used for antibiotics from microorganisms and different procedures have been used to improve isolation by the contact bioautography.

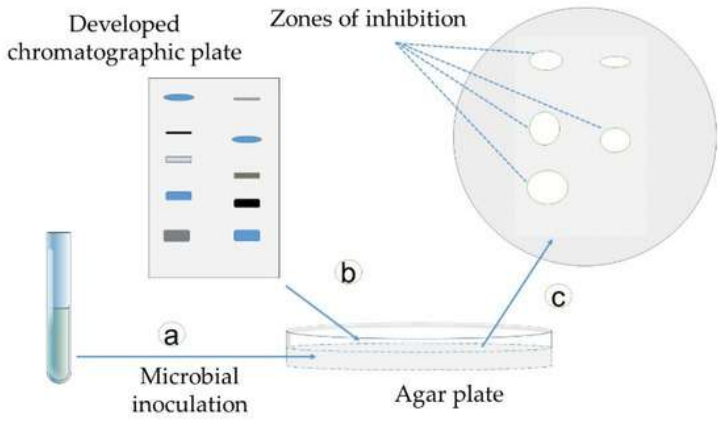


Figure 15: Schematic overview of contact bioautography

1.4.2 Direct TLC bioautographic detection

In direct thin layer bioautography, which developed from the TLC plate was spray or dipped into a bacterial suspension. The agar plate was incubated at 25°C for 48 hours under humid conditions. For visualizing the growth of microorganisms, tetrazolium salt is used. These salts are converted by dehydrogenases of living microorganisms to give formazan color [22]. The advantages of the method are speedy and versatility. However, it also points to difficulties in the quantitative interpretation of the results obtained. Influence of factors such as microorganisms tested, medium composition, pH, and solubility of samples in cultures on biological detection and in summary on screening methods for testing antimicrobial activity in natural products. According to Figure 16, the diagram of a direct bioautographic process begins from spraying with microbial strain or dipping the chromatogram in microbial strain and spraying with the tetrazolium salt with incubation under the moist condition for 48 hours or 2 days. Then, the result will exhibit the white zone of inhibition against the background which indicates antimicrobial. This is the direct thin layer bioautography assays that are especially suitable for selective detection which combines with the chromatography [23].

