EFFECTS OF THE ZINGIBERACEAE SPICE EXTRACTS ON GROWTH AND MORPHOLOGICAL CHANGES OF FOODBORNE PATHOGENS

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ผลของสารสกัดจากเครื่องเทศตระกูลขิงข่า ต่อการยับยั้งการเจริญของจุลินทรีย์และการเปลี่ยนแปลงสัณฐานวิทยาของแบคทีเรียที่พบในอาหาร

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

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สารสกัดหยาบจากเครื่องเทศ 4 ชนิดในตระกูล Zingiberaceae ได้แก่ ข่า ขิง ขมิ้น และกระชาย ได้นำมาทำการหาค่าความสามารถในการยับยั้งการเจริญเติบโตของจุลินทรีย์ และประเมินการเปลี่ยนแปลงทางสัณฐานวิทยาของเซลล์ สารสกัดหยาบจากเครื่องเทศเตรียมจากตัวทำละลายได้แก่ น้ำ เมทานอล เอทานอล และเอธิลอะซีเตท และทดสอบผลของการยับยั้งตัวแทนของเชื้อแบคทีเรีย 2 กลุ่ม ได้แก่ *Staphylococcus aureus* และ *Escherichia coli* สารสกัดจากข่าด้วยเมทานอล และเอทานอล ได้นำไปทดสอบกับเชื้อจุลินทรีย์อื่นๆเช่น *Staphylococcus epidermidis*, *Streptococcus lactis*, *Bacillus cereus*, *Bacillus megaterium*, *Salmonella* sp., *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae* **แ ล ะ** Methicillin Resistant *Staphylococcus aureus* วิธีการ broth dilution เป็นวิธีการซึ่งใช้ในการทดสอบผลของการยับยั้งเชื้อแบคทีเรียของสารสกัดจากน้ำ พบว่าสารสกัดจากขิงด้วยน้ำที่ความเข้มข้น 5.00 มิลลิกรัมต่อมิลลิลิตร ให้ผลดีที่สุดต่อการยับยั้งการเจริญเติบโตของตัวแทนของเชื้อแบคทีเรีย 2 กลุ่ม ได้แก่ *S. aureus* และ *E. coli* และสารสกัดจากเครื่องเทศชนิดอื่นที่ความเข้มข้นเดียวกันคือที่ 5.00 มิลลิกรัมต่อมิลลิลิตรให้การยับยั้งการเจริญเติบโตของแบคทีเรียทั้งสองชนิด วิธีการ agar disc diffusion ถูกใช้เพื่อตรวจสอบคุณสมบัติการยับยั้งการเจริญเติบโตของเชื้อของสารสกัดจากเมทานอล เอทานอล และเอธิลอะซีเตท สารสกัดจากข่าด้วยเมทานอล เอทานอล และเอธิลอะซีเตท ให้ผลในการยับยั้งเชื้อ *S. aureus* ได้ดีที่สุดเมื่อเปรียบเทียบกับสารสกัดจากเครื่องเทศชนิดอื่น จากการวิเคราะห์ปริมาณความเข้มข้นต่ำสุดของสารสกัดจากข่าด้วยเมทานอลที่สามารถยับยั้งการเจริญของเชื้อ *S. aureus* (Minimum inhibitory concentration) คือ 0.800 มิลลิกรัมต่อมิลลิลิตร และความเข้มข้นต่ำสุดที่สามารถฆ่าเชื้อ *S. aureus* (Minimum bactericidal concentration) คือ 1.600 มิลลิกรัมต่อมิลลิลิตร ความเข้มข้นต่ำสุดของสารสกัดจากข่าด้วยเอทานอลที่สามารถยับยั้งการเจริญของเชื้อ *S. aureus* คือ 0.325 มิลลิกรัมต่อมิลลิลิตร และความเข้มข้นต่ำสุดที่สามารถฆ่าเชื้อ *S. aureus* คือ 1.300 มิลลิกรัมต่อมิลลิลิตร นอกจากนี้สารสกัดจากข่ายังสามารถยับยั้งการเจริญเติบโตของเชื้อ *Staphylococcus epidermidis* และ *Saccharomyces cerevisiae* สามารถยับยั้งการเจริญเติบโตของเชื้อ *Bacillus cereus* และ *Bacillus megaterium* ได้เพียงเล็กน้อย ในขณะที่ไม่สามารถยับยั้งการเจริญเติบโตของเชื้อแกรมลบบางชนิด ได้แก่ *Salmonella* sp., *Enterobacter aerogenes* และ *Pseudomonas aeruginosa* ได้เลย

จากการวิเคราะห์องค์ประกอบทางเคมีในสารสกัดจากข่าด้วยเมทานอล และเอทานอล ด้วยการใช้วิธีทินแลเยอร์โครมาโตกราฟี ก๊าซโครมาโตกราฟี-แมสสเปรคโตเมทรี และนิวเคลียร์แมกเนติกเรโซแนนซ์ พบสารประกอบในสารสกัดจากข่าด้วยเมทานอล และเอทานอล ได้แก่ ดีแอล อะซีทอกซีคาวิคอล อะซีเตท (D, L-1'-acetoxychavicol acetate) พาราคูมาริล ไดอะซีเตท (*p*-coumaryl diacetate) อะซีทอกซียูจีนอล อะซีเตท (acetoxyeugenol acetate) และ เมื่อนำสารสกัดจากข่าด้วยเมทานอลและเอทานอล มาทำการวิเคราะห์หากลไก และบริเวณที่สารสกัดเข้าทำปฏิกิริยากับส่วนประกอบต่างๆของเซลล์เบื้องต้นด้วยการส่องด้วยกล้องจุลทรรศน์อิเลคตรอนแบบส่องผ่าน การตรวจสอบปฏิกิริยาการทำงานของเอนไซม์อาร์จินีน ไดไฮโดรเลส และยูรีเอส การวัดปริมาณการนำเข้าของสารเรืองแสงโพรพิเดียม ไอโอไดด์ เมื่อสังเกตุด้วยกล้องจุลทรรศน์อิเลคตรอน แบบส่องผ่าน พบว่าสารสกัดจากข่าด้วยเมทานอล ก่อให้เกิดการรวมตัวของไซโตพลาสซึม และการขาดหายของไซโตพลาสซึมในเซลล์ สารสกัดจากข่าด้วยเอทานอล แสดงการเปลี่ยนแปลงความแข็งแรงของเยื่อหุ้มเซลล์และผนังเซลล์ สารสกัดทั้งสองชนิดสามารถชะลอการใช้น้ำตาลและยับยั้งการทำงานของเอนไซม์อาร์จินีน ไดไฮโดรเลส และเอนไซม์ยูรีเอส ภายใน 48 ชั่วโมง และ 24 ชั่วโมงตามลำดับ *S. aureus* ซึ่งถูกกระทำด้วยสารสกัดแสดงการนำเข้าของโพรพิเดียม ไอโอไดด์เพิ่มขึ้น ซึ่งบ่งชี้ว่ามีการนำเข้าสารผ่านเยื่อหุ้มเซลล์แบคทีเรียเพิ่มขึ้น