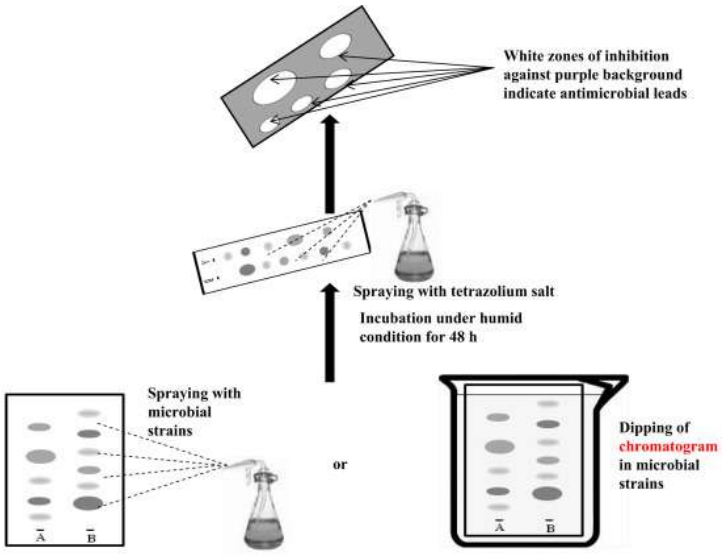


Figure 16: Diagram of the direct bioautographic process

1.4.3 Immersion or agar overlay bioautography

In agar overlay or immersion bioautography, it is the combination of contact and direct bioautography. The immersion bioautography assay has been used for yeasts and can also be applied to bacteria for testing the antimicrobial compounds. For gram-negative bacteria, an agar solution containing red bacteria is used. The red gel is incubated overnight at room temperature, and the inhibitory zone appears as a white or pale-yellow area on a red background. With other colorless microorganisms, the inhibitory zone of microbial growth is viewed. This can be seen with the help of reagents that detect dehydrogenase activity. According to the figure below, the diagram showed TLC plates are covered with molten agar containing molten and seeds. After agar solidification, the plates are incubated for 24 hrs at 37°C [24]. Compounds are eliminated from the chromatogram into the agar media. The agar layer remains on the surface of the TLC plate during curing, so the compound transfer is not limited to contact time. The inhibitory zone exhibits the reduced fluorescence signal visible on the agar layer in the area where the anti-active ingredient is on the TLC plate. The disadvantage of this method is the influence of the polarity of the compound on the adhesion known as the apolar compounds which can cause the agar layer and the TLC surface to disconnect.

Figure 17: Diagram of immersion bioautography process

1.5 The research objectives

There were many people infected and killed by the modern drug resistance problem. Therefore, Thai herbs which were one of the alternative drugs were assayed antimicrobial activity to solve drug resistance problems. In this study, we would like to screen the antibacterial activity of 10 Thai plants that were chosen from the Samyan Market as there were none previous studies. Therefore, the goal of this project is:

1.5.1 To screen the antibacterial activity of each 10 plant extracts by using the direct-TLC bioautography method

1.5.2 To identify the plant extracts that exhibit antibacterial properties

Chapter 2
Experimental

2.1 List of equipment and instrument

- Mortar and pestle
- Erlenmeyer flask
- Glass funnel
- Glass vial
- Dropper
- Graduated glass cylinder
- Evaporating flask
- Capillary tube
- TLC aluminum sheet gel 60
- Test tubes
- Rotary evaporator
- Sonicator bath
- UV lamp
- Laboratory round heating plate
- Chromatography column
- Micro-centrifuge tube
- Micropipette
- Micro vial
- Parafilm

2.2 List of chemicals and materials

2.2.1 Chemicals

- Methanol (MeOH)
- Acetone
- Ethyl acetate (EtOAc)
- Dichloromethane (CH_2Cl_2)
- Hexanes

2.2.2 Plant materials

Cymbopogon citratus (Lemongrass)

KF-7

Zingiber officinale (Ginger)

KF-01

Lycopersicon esculentum Mill (Tomato)

KF-8

Garcinia mangostana (Mangosteen)

KF-06

Andrographis paniculata (Green chiretta)

KF- 03

Centella asiatica (Gotu Kola)

KF-04

Curcuma longa (Turmeric)

KF-05

Piper nigrum (Black pepper)

KF-02

Allium sativum (Garlic)

KF-9

Allium ascalonicum (Shallot)

KF-10

2.3 Experimental procedure

2.3.1 Collection and preparation of plant materials

Ten Thai plants as shown in 2.2.2 were selected to examine the antimicrobial activities in this experiment. Each plant was used for about 200-400 g in the experiment. Most of them were

collected at the supermarket in the department store and some of them were collected at Samyan market in Bangkok, Thailand. All of the plants were cut into tiny pieces and air-dried at room temperature under the shade of sunlight for several days. Then, each plant was stored in the sterile Erlenmeyer flask and covered the mouth of the flask with foil to prevent the undesirable substances until further use.

2.3.2 Preparation of methanol crude plant extracts

Each type of plant sample was extracted with methanol in the Erlenmeyer flask. The methanol was poured into each flask and waited until each dried plant was soaked for about 2-3 days. However, the amounts of suitable solvents of each plant after 2-3 days as shown in Table 3 were lesser than previous as methanol was easily volatile. Afterward, the methanol crude plant extracts in each flask were filtered using sterile cotton. Then, the rotary evaporator was used at 50°C with reduced pressure to remove the solvent to have a higher concentration of crude methanol extracts. The overall process was repeated for 3 times continually and combined each of the plant extracts all together. Then, each extract was stored in the transparent vial and able to find a suitable mobile phase for TLC detection in further processes.