จากผลการทดลองเหล่านี้พบว่าสารสกัดหยาบจากข่าด้วยเมทานอลและเอทานอลสามารถยับยั้งการเจริญเติบโตของเชื้อแบคทีเรียแกรมบวกบางชนิดและยีสต์ เป็นสาเหตุให้เกิดการรบกวนความแข็งแรงของผนังเซลล์และเยื่อหุ้มเซลล์ และรบกวนการทำงานของเอนไซม์ซึ่งเป็นไปตามสมมติฐานที่คาดไว้

สาขาวิชา เทคโนโลยีอาหาร ลายมือชื่อนักศึกษา ศิราวรรณ อ่านเจริญดอกดาว

ปีการศึกษา 2548 ลายมือชื่ออาจารย์ที่ปรึกษา

JIRAWAN OONMETTA-AREE : EFFECTS OF THE ZINGIBERACEAE SPICE EXTRACTS ON GROWTH AND MORPHOLOGICAL CHANGE OF FOODBORNE PATHOGENS. THESIS ADVISOR : ASST. PROF. PIYAWAN GASALUCK, Ph.D., 137 P.P. ISBN 974-533-463-4

ZINGIBERACEAE/*STAPHYLOCOCCUS AUREUS*/ANTIMICROBIAL ACTIVITY/D, L-1'-ACETOXYCHAVICOL ACETATE/MEMBRANE INTEGRITY

Crude extracts of four spices in the Zingiberaceae (galangal, ginger, turmeric and krachai) were evaluated in their ability to inhibit the growth of test microorganisms and to assess cell morphological changes. Crude extracts were prepared by distilled water-, methanol-, ethanol- or ethyl acetate-soluble extraction and tested for their inhibitory effects against representative foodborne pathogens—*Staphylococcus aureus* and *Escherichia coli*. They were also tested galangal methanol and ethanol against other microorganisms both gram positive and gram negative bacteria such as *Staphylococcus epidermidis*, *Streptococcus lactis*, *Bacillus cereus*, *Bacillus megaterium*, *Salmonella* sp., *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae* and Methicillin Resistant *Staphylococcus aureus*. The broth dilution method was used in testing the antibacterial effects of the lyophilized water extract. By using a broth dilution method, the lyophilized ginger water-extracted at a concentration of 5.00 mg/ml was found to have strong inhibitory effects on the growth of representative foodborne pathogens—*Staphylococcus aureus* and *Escherichia coli*, whereas the same extracts of other spices at concentration of 5.00 mg/ml marginally inhibited the growth of both bacteria. The dried spices methanol, ethanol, and ethyl acetate extracts were determined and ethyl acetate galangal extracts were found to have the strongest inhibitory effects on

the growth of *S. aureus*. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) against *S. aureus* of dried galangal methanol and ethanol extracts were 0.800 mg/ml and 0.325 mg/ml and 1.600 mg/ml and 1.300 mg/ml, respectively. The dried galangal methanol and ethanol extracts also inhibited the growth of *Staphylococcus epidermidis* and *Saccharomyces cerevisiae*. The extracts were less antimicrobial activity against *Bacillus cereus* and *Bacillus megaterium* and there were no antimicrobial activity against some gram negative bacteria: *Salmonella* sp., *Enterobacter aerogenes* and *Pseudomonas aeruginosa*. Chemical analysis of the galangal methanol and ethanol extract compounds were D, L-1'-acetoxychavicol acetate (ACA), *p*-coumaryl diacetate, 1'-acetoxyeugenol acetate, and trace compounds, which were determined by using TLC, GC-MS and NMR.

The modes of action and target sites of compounds in crude galangal methanol and ethanol extracts against *S. aureus* were investigated by using transmission electron microscopy (TEM), determining arginine dihydrolase, urease activity and measuring an uptake of fluorescent nuclear stain propidium iodide. The dried galangal methanol extract caused cytoplasm coagulation and some lack of cytoplasm. Electron micrographs showed that the dried galangal ethanol extract showed alteration in membrane's integrity with plasma membranes and cell walls were disrupted and damaged resulting in a release of cell materials from cytoplasm. The extracts were also able to delay the sugar utilization, inhibit arginine dihydrolase and urease activities within 48 h and 24 h, respectively. *S. aureus* treated with crude extracts showed an increase in the uptake of the nucleic acid stain propidium iodide indicating an increase of cytoplasmic membranes permeability.

These results found that the crude galangal methanol and ethanol extracts were able to inhibit the growth of some gram positive bacteria and yeast, causing interfere the

cell wall and cell membrane integrity and enzymatic function that relevant to the hypothesis.

School of Food Technology Student's Signature Jiraporn Dommeharee

Academic Year 2005 Advisor's Signature

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Jirawan Oonmetta-aree

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LIST OF ABBREVIATIONS

SFP	=	Staphylococcal food poisoning
SEs	=	Staphylococcal enterotoxins
TNase	=	Thermonuclease
DNase	=	Deoxyribonuclease
DNA	=	Deoxyribonucleic acid
RNA	=	Ribonucleic acid
SEB	=	Staphylococcal enterotoxin B
EPEC	=	Enteropathogic *Escherichia coli*
ETEC	=	Enterotoxigenic *Escherichia coli*
LT	=	Heat labile toxin
ST	=	Heat stable toxin
EAEC	=	Enteroaggregate *Escherichia coli*
EHEC	=	Enterohemorrhagic *Escherichia coli*
LPS	=	Lipopolysaccharides
PMF	=	Proton motive force
CFDA-SE	=	Carboxyfluorescein diacetate succinimidyl ester
NA	=	Nutrient agar
NB	=	Nutrient broth
TSB	=	Trypticase soy broth
MHB	=	Muller Hinton broth
CFU	=	Colony forming unit
MIC	=	Minimum Inhibitory Concentration
MBC	=	Minimum Bactericidal Concentration

LIST OF ABBREVIATIONS (Continued)

GMet	=	Galangal methanol extract
GEt	=	Galangal ethanol extract
LB	=	Luria-Bertani medium
TLC	=	Thin Layer Chromatography
GC-MS	=	Gas Chromatography-Mass Spectrophotometry
Mu	=	Mass unit
NMR	=	Nuclear Magnetic Resonance
$CDCl_3$	=	Deuterate chloroform
ACA	=	D, L-1'-Acetoxcychavicol acetate
TEM	=	Transmission electron microscopy
PI	=	Propidium iodide

CHAPTER I

INTRODUCTION

Foodborne pathogens are a group of microorganisms that are capable of giving rise to foodborne diseases in human. A foodborne disease is defined by the World Health Organization (WHO) as 'a disease of an infectious or toxic nature caused by, or thought to be caused by the consumption of food or water' (Adam and Moss, 1995). Bacterial foodborne pathogens have had a tremendous impact on public opinion as the most critical food-related risk factor affecting consumers. Since the early 1980s, numerous outbreaks involving foodborne pathogens; *Salmonella* spp., *Listeria* spp. and *Escherichia coli* (*E. coli*) have demonstrated the importance of food safety, including, proper food handling and cooking procedures at home, food service establishments and commercially available products (Muriana, 1996). Some of the food spoilage and foodborne pathogens are often found in the food processing environments, especially when dealing with raw animal products. They cause problems that are not yet under adequate control despite of the range of strong preservation techniques available (e.g., freezing, sterilizing, drying and adding preservatives). In fact, food manufacturers are increasingly relying on milder preservation techniques to comply with the consumers' demand for foods with a more natural appearance and nutritious quality than those achieved by the robust techniques. In addition, consumers are increasingly refusing foods prepared with preservatives of chemical origin to achieve sufficiently long shelf life and a high degree of safety with

respect to foodborne pathogenic microorganisms. Many researchers, therefore, attempt to look for new natural preservatives that sufficiently assure the safety of the food products. Spices are one of the natural preservatives under consideration because they contain compounds particularly the essential oils that have some antimicrobial activity to inhibit bacteria, yeasts, and molds. Spices have traditionally been found to be useful for food preservation as well as for medicinal purposes (Burt, 2004; Marino, 1999).

In Asian countries, various spices, herbs and condiments have been utilized as important ingredients for the preparation of unique gastronomic dishes since ancient times. Many of the commonly consumed spices in the world such as ginger, cinnamon, clove, garlic, etc. originated from Asia. Spices are not only used for food flavoring but also to help maintain health and promote wellness.

The Zingiberaceae Family such as galangal, ginger, turmeric and krachai are the main spices in Thai cuisine. Several studies showed that the essential oils in these spices: zingiberene and gingerol in ginger, methyl-cinnamate and eugenol in galangal, turmerone in turmeric all possess inhibitory effects on the growth of some bacteria. These compounds including flavonoids have antimicrobial and antioxidative properties. The essential oils from both fresh and dried rhizomes of galangal have antimicrobial activities against bacteria, fungi, yeast and parasite (Fransworth and Bunyapraphatsara, 1992). Janssen and Scheffer (1985) have reported that terpinen-4-ol, one of the monoterpenes in the essential oil from fresh galangal rhizomes, contains an antifungal activity against *Trichophyton mentagrophytes*. Acetoxychavicol acetate (ACA), a compound isolated from an n-pentane/ diethyl ether-soluble extract of dried galangal rhizomes, is active against some bacteria and many dermatophyte species (Janssen and Scheffer, 1985). In addition, the ability of ACA to act as an anti-ulcer (Mitsui, Kobayashi, Nagahori and

Ogiso, 1976) and anti-tumor agents (Itokawa, Morita, Sumitomo, Totsuka and Takeya, 1987) as well as an inhibitor of chemically induced carcinogenesis is an event (Murakami, Ohura, Nakamura, Koshimizu and Ohigashi, 1996; Ohnishi et al., 1996).

Spices possess a number of antimicrobial activities. These spices are used as protection against the growth of pathogens, particularly bacteria and molds, which produce bacterial toxins and mycotoxins causing food poisoning and spoilage. Their compounds have different modes of action on microbial cells. They could disrupt the cell membrane by increasing permeability and changing the bacterial metabolism, causing the above mentioned organisms to become susceptible to the antimicrobial effect of spices.

Mechanisms of being natural preservative agents for the purpose of antimicroorganisms or extending shelf life of foods are very interesting, and the information on the mode of action of Thai spices is restricted to merely a few spices (Gould, 1989; Meena, 1994). This study is performed to monitor and investigate the effects of some spices in the Zingiberaceae Family on the growth and the morphological changes against selected foodborne pathogens.

1.1 Research objectives

1. To investigate antimicrobial activities in methanol and ethanol extracts of selected spices in the Zingiberaceae Family against foodborne pathogens.
2. To study the primary effect(s) of selected spice extracts on morphology of foodborne pathogens.
3. To determine and analysis the main component of the extracts of the selected spices.

1.2 Research hypothesis

1. The crude extracts of the Zingiberaceae spice extracts can inhibit microbial growth by causing morphological damages to gram-positive bacteria observable Transmission Electron Microscopy (TEM).

2. The crude extracts of galangal interfere with the metabolic and/ or enzymatic function of the cells.

1.3 Scope and limitation of the study

The crude aqueous, ethanol, methanol and ethyl acetate extracts from root and rhizomes of some members of Zingiberaceae family will be selected as natural antimicrobial agents for inhibiting the growth of selected foodborne pathogens. These extracts will be determined as minimum.

In this study, some spices in the Zingiberaceae Family namely galangal, ginger, turmeric and krachai were selected as candidates for natural antimicrobial agents. Crude extracts of those spices were prepared from their roots and/ or rhizomes in various solvents, including ethanol, methanol, ethyl acetate and distill water to test for their antimicrobial activities. To investigate the inhibitory effects of these spice crude extracts on certain foodborne pathogens, by using broth dilution and agar disc diffusion assay. The microbes used as representatives of commonly found foodborne pathogens were *S. aureus* and *E. coli.* The best antimicrobial activity of selected crude spice extract compounds was determined and identified by TLC, GC-MS and NMR. Effects of crude galangal methanol and ethanol extracts on bacterial cell by using TEM and biochemical assay were observed the primary and main target site.

1.4 Expected results

1. To gain knowledge of the dose-response of spice extract on selected foodborne pathogens.
2. To understand the primary and main targets of spice extract on bacterial cell.
3. To know the main constituent of spice extract which is an active compound.

1.5 References

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CHAPTER II

LITERATURE REVIEWS

2.1 Foodborne pathogens

Microorganisms are important since they can cause diseases and food spoilage as well as can produce food and food ingredients. Human illness from the consumption of foods contaminated with factors other than poisons or chemical toxic agents was recognized long before the understanding of the role of pathogens in foodborne disease. In the Middle Ages, several mass food poisoning incidences in Europe from the consumption of grains infested with toxin-producing fungi were recorded. *Salmonella* spp. and *Staphylococcus aureus*, due to the high incidence of salmonellosis and staphylococcal poisoning, and *Clostridium botulinum*, due to the high fatality rate from botulism, were isolated from foods incriminated with foodborne disease before the 20th century (Ray, 1996). Many bacterial species, some molds and viruses, but not yeasts, involve in foodborne diseases. Most bacteria, molds and yeasts, due to their ability to grow in foods are potentially capable of causing food spoilage. Microbial contamination in food can contaminate food during various stages of handling from food processing to consumption, which can cause large economic losses. Foods of the animal origin are associated more with foodborne disease than foods of the plant origin. On the basis of types of illnesses, pathogenic effects can be divided into three groups: (1) intoxication involving food poisoning in which the organism grows in the food and releases a toxin from the cells. These can be either enterotoxin

affecting the gut as caused by Staphylococcus food poisoning; (2) infection involves food poisoning caused by the ingestion of live organisms when the organisms grow in the gastrointestinal tract and cause diseases such as Salmonellosis; and (3) toxicoinfection involves food poisoning caused by the ingestion of a large number of viable cells of some pathogenic bacteria via contaminated food and water. Generally, the bacterial cells either sporulate or die and release toxins to produce the symptoms. This normally requires pathogens to be alive and present in large numbers when the contaminated food is consumed. These bacteria are *Cl. perfringens* gastroenteritis, *Bacillus cereus* gastroenteritis (Garbutt, 1997; Ray, 1996).