Plant materials	Weight of plants (g)	Amount of MeOH after 2-3 days (mL)
Cymbopogon citratus	200	500
Zingiber officinale	320	350
Lycopersicon esculentum Mill.	250	100
Garcinia mangostana	350	750
Andrographis paniculata	360	1000
Centella asiatica	325	1100
Curcuma longa	180	450

Piper nigrum	200	230
Allium sativum	200	500
Allium ascalonicum	200	230

Table 3: The weight of each plant and the average amount of Methanol after 2-3 days

2.3.3 Techniques for compound purification

2.3.3.1 Column chromatography

The column chromatography technique was used to separate compounds due to the ability to absorb the inequitable substances on the stationary phase. This technique used the silica gel to load into the column the suitable amount of silica gel with a suitable proportion of the amount required of each chemical substance and not allow the silica gel to dry by adding the solvent into the column chromatography. In this study, there were three plants including *L. esculentum Mill* (Tomato), *A. sativum* (Garlic) and *A. ascalonicum* (Shallot) that used column chromatography for separation. The suitable solvent which was methanol being prepared to separate the desired compounds and wait for the crude plant was exhausted from the column chromatography.

2.3.3.2 Thin layer chromatography (TLC)

Thin Layer Chromatography is one of the separation techniques which is used to isolate non-volatile mixtures. This technique is conducted on a sheet of aluminum which is covered with a thin layer of adsorbent material that is made up of silica gel. The TLC plate is being cut into the proper size to identify the distance of the solvent motion. The small size of the capillary is being used to transfer the chemical compound onto the TLC plate and find a suitable mobile solvent. Then, the TLC plate is put into a closed container followed by an enclosed container with a glass plate, and waits for the solvent to move up from the bottom to the solvent front. After the solvent reaches the top of the TLC plate, remove it and dry before checking the TLC with UV light at 256

nm wavelength. Then, dipping it into ammonium molybdate in 5% H_2SO/EtOH as this dipping agent helps to observe most of the compound on TLC plates such as organic compounds. Lastly, heat the TLC plate onto the hot plate for 1-2 minutes and notice the distance of the spot.

2.3.3.3 Determination of suitable mobile phase for TLC detection of each extract

In this experiment, five mobile phase systems were used to screen the appropriate mobile phase which arranged from the lowest to the highest polarity known as hexane, dichloromethane, ethyl acetate, acetone, and methanol. In this step, each of the crude methanol extracts achieved from the previous step (2.3.3.1) was spotted on the thin layer chromatography (TLC) plates followed by selecting the appropriate solvents. For choosing the suitable mobile phase, firstly began with low polar solvents. However, more than one solvent was used due to the higher ability to separate as they had different polarities. The suitable mobile phases or solvents will show the good separation of spots on the TLC plate.

2.3.4 Examination of antibacterial activity of plant extracts

2.3.4.1 Antibacterial activity test by the agar diffusion method

Antibacterial activity against *Staphylococcus aureus* ATCC 25923 was carried out by using the standard disk diffusion method [25]. *S. aureus* was grown on a tryptic soy agar overnight at 37 °C. Single colony was inoculated into tryptic soy broth, then the culture was incubated at 37 °C until the turbidity of the culture to a 0.5 McFarland standard. The culture broth was spread over the entire surface of the Mueller Hinton agar. The agar petri dish was air dried under sterile laminar flow before a 6- mm filter paper disk impregnated with suitable concentration/ volume of sample to be tested was placed on the surface of the agar. The agar plate was incubated at 37 °C for a total of 24 hours before the zone of inhibition was measured using a ruler or a caliper.

2.3.4.2 Bioautography detection in thin-layer chromatography

The direct thin-layer bioautography method is the developed TLC which is dipped in a suspension of a microorganism growing in a proper broth and incubated in a humid environment. The procedure in bioautographic method is similar to the one used in agar diffusion method as previously described. This protocol is based on agar-overlay technique. Sterile molten Mueller Hinton agar (MHA) was poured into a petri dish and allowed to solidify. Then, a dried TLC plate was placed face up on the agar base, and melt MHA inoculated with *S. aureus* (10^8 CFU/ml) was poured across the top of the TLC plate. The plate was allowed to solidify and incubate at 37 ^{o}C for 24 hours before the antibacterial zone appeared as a clear spot against a background of bacterial colonies was visualized.