2.1.1 Staphylococcal food poisoning; Staphylococcal gastroenteritis

Staphylococcal food poisoning (SFP) or food intoxication syndrome was first recognized as a separate genus in 1894 by Denys, J. (Bergdoll, 1989; Jay, 2000). Since that time they have been subdivided into many species, with the most important being *S. aureus*. Staphylococcal food poisoning, caused by toxins of *S. aureus*, is considered to be one of the most frequently involved in staphylococcal diseases. This foodborne disease is a common cause of gastroenteritis worldwide. Unlike many other forms of gastroenteritis, SFP is not due to the ingestion of live microorganisms but rather results from one or more performed staphylococcal enterotoxins (SEs) in staphylococci-contaminated food. This form of food poisoning is considered to be intoxication because it does not require growth of the bacterium in the host (Jablonski and Bohach, 2001). Enterotoxins are produced only by some staphylococcal species and strains. Enterotoxin production is believed generally to be associated with *S. aureus* strains that produce coagulase and thermonuclease (TNase), many species of Staphylococcus that produce neither coagulase

nor TNase are known to produce enterotoxins (Jay, 2000).

2.1.1.1 Characteristics of *Staphylococcus aureus*

S. aureus is gram-positive and catalase-positive cocci, which is morphologically similar the genus Micrococcus but grows anaerobically and demonstrates facultative anaerobic metabolism, in contrast to the strictly respiratory metabolism of aerobic micrococci. Colonies are smooth, raised, glistening, circular, entire and translucent and single colonies may obtain a size of 6-8 mm in diameter on nonselective media used for the propagation of staphylococci (Kloos and Schleifer, 1975; Sneath, Mair, Sharpe, and Holt, eds., 1984). Colonial pigment is variable; however, most strains demonstrate some degree of colony or cell pigmentation, ranging from gray or gray-white with a yellowish tint, through yellow-orange to orange.

2.1.1.2 Growth and its control

Most strains ferment mannitol and produce coagulase, thermonuclease and hemolysin but differ in their sensitivity to bacteriophages. The cells are killed at 66°C in 12 min and 72°C in 15 s. They can ferment carbohydrates and also cause proteolysis by the extracellular proteolytic enzymes (Ray, 1996). They are mesophiles with growth temperature range of 10-45°C, with optimum temperature at 30-37°C. Upper limit of growth can be extended above 44°C by addition of NaCl, monosodium glutamate (MSG) and soy sauce. Optimum pH for growth is 7.0-7.5. Minimum pH for growth is 4.2, maximum pH is 9.3. Growth was inhibited in the presence of 0.1% acetic acid (pH 5.1). The low water activity at which *S. aureus* grows is particularly significant. The organism

is resistant to drying and may grow and produce enterotoxins in foods with a_w as low as 0.85. Growth is good at NaCl concentrations up to 10% and is relatively poor at 15%. Optimum a_w for growth is 0.99. Its ability to grow at low a_w means that it has a competitive advantage on low a_w foods (Sneath et al., eds., 1984; ESR, www, 2001).

S. aureus produced aerobically and anerobically acid from glucose, lactose, maltose and mannitol. Acid is produced aerobically from fructose, galactose, mannose, ribose, sucrose, trehalose, turanose and glycerol. Esculin and starch are not usually hydrolyzed. Hyaluronic acid may be hydrolyzed by hyaluronic acid lyase. Ammonia is produced from arginine by arginine dihydrolase and from urea by urease (Krasuski, 1981). Lysine and ornithine are not decarboxylated. Heat-resistant staphylococcal nuclease (thermonuclease, TNase, DNase, phosphodiesterase) having endo- and exonucleolytic properties, which can cleave either DNA or RNA. This nuclease may be used as an indicator for the detection of *S. aureus* in foods.

In general, enterotoxin production tends to be favored by the optimum growth conditions of pH, temperature, a_w etc. Staphylococcal enterotoxins have been reported to appear in cultures as early as 4-6 hours and to increase proportionately through the stationary phase and into the transitional phase. Enterotoxin production has been shown to occur during all phases of growth, although earlier studies revealed that with strain S-6, 95% of staphylococcal enterotoxin B (SEB) was released during the latter part of the log phase of growth. In some study, thermonuclease (TNase) was detectable before enterotoxin production (Jay, 2000).

2.1.1.3 Sources of Staphylococcal food contamination

Staphylococci are ubiquitous, although their major reservoirs are human and animals. Humans are the main reservoir for staphylococci involved in human disease. The percentage of healthy humans who carry staphylococci at any one time varies from 30 to 50%, with 15-35% being persistent carriers (Williams, 1963). Colonized individuals are carriers and provide the main source for the dissemination of staphylococci and to food. Dissemination of *S. aureus* among humans and from humans to food can occur by direct contact, indirectly by skin fragments, or through respiratory tract droplet nuclei. Food poisoning outbreaks occur from foods that have been contaminated by healthy staphylococcal carriers such as the outbreak from packaged ham that contaminated from person who packaged it.

It is well known that animals can also develop staphylococcal infections. Mastitis is a typical example of a staphylococcal disease occurring in animals, particularly cows (Olson, Casman, Baer, and Stone, 1970) and sheep (Guitierrez, Menes, Garcia, Moreno, and Bergdoll, 1982). Colonization of animals by this bacterium is also a public health concern because it may result in contamination of food and milk with *S. aureus* before or during processing. Animal carriers are of less importance in the staphylococcal food poisoning than human carriers because most food poisoning outbreaks result from contamination by food handlers. This is because most outbreaks result from staphylococci growing in foods after the food has been heated. Heat treatment generally will destroy any staphylococci present before there is adequate growth for enterotoxin production. Enterotoxin produced in the food by staphylococci introduced onto the food after heating.

Foods commonly associated with staphylococcal food poisoning are meat (beef, pork and poultry) and meat products (ham, salami, hotdogs), salads (ham, chicken, potato),

cream-filled bakery products and dairy products (cheese). Raw meats are usually contaminated with staphylococci, but these organisms usually are destroyed when the meat is processed or heated before eating. Furthermore, even though raw foods such as uncooked meat and unpasteurized milk may be contaminated with staphylococci, these foods also are contaminated with other organisms that inhibit the growth of the staphylococci because they are not good microbial competitors. The only time that unpasteurized milk is likely to be involved in food poisoning is when the milk contains a large inoculum, which can occur if it comes from a mastitic animal. Foods which present the greatest risk are those in which the normal flora has been destroyed (e.g. cooked meats) or inhibited (e.g. cured, salted meats). Staphylococci grow well in cooked foods, which are high in protein, sugar or salt, low in acid, or food with moist fillings (ESR, www, 2001).

2.1.1.4 Characteristics of disease

SFP is usually described as a self-limiting illness presenting with emisis following a short incubation period at 30 min to 7 hours after eating food containing enterotoxins (Jablonski and Bohach, 2001). Other common symptoms usually include nausea, vomiting, abdominal cramps, diarrhea, headaches, muscular cramping, and/ or prostration. In mild cases, there may be nausea and vomiting without diarrhea, or cramps and diarrhea without vomiting. Recovery is rapid, usually within 2 days.

According to the FDA, effective doses of Staphylococcal enterotoxins may be achieved when populations of *S. aureus* are greater than 10^5 cells/ g of contaminated food (Bender, Hedberg, Besser, Boxrud, MacDonald, and Osterholm, 1997). The toxin level is less than 1.0 μg of toxin in contaminated food can produce symptoms. Small numbers of

S. aureus in food are not a direct hazard to health.

2.1.2 Foodborne Gastroenteritis Caused by *Escherichia coli*

Escherichia coli was first isolated from children's feces and described in 1885. Strains of *E. coli* were considered as a foodborne pathogen in the early 1940s. It is a common facultative anaerobic normal flora in the intestinal tract in human and warm-blood animals.

2.1.2.1 Characteristics of *Escherichia coli*

E. coli is classified in the family Enterobacteriaceae, a gram negative, facultative, non-spore forming coccobacilli. Distribution of *E.coli* in the environment is determined by its presence in the bowel of humans and animals. Its presence in water supplies is an indication of recent fecal contamination and the potential presence of enteric pathogens. *E. coli* is easily grown in/ on culture media. This microorganism is often referred to as a "coliform bacterium" which is able to ferment lactose with the production of acid and gas within 48 h at 35˚C (Molenda, 1994).

E. coli is a harmless commensal, some strains are pathogenic and cause diarrheal disease, which it can become an opportunistic pathogen causing a number of infections such as gram negative sepsis, urinary tract infections, pneumonia in immunosuppressed patients, and meningitis in neonates. It is commonly found in feces, and is ready to be cultured with generally non pathogenic character. Therefore, it is used as an indicator of fecal contamination for the possible presence of other enteric pathogens such as *Salmonella typhi*. This usage has been transferred to foods where greater

circumspection is required in interpreting the significance of positive results (Adams and Moss, 1995).

E. coli isolates are serologically differentiated based on three major surface antigens, which enable serotyping: the lipopolysaccharide somatic O, flagellar H, and polysaccharide, capsular K antigens was proposed by Kauffman in the 1940s. As currently applied in the O:H system, principal serogroups are defined by O antigens and then subdivided into serovars on the basis of H antigens. Strains of each category of pathogenic *E. coli* tend to fall within certain O:H serotypes, so the scheme plays an important role in detecting pathogens as well as in epidemiological investigations.

2.1.2.2 Pathogenesis and Clinical Features

Diarrheagenic *E. coli* can be categorized into specific groups based on virulence properties, mechanisms of pathogenicity, clinical syndromes and distinct O: H serotypes. These categories include enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregrative *E. coli* (EAEC) and enterohemorrhagic *E. coli* (EHEC) (Padhye and Doyle, 1992; Molenda, 1994).

2.1.2.2.1 Enteropathogenic *E. coli* (EPEC)

Enteropathogenic *E. coli* (EPEC) can cause severe diarrhea in infants, especially in developing countries. They are transmitted directly or indirectly through human carriers. Several serotypes are implicated in waterborne and foodborne disease outbreaks in different countries. The major O serogroups associated with illness are O55, O86, O111ab, O119, O125ac, O126, O127, O128ab and O142 (Meng, Doyle, T. Zhao, and S. Zhao, 2001). The original definition of EPEC is diarrheagenic *E. coli* belonging to

serogroups epidemiologically incriminated as pathogens but whose pathogenic mechanism has not been proven to be related to either enterotoxins or *Shigella*-like invasiveness. One needs to ingest high numbers of cells (10^6 to 10^9) to develop the symptoms. Symptoms of EPEC infection include malaise, vomiting and diarrhea with stools containing mucus but rarely bloody. They appear 12-36 h after ingestion of the organism. In infants, the illness is more severe than many other diarrheal infections and persist for longer than two weeks in some cases (Molendar, 1994).

2.1.2.2.2 Enterotoxigenic *E. coli* (ETEC)

These strains are the major cause of diarrhea among travelers, as well as infants in many developing countries with poor sanitation. ETEC produce enterotoxin which is defined as an exotoxin produced by bacteria which has a detrimental effect on the intestine. There are two types of enterotoxin produced by ETEC strains. One is heat labile toxin (LT) which is inactivated at 60˚C after 30 min and at low pH, the other is heat stable toxin (ST) which can withstand heating at 100˚C for 15 min and is acid resistant. The symptoms of ETEC enteritis resemble those of cholera. The pathogen is spread directly or indirectly by human carriers. Both food and water have been implicated in outbreaks and sporadic cases in humans. Ingestion of large numbers of cells (10^8 to 10^9) are necessary for an individual to develop the symptoms.

2.1.2.2.3 Enteroinvasive *E. coli* (EIEC)

These strains are known to cause dysentery, like shigellosis. EIEC invades and multiplies within the epithelial cells of the colon causing ulceration and inflammation. Symptoms include diarrheal stools, which contain blood, mucus and fecal leukocytes.

Human carriers, directly or indirectly, spread the disease. Ingestion of as many as 10^6 cells may be necessary for an individual to develop the symptoms.

2.1.2.2.4 Enteroaggregative *E. coli* (EAEC)

EAEC recently have been associated with persistent diarrhea in infants and children in several countries worldwide. It is different from the other types of pathogenic E. coli because of their ability to produce a characteristic pattern of aggregative adherence on HEp-2 cells. It adheres in an appearance of stacked bricks to the surface of HEp-2 cells.

2.1.2.2.5 Enterohemorrhagic *E. coli* (EHEC)

EHEC also known as verotoxin producing *E. coli*, was first recognized as human pathogens in 1982 (Molendar, 1994). A number of serotype have this capacity but O157:H7 is the one most frequently isolated from humans It has attracted attention not only because foodborne transmission is more common than with other diarrheagenic *E. coli*, but because it can also cause the life-threatening conditions, hemorrhagic colitis; a self-limiting, acute, bloody diarrhea that begins with stomach cramps and watery diarrhea after incubation period of 3-8 days, hemolytic uremic syndrome; cause acute renal failure, hemolytic anemia, and thrombotic thrombocytopenic purpura; that is related to hemolytic uremic syndrome including fever and neurological syndrome (Adams and Moss, 1995). Animals, particularly dairy cattle, are thought to be the carriers. Ingestion of as few as 10 to 100 cells can produce the disease. The ability of this serotype to produce three enterotoxins (verotoxins) has been recognized to be the causative agent of the disease symptoms.

2.1.2.3 Sources of *Escherichia coli* Food Contamination

Fecal contamination of water supplies and contaminated food handlers have been most frequently implicated in outbreaks caused by EPEC, EIEC and ETEC. A number of foods have been involved, including a coffee substitute in Romania in 1961, vegetable, potato salad and sushi. The mold-ripened soft cheese have been responsible for outbreak in 1971, associate with EIEC in which more than 387 people were affected, and in 1983, caused by ETEC (ST). *E. coli* would not be expected to survive well in a fermented dairy product with a pH below 5 but, where contamination is associated with mold-ripening, the local increase in pH as a result of lactate utilization and amine production by the mold would allow the organism to go.

EHEC is thought to be present in the intestine of animals, particularly in dairy cattle, without producing symptoms. Food of animal origin, especially ground beef, has been implicated in many outbreaks in the United States, the U.K., and Canada (Ray, 1996). The affected people were found to consume contaminated hamburgers. The hamburgers, contaminated with *E. coli* O157:H7, were cooked at a temperature that failed to kill the pathogen. In addition to ground beef, other foods, such as apple cider, raw milk and uncooked sausages, have been implicated. Investigations revealed the presence of *E. coli* O157:H7 in many different types of foods of animal origin, such as ground beef, pork, poultry, lamb and raw milk, in low percentages. The organism was isolated in low frequencies from dairy cows as well as calves and chickens.

The pathogen is sensitive to pasteurization temperature. Thus proper heat treatment, elimination of post heat contamination for a ready-to-eat food and refrigeration of a food soon after preparation are necessary to control the disease. Proper sanitation at all stages of food processing and handling, cooking or heating at appropriate temperature,

proper refrigeration and prevention of cross-contamination should be practiced in order to control the presence of pathogenic *E. coli* include *E. coli* O157:H7 in ready-to-eat food.