Chapter 3
Results and discussion

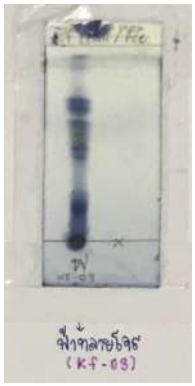
3.1 Preparation of plant extracts

Ten Thai herbal plants, *Z. officinale*, *P. nigrum*, *A. paniculata*, *C. asiatics*, *C. longa*, *G. mangostana*, *L. esculentum*, *C. citratus*, *A. sartivum* and *A. ascalonicum*, were selected to study the antimicrobial activity of their constituents on the basis of their biological properties. First of all, the MeOH crude extracts were prepared by soaking plant samples in MeOH, then removing the solvent under reduced pressure and drying under vacuum. All crude extracted appeared as dark brown gums.

3.1.1 Determination of suitable mobile phase conditions for TLC

Since the present study aimed to identify the antibacterial metabolites of *Thai plant* extracts using TLC bioautographic assay, the suitable mobile phases for separation of components in the extracts are necessary. This method uses the TLC plate to be the main equipment to perform the separation of the compounds of each crude plant extracts. Five organic solvents were used to make mixtures as mobile phases in this experiment, known as methanol (MeOH), acetone, ethyl acetate (EtOAc), dichloromethane (CH_2Cl_2), and hexanes. For the condition of mobile phases that were used to consider, the mobile phase providing the good separation of the compounds in each would be selected for further antimicrobial assay. As shown in Table 3.1, the suitable mobile phase for *Z. officinale* (KF-01) and *P. nigrum* (KF-02) extracts was 20% Acetone/Hexane, while that for *A. paniculata* (KF-03) was the same solvent mixture but in a different ratio which was 30% Acetone/Hexane. It could be identified that the composition of these three plant extracts were having similar polarity. Besides, it was found that a mixture of ethyl acetate and hexanes was good for metabolite separation of *C. asiatica* (KF-04)*, C. longa* (KF-05) and *G. mangostana* (KF-06), at 30%, 30% and 25% EtOAc in hexanes, respectively. On the other hand, *L. esculentum* Mill (KF-08) was the only fruit among others that had high polarity as it used 2% MeOH/EtOAc. However, the remaining three plant extracts which were *C. citratus* (KF-07), *A. sativum* (KF-09), and *A.*

ascalonicum (KF-10) were not having good separation in any mobile phase conditions, as well as they were not subjected to further TLC bioautographic assay because they did not show antibacterial activity in disc diffusion model shown in section 3.2.

Plant extracts	Mobile phase conditions	Suitable condition
Zingiber officinale (Ginger) and *Piper nigrum* (Black pepper)	10 % Acetone/ Hexanes 20% Acetone / Hexanes	20% Acetone/ Hexanes 
Andrographis paniculata (Green chiretta)	20% EtOAc/ Hexanes 20% Acetone/ Hexanes 30% Acetone/ Hexanes 35% Acetone/ Hexanes 40% Acetone/ Hexanes	35% Acetone/ Hexanes 

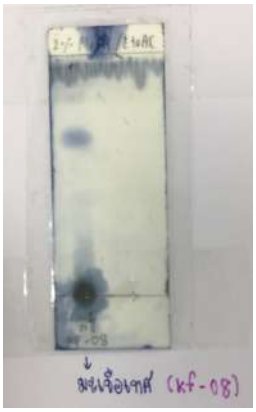
Centella asiatica (Gotu Kola) and *Curcuma longa* (Turmeric)	10% EtOAc/ Hexanes 20% EtOAc/ Hexanes 30% EtOAc/ Hexanes 50% EtOAc/ Hexanes	30% EtOAc/ Hexanes 
Garcinia mangostana (Mangosteen)	10% EtOAc/ Hexanes 30% EtOAc/ Hexanes 20% EtOAc/ Hexanes 25% EtOAc/ Hexanes	25% EtOAc/ Hexanes 
Lycopersicon esculentum Mill. (Tomato)	5% MeOH/ EtOAc 2% MeOH/ EtOAc 3% MeOH/ EtOAc	2% MeOH/ EtOAc 

Table 4: Determination of suitable mobile phase conditions for TLC

3.2 Antimicrobial assays of plant extracts

In the present study, in order to look at the difference of the results from the classical method, agar disk diffusion, and the directed TLC-bioautography, our crude extracts were subjected to both assays.

The antibacterial activity of the crude extracts was assayed against a resistant gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA). This strain is a type of microorganism in which approximately 30% of people present in their nose and 20% occur on the skin. It can cause various diseases such as bloodstream infections, lung infections and it also can occur from common to serious diseases. Moreover, the problem of these strains is because it becomes drug-resistant to all antibiotics against infections.

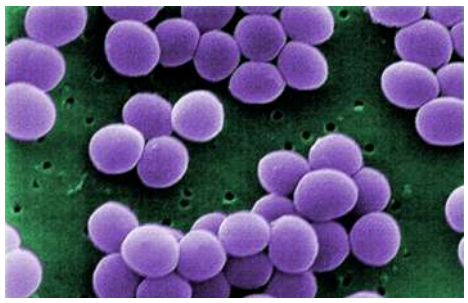


Figure 18: Methicillin-resistant *Staphylococcus aureus* (MRSA)

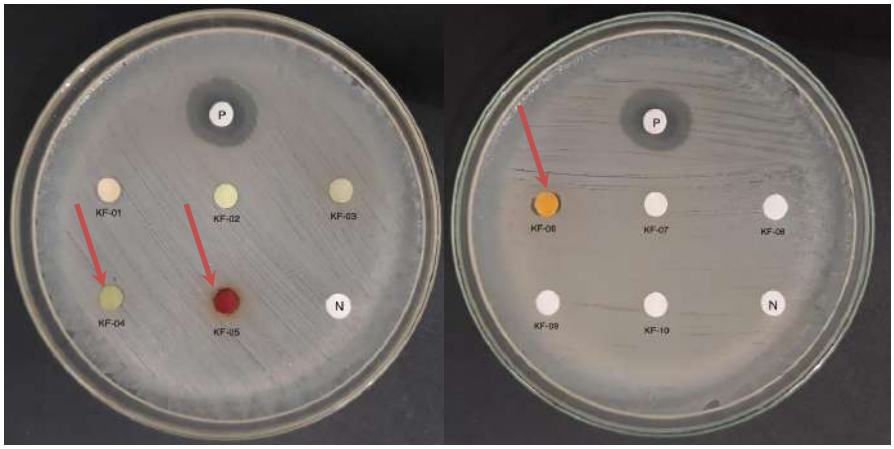
3.2.1 Agar disk diffusion assay

Agar disk diffusion test is one of the common antibacterial assays of the compound/the extracts on a specific microorganism. An agar plate is first spread with bacteria, then paper disks containing compounds/extracts are placed atop of it. The bacteria is then allowed to grow on the agar media and then observed for growth and effect of the antibiotic on it.

In this study, the agar disk diffusion method has separated into 2 trials with the different amounts of plant extracts, 300 and 700 ug respectively. The reason that we use this amount is

because it will show the different specific appearance of the inhibition zone as the amount of plant extracts increased. The paper disc was being used to test the antimicrobial activity by placing it on the agar surface in a Petri dish. In this experiment, the antibacterial activity of each plant was examined against *S. aureus* after incubating for 24 hours.