2.2 Thai spices

Thai spices have been used for centuries as food preservatives or to make foods more appetizing by improving taste and/ or aroma. Many spices used in Thai cuisine have beneficial medicinal properties such as carminative, antidiarrhea and antimicrobial digestant activities, which indicated that they contain antimicrobial agents.

Herbs in the Zingiberaceae family (e.g. galangal, ginger, turmeric and krachai) are the main spices in Thai cuisine. Several studies showed that essential oils and some compounds in these spices -zingiberene and gingerol in ginger, methyl cinnamate and eugenol in galangal, turmerone in turmeric- possess an inhibitory effect on the growth of some bacteria.

2.2.1 Botanical Description of *Alpinia galanga* (Linn.) Sw. [*Languas galanga* (Linn.) Stuntz. (Syn.)]

Alpinia galanga (Linn.) Sw. or *Languas galanga* (Linn.) Stuntz. (Syn.) (*A. galanga*) is a perennial herb in the Zingiberaceae family, shown in Figure 2.1. The common names of this plant are galangal, Katuk karohinee (Central), Khaa (general), Khaa yauk, Khaa Luang (Northern), Seh-ae-khoei, Sa-e-choei (Karen-Mae Hong Son) (Farnsworth and Bunyapraphatsara, 1992).

Galangal is an underground stem herb, 2-2.5 m high, having aerial stem leafy; rhizome with conspicuously nodes and internodes and slightly aromatic. Leaves, slightly

hairy with leaf-sheaths, are simple, rising from the underground stem. Fruit is globose or ellipsoid capsule containing 2-3 fully matured seed (Farnsworth and Bunyapraphatsara, 1992). It has been used for medicinal purposes and possesses similar properties to ginger: aromatic stimulant, carminative and digestant. It has the ability of microbial inhibition against bacteria such as Streptococci, Staphylococci, coliform bacteria, fungi, yeast and parasite (Farnsworth and Bunyapraphatsara, 1992).

Figure 2.1 *Alpinia galanga* (Linn.) Sw.

Specific nutritional facts: 100 g of edible ginger contain 20 Kcal of total energy, 1.3 g of protein, 0.3 g of lipid, 3.1 g of carbohydrate, 5 g of calcium, 27 g of phosphorous, 0.1 g of iron, 0.24 g of vitamin B1, 0.06 g of vitamin B2, 0.4 g of niacin, 22 g of vitamin C and 2.41 g of β-carotene (มูลนิธิโตโยต้าแห่งประเทศไทย, 2543).

Chemical constituents of galangal are (1'S)-1'-acetoxychavicol acetate, (1'S)-1'-acetoxyeugenol acetate, 1'-acetoxychavicol acetate, 1'-acetoxyeugenol acetate, D-camphore, chavicol, chavicol acetate, 1,8-cineole, trans-coniferyl diacetate, trans-p-coumaryl diacetate, di-(p-hydroxy-cis-styryl) methane, essential oil, eugenol, eugenol

acetate, trans-β-farnesene, galangin, 7-hydroxy-3,5-dimethoxy flavone, 4-hydroxybenzaldehyde, 1'-hydroxychavicol acetate, p-hydroxycinnamaldehyde, isorhamnetin, kaempferol, kaempferol-4'-methyl ether, kaempferol-7-methyl ether, methylcinnamate, methyleugenol, pinenes, quercetin, quercetin-3-methyl ether, resins and sesquiterpenoids (Farnsworth and Bunyapraphatsara, 1992).

The ethereal galangal extract exhibited antibacterial activity against several bacteria. *Bacillus subtilis* was reported to be the most sensitive bacteria, followed by *E. coli*, *S. aureus* and *P. aeruginosa*, respectively (Iamthammachard, 1982). The essential oil was found to be effective against many gram positive bacteria (Janssen and Scheffer, 1985). Both water and alcohol extracts showed significant inhibition against *S. aureus* 6538, *S. aureus* 25923, β-streptococcus group A, β-streptococcus group B, *Sarcina lutea*, *B. subtilis*, *Mycobacterium smegmatis*, *P. aeruginosa* and *Aeromonas hydrophila* (Chutiyasantayanon, Sirikul, Buntaweekul, and Temrattanasirikul, 1985). The acetone extract showed antioxidant and antimicrobial effects in minced raw beef. It was able to extended the shelf life of raw minced beef up to 4 days of storage at 4±1˚C (Cheah and Gan, 2000).

It was reported that alcohol and chloroform extracts possessed antifungal activity against *Microsporum gypsum*, *Trichophyton rubrum*, *Epidermophyton floccosum*, *Candida albicans*, *Crytococcus neoformans* and *Saccharomyces* sp. Low inhibitory action was shown by the water extract. No activity was found in fresh juices (Achararit, Panyayong, and Ruchatakomut, 1984). In another study, petroleum ether, chloroform and 95% alcohol extracts were found to exhibit antifungal activities against *M. gypseum* and *T. rubrum* (Limsrimanee and Siriratana, 1983).

2.2.2 Botanical Description of *Zingiber officinalis* Roscoe

Zingiber officinalis (*Z. officinalis*) or ginger rhizome is the knotted, thick tuber, which grows underground. The actual root sprawls out from knots on the rhizome. Above the ground are 12-inch long stems with long, narrow, ribbed, green leaves and white or yellowish-green flowers (Health and age, www, 2002). It is an excellent remedy for digestive problems such as flatulence, nausea, indigestion, intestinal infections and certain types of food poisoning. Ginger inhibits platelet aggregation. It acts against bacteria, yeast, mold, virus including protozoa. It has antioxidative, antimutagenic and anti-inflammatory properties.

Specific nutritional facts: 100 g of edible ginger contain 25 Kcal of total energy, 0.4 g of protein, 0.6 g of lipid, 4.4 g of carbohydrate, 18 g of calcium, 22 g of phosphorous, 1.2 g of iron, 0.02 g of vitamin B1, 0.02 g of vitamin B2, 1 g of niacin, and 1 g of vitamin C (มูลนิธิโตโยต้าแห่งประเทศไทย, 2543).

Chemical constituents of ginger are 3% essential oils (bisabolone, phellandrene, citral, borneol, citonellol, etc.), 20-25% of the oleoresin (gingerol, shogaol), phenol, vitamins and minerals (นันทวัน และอรนุช, 2539; Chrubasik, Pittler, and Roufogalis, 2005).

Ginger exerts *in vitro* antioxidative, antitumorigenic and immunomodulatory effects and is an effective antimicrobial and antiviral agent. Animal studies demonstrate effects on the gastrointestinal tract, the cardiovascular system, on experimental pain and fever, antioxidative (Chung, Jung, Surh, Lee, and Park, 2001), antilipidemic (Ahmed and Shama, 1997) and antitumor effects (Banerjee, Sharma, Kale, and Rao, 1994; Nagasawa, Watanabe, and Inatomi, 2002), as well as central and other effects.