3.2.1.1 The first trial at 300 ug concentration of crude extracts

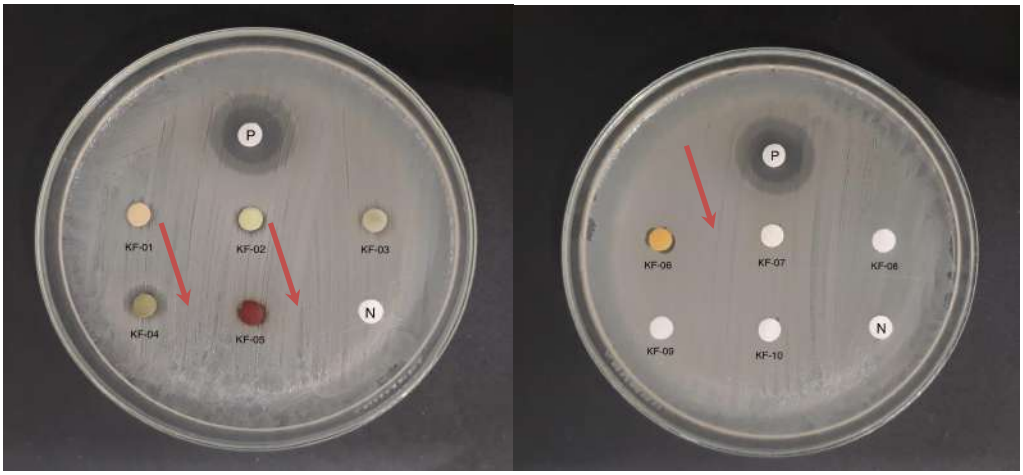


(P = positive control, N = methanol)

Figure 19: Antibacterial activity against *S. aureus* in the amount of 300 ug crude extract (incubated for 24 hours)

For the first trial, 300 ug of each crude extract was applied to examine the antibacterial activity against *S. aureus*. It has been observed that only *C. asiatica* (KF-04), *C. longa* (KF-05), and *G. mangostana* (KF-06) exhibited weak antimicrobial activity from the small size of clear zone. However, *G. mangostana* (KF-06) showed the highest inhibit zone compared with other crude extracts. The antimicrobial activity could be determined by measuring the diameter of the inhibition zone. Then, the measured diameter needs to be compared with a database of zone standards known as the positive control. Overall, each crude extract still had very weak antimicrobial activity.

3.2.1.2 The second trial 700 ug concentration of crude extracts



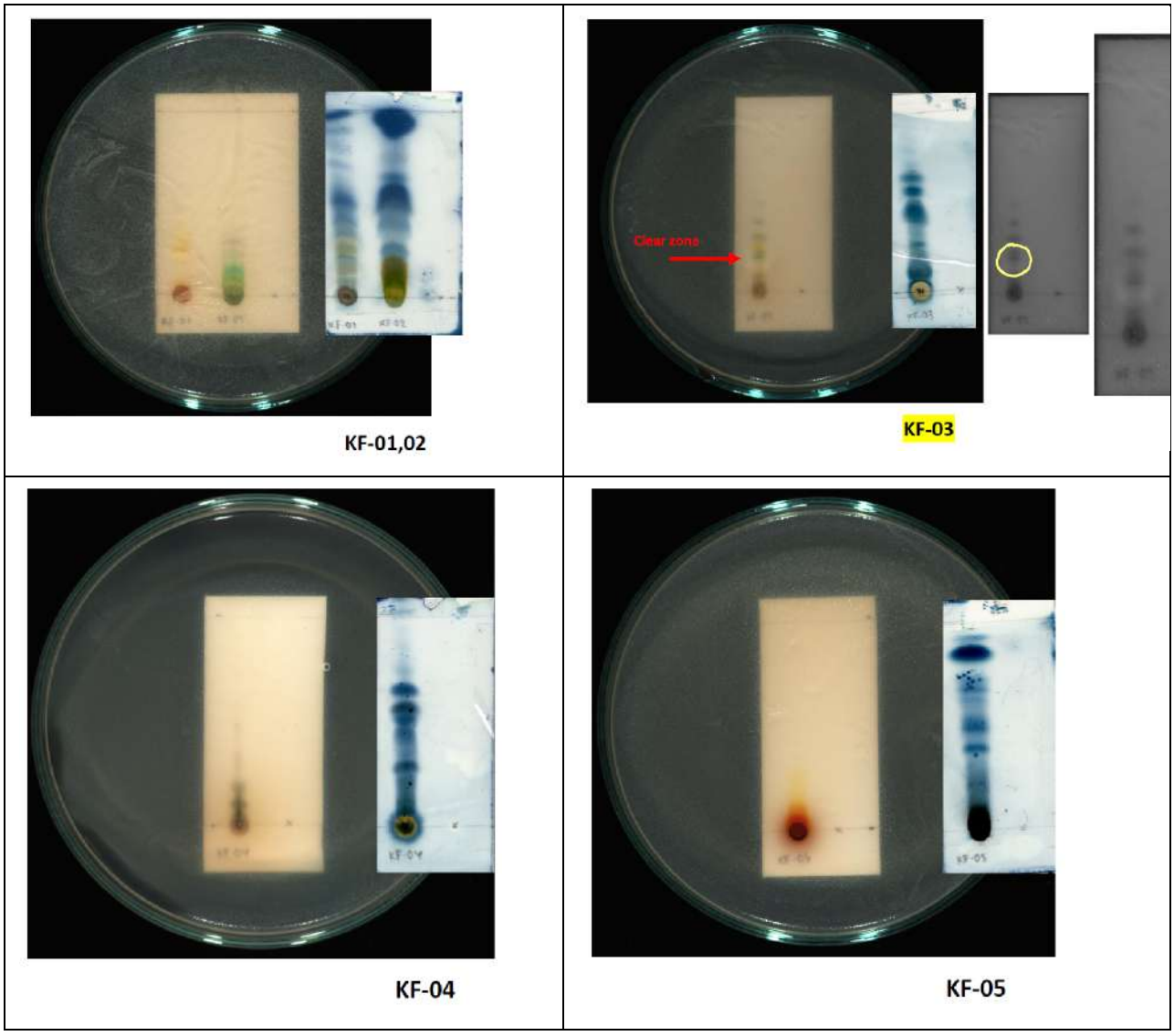
(P = positive control, N = methanol)