Figure 2.2 *Zingiber officinalis*

Hydroethanolic ginger extract exhibited potent antibacterial activity against gram positive and gram negative bacteria (Mascolo, Jain, Jain, and Capasso, 1989) including *S. aureus*, *S. pyogenes*, *S. pneumoniae* and *Haemophilus influenzae* (Akoachere, Ndip, Chenwi, Ndip, Njock, and Anong, 2002), *P. aeruginosa*, *S. typhimurium* and *E. coli* (Jagetia, Baliga, Venkatesh, and Ulloor, 2003). A methanolic extract of the dried powder ginger rhizome and isolated constituents ([6]-, [8]-, [10]-gingerol and [6]-shogaol) inhibited the growth of 19 strains of *Helicobacter pylori* (including five CagA+ strains) in vitro (Mahady, Penland, Yun, Lu, and Stoia, 2003). The fraction of the crude extract containing the gingerols was the most effective.

Treatment of ginger root with boiling water abolished the antibacterial effect against *E. coli*, *S. typhimurium*, *V. parahaemolyticus*, *P. aeruginosa*, *P. vulgaris*,

S. aureus, *Mycobacterium phlei*, *S. faecalis* and *B. cereus* except that against the growth of *Micrococcus luteus*. This indicates that the antibacterial principle is heat labile (Chen, M.D. Chang, and T.J. Chang, 1985). [10]-Gingerol and [6]-gingerdiol had potent antifungal activity against 13 human pathogens at concentrations below 1 mg/ ml (Ficker *et al*., 2003). Hydroethanolic ginger extract inhibited does dependently the growth of *Candida albicans* (Jagetia *et al*., 2003). Constituents of ginger oil exhibited moderate growth regulatory and antifeedant activity against Spilosoma oblique (especially [6]-dehydroshogaol and significant antifungal activity against *Rhizoctonia solani* (especially dehydrozingerone) (Agarwal, Walia, Dhingra, and Khambay, 2001).

In the plaque reduction test, several sesquiterpenes with antirhinoviral activity were isolated from dried ginger rhizomes, the most active being β-sesquiphellandrene (Denyer, Jackson, Loakes, Ellis, and Young, 1994)

2.2.3 Botanical Description of *Curcuma longa* Linn. [*Curcuma domestica* Valeton]

Figure 2.3 *Curcuma longa* Linn.

Curcuma longa Linn. is a perennial rhizomatous herb in the family of Zingiberaceae as shown in Figure 2.3. The common names of this plant are turmeric, Khamin (general), Khamin kaeng, Khamin yok, Khamin hua (Chiangmai), Khamin chan (Central, Peninsular), Kheemin, Min (Peninsular), Taa-yo (Karen Kamphaeng Phet), Sa-yo (Karen-Mae Hong Son) (เต็ม สมิตินันท์, 2523).

Turmeric is a stemless rhizomatous herb, having flesh rhizome. Rootstock is large, ellipsoid-ovate with sessile tubes with orange color inside, giving rise of the short blunt daughter rhizomes called fingers. Leaves are large up to 1.2 m or more long including petiole which is about as long as the blade, oblong-lanceolate tapering to the base. It is cultivated throughout tropical of Asia such as India, China, Ceylon, Thailand, and other tropical countries as well as Africa and South America (Fransworth and Bunyapraphatsara, 1992).

The rhizome of *Curcuma longa* Linn. is yellow and used as a coloring agent, household medicine and food flavoring spice. For medicinal purpose, fresh and dried rhizomes are used internally as treatment of peptic ulcer and dyspepsia and applied externally for relieving inflammatory, itching and infected wounds. It is also used as aromatic, stimulant and carminative drugs. In Thai folklore medicine, powdered turmeric is heated with coconut oil or lard until yellow oil is obtained. This oil is then applied to wounds for its curing effect (Fransworth and Bunyapraphatsara, 1992). Moreover, it has been used as antidiarrhea, antiemetic, antipyretic, aromatic stomachic and anti-infection of skin diseases. It also exhibits antibacterial activities against *S. aureus*, *E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa*, *C. botulinum*. In India, turmeric is also employed as a condiment and curry powder. It is used for dying clothes, employed in pharmacy for

coloring agents in ointments and other preparations (Bentley and Trimen, 1983). Curcumin is a compound in turmeric, exerts *in vivo* an antiviral properties against HIV (Mazumder, Raghavan, Weinstein, Kohn, and Pommier, 1995) and also provides significant protection ($P< 0.05$) against disease caused by intravaginal HSV-2 challenge (K.Z. Bourne, N. Bourne, Reising, and Stanberry, 1999).

Specific nutritional facts: 100 g of edible turmeric contain 65 Kcal of total energy, 1.2 g of protein, 1.4 g of lipid, 11.4 g of carbohydrate, 9 g of calcium, 41 g of phosphorous, 2.3 g of iron, 0.02 g of vitamin B1, 0.03 g of vitamin B2, 1.3 g of niacin, and 12 g of vitamin C (มูลนิธิโตโยต้าแห่งประเทศไทย, 2543).

Chemical constituents of *Curcuma longa* Linn. are at least 5% curcuminoid and 7% volatile oil contents, oleoresins. Turmeric contains approximately 7 percent of a yellow volatile oil comprising of turmerone and zingiberene as major components as well as many other sesquiterpenes and monoterpenes; yellow coloring matter including curcumin or diferuloymethane (1.8 % - 5.4 %), desmethoxycurcumin, and bisdesmethoxycurcumin (Thai Herbal Pharmacopoeia, 1995).

2.2.4 Botanical Description of *Boesenbergia rotunda* (L.) Mansf. or *B. pandurata* (Roxb.) Schltr. [*Gastrochillus panduratus* (Ridl.) Schltr].

Boesenbergia rotunda (L.) Mansf. is a rhizome, which is a member of the Zingiberaceae family, shown in Figure 2.4. The common names of this plant are Ka-an, Jeeb, See-fu and Ra-an. It is probably native to Java and Sumatra. It now grows readily throughout Sri Lanka, India, Southeast Asia including Thailand. krachai looks like a cluster of long orange-brown fingers; when the skin is scraped off, the yellow interior is

revealed. They are 1-3% of long tubes sprouting from the central part all in the same direction (like the fingers of a hand). This spice has an aromatic, spicy flavor (Tropical herbs& spices of Thailand, 1997).

Figure 2.4 *Boesenbergia rotunda* (L.) Mansf.

Specific nutritional facts: 100 g of edible krachai contain 49 Kcal of total energy, 1.3 g of protein, 0.8 g of lipid, 9.2 g of carbohydrate, 80 g of calcium, 71 g of phosphorous, 2.3 g of iron, 0.07 g of vitamin B1, 0.3 g of vitamin B2, 3.5 g of niacin, and 2 g of vitamin C (มูลนิธิโตโยต้าแห่งประเทศไทย, 2543).

Krachai contains 1-3 % of essential oils. Several compounds have been identified, alpinetin, boesenbergin A, boesenbergin B, cardamomin, 2'-4'-dihydroxy-6'-methoxy chalcone, 2'-6'-dihydroxy-4'-methoxy chalcone, flavone, panduratin A, panduratin B-1, panduratin B-2, pinostrobin (นันทวัน และอรนุช, 2539). Krachai has an antifungal, antibacterial (Achararit et al., 1983), antitumor (Murakami, Kondo, Nakamura, Ohigashi, and Koshimizu, 1993), antimutagenicity (Ungsurungsie, Suthienkul, and Paovalo, 1982), anti-inflammatory (Mahidol, Tuntiwachwuttikul, and Reutrakul, 1982)

and pesticide properties (Areekul, Sinchaisri, and Tingvatananon, 1987). Cardamomin is reported that is antifungal (Achararit et al., 1983). Chloroform extract showed significant topical anti-inflammatory activity in the assay of TPA-induced ear edema in rats (Tuchinda, Reutrakul, Claeson, Pongprayom, Sematong, Santisuk, and Taylor, 2002).