Figure 20: Antibacterial activity against *S.aureus* in the amount of 700 ug crude extract (incubated for 24 hours)

For the second trial, the amount of each crude extract was used higher than the first trial which was 700 ug. In this case, the antimicrobial activity of each plant against *S. aureus* has been observed more obviously which might due to the higher amount of crude extract.s There were 6 plant extracts that have been found to have antimicrobial agents as it showed the larger diameter of the inhibition zone. These were known as *Z. officinale* (KF-01), *P. nigrum* (KF-02), *A. paniculata* (KF-03), *C. asiatica* (KF-04), *C. longa* (KF-05), and *G. mangostana* (KF-06). However, the *C. asiatica* (KF-04) has been observed to have the highest antimicrobial activity against *S. aureus* with the amount of 700 ug crude extract.

3.2.2 Directed TLC-bioautography method

For the directed TLC –bioautography assay, seven crude extracts were run on TLC by loading 2 mg of extract on it, and run with suitable mobile phase for each extract from 3.1. The results are shown in Figure 3.4. Only compounds in *A. paniculata* (KF-03) and *G. mangostana* (KF-06) displayed the clear zone on TLC around some spots (as pointed by red arrow). This could let us know the metabolites displaying anti-MRSA when we do the isolation by column chromatography.



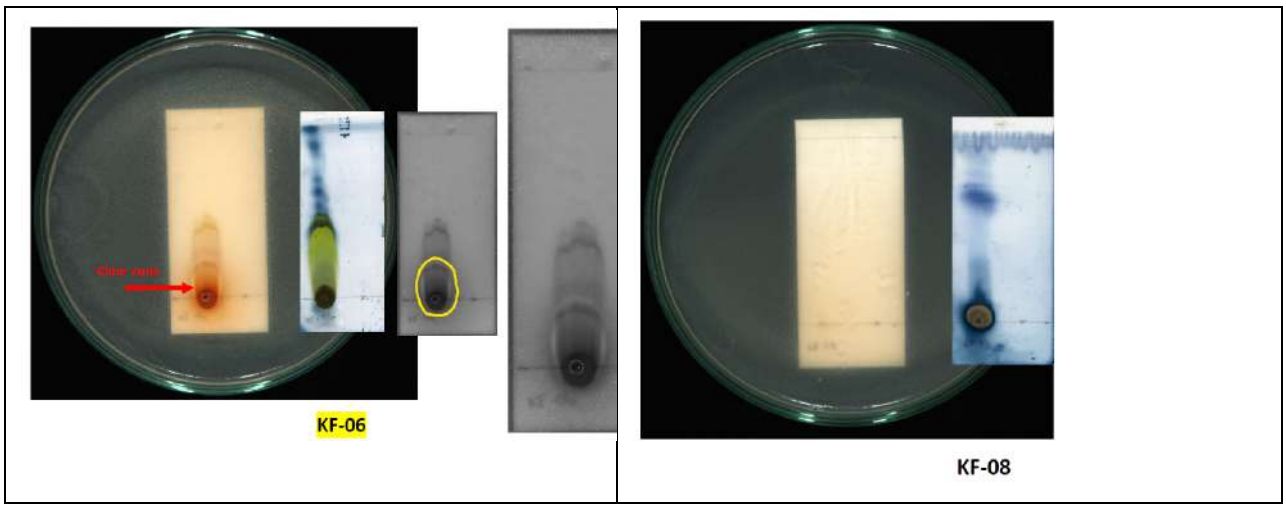


Figure 21: Antibacterial activity against *S. aureus* by directed TLC –bioautography assay (incubated for 24 hours)

3.3 Comparison of agar disk diffusion and TLC-bioautography assays

From the results, the difference has been observed, the results from agar disk diffusion told which crude extracts showed to be active, but when the extracts were subjected to TLC–bioautography, no active composition was observed. This might be because the test by agar disk the whole compounds in the extract was loaded at the same position and it might give the synergistic effect to display the activity. On the other hand, the test by TLC–bioautography containing each compound in a different place, so the real activity of each compound could be observed. Thus, the TLC–bioautography method gave more benefit because it helps to pursue the active compounds when we do isolation in quantitative and obtain the right compound displaying antibacterial activity.

Chapter IV
Conclusion

In this study, 10 Thai plants were selected to examine the antimicrobial activity by 2 different methods which were agar disc diffusion and directed TLC bioautography. The methanol was the solvent that was being used for crude plant extracts due to the high polarity of methanol would dissolve more bioactive compounds in the plant. The methanol crude plant extract was purified by evaporation. Finally, there were 7 out of 10 plant extracts that show the best separation of the spot on the TLC plate. Therefore, the bioactive compound of 7 plant extracts known as *Zingiber officinale* (KF-01), *Piper nigrum* (KF-02), *Andrographis paniculata* (KF-03), *Centella asiatica* (KF-04), *Curcuma longa* (KF-05), *Garcinia mangostana* (KF-06), and *Lycopersicon esculentum Mill* (KF-08) was identified by agar disc diffusion and directed TLC bioautography.

For the agar disc diffusion method, it was classified into 2 trials in the different amounts of crude extract which were 300 ug and 700 ug respectively. The antibacterial activity of each plant extract was investigated against *S.aureus* and incubated for 24 hours to observe the result. For 300ug of crude extract, KF-04 to KF-06 were shown antibacterial properties. However, KF-01 to KF-03 did not see any antibacterial activity. Therefore, the amount of crude extract was increased to 700 ug. Finally, the *Centella asiatica* (KF-04) has been observed to have the highest antimicrobial activity against *S.aureus*.

For the directed TLC bioautography method, *Andrographis paniculata* and *Garcinia mangostana* have been performed to have clear zones on the TLC plate to represent antimicrobial. However, the highest inhibition zone was presented in *Curcuma longa* on a Thin-layer chromatography plate.

However, some of the crude extract that was active in disc diffusion may not be observed in the TLC-bioautography as the crude extract was loaded at the same position instead of showing clear separation on the TLC plate. Therefore, TLC-bioautography was provided more benefits as it can achieve the correct compound that shows antibacterial activity.

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