2.3 Antimicrobial activities of plant and/ or spices and their essential oils

Spices are roots, bark, seeds, buds, leaves or fruit of aromatic plants that have been used for thousands of centuries, are added to foods as flavoring agents. Spices have used for food preservations and for their medicinal value since the ancient times (Davidson, 2001). Scientific experiments since the late 19^{th} century have documented the antimicrobial properties of some spices and their components (Shelef, 1983). For examples, Extracts of mace, bay leaf and nutmeg added to turkey frankfurter slurries inhibited toxin production by *C. botulinum* (Hall and Maurer, 1986). Many researchers investigated each antimicrobial activity of spice and studied spice combinations. Clove, cinnamon, oregano and thyme and to a lesser extent, sage and rosemary have strongest antimicrobial activity among spices (Davidson, 2001).

The antimicrobial activities of extracts obtained from spice, herbs and other aromatic plants or parts thereof using organic solvent or steam distillation have been recognized for many years. Plants and plant extracts have been used since antiquity in folk medicine and food preservation, providing a range of compounds possessing pharmacological activities. Most commonly, the active antimicrobial compounds are found in the essential oil fraction. Essential oils (also called volatile or ethereal oils) are aromatic oily liquids obtained from plant material. They can be obtained by expression,

fermentation, effleurage or extraction but the method of steam distillation is most commonly used for commercial production of essential oils (Van de Braak and Leijten, 1999). Essential oils are mostly soluble in alcohol and to limited extent in water. They consist of mixtures of esters, aldehydes, ketones and terpenes (Cowan, 1999; Harborne, 1998). It has long been recognized that some essential oils and components have a wide spectrum of antimicrobial effects and these have been reviewed, as have the antimicrobial properties of spices (Shelef, 1983). Besides antibacterial properties (Carson, 1995), essential oils or their components have been shown to exhibit antiviral (Bishop, 1995), antimycotic (Janssen and Scheffer, 1985), antitoxigenic (Ultee and Smid, 2001) antiparasitic (Pandey, Kalra, Tandon, Mehrotra, Singh, and Kumar, 2000) and insecticidal properties (Karpouhtsis, Pardali, Feggou, Kokkini, Scouras, and Mavragani-Tsipidou, 1998) properties.

The impact of essential oils on bacteria, especially on pathogens, has been extensively studied in the laboratory. *E. coli* was found to be more vulnerable to the essential oils of sage, rosemary, cumin, caraway, clove and thyme than *P. fluorescens* or *Serratia marcescens* (Farag, Daw, Hewedi, and El-Baroty, 1989). *S.* Typhimurium was also more sensitive to oregano and thyme oils than *P. aeruginosa* (Paster, Juven, and Harshemesh, 1988). Nychas (1995) summarized findings that essential oil compounds from many different plant sources inhibit many foodborne pathogens. *S. aureus*, *L. monocytogenes*, *A. hydrophila*, *S.* Typhimurium and *C. botulinum* were to some degree sensitive to extracts from linden flower, orange, lemon, grapefruit, mandarin, sage, rosemary, oregano, thyme, cinnamon, cumin, caraway, clove, thyme, allspice, mastic gum and onion. However, most researchers inevitably came to conclude that the effectiveness

of essential oils decreased when experiments were conducted in vivo. This could well be due to specific components of the food matrix, such as proteins and fats, which immobilize and inactivate the essential oil components.

2.4 Mechanisms of antibacterial action

Antimicrobial activity varies greatly between different types of microorganisms and it might differ between different strains of the same species. The ability of each antimicrobial agent to penetrate to the bacterial cells is due to the composition and structure of the cell and outer walls of the microorganisms. The fundamental divisions of the bacterial cells, i.e. cell wall, cytoplasmic membrane and cytoplasm, occur in all species. The basic structure and composition of the cytoplasm and the cytoplasmic membrane are largely conserved between different types of bacteria although subtle differences may occur. In terms of susceptibility to antimicrobial agents, the structure and composition of the outer envelope are more interesting as they differ widely between microorganisms (Figure 2.5). It is generally accepted that the bacterial outer envelope is responsible for the different microbial responses to biocide challenges. For example, the outer membrane of Gram negative bacteria acts as permeability barriers and is responsible for the intrinsic resistance of the microorganisms to antimicrobial compounds (Gilbert, Pemberton, and Wilkinson, 1990; Nikaido and Vaara, 1985).

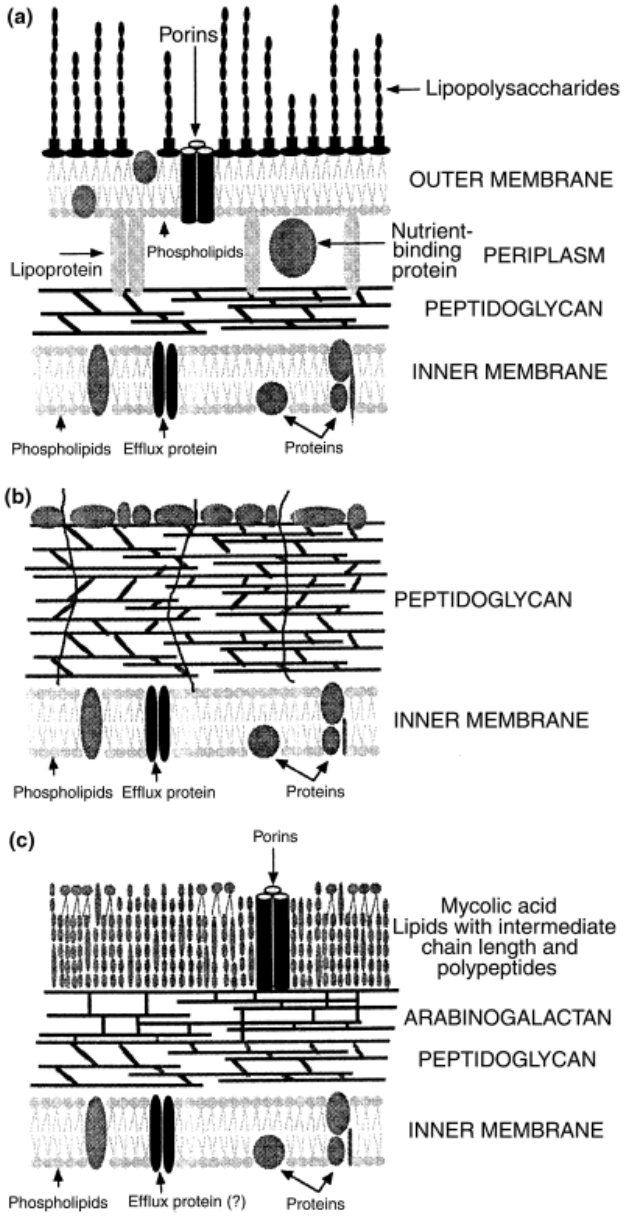

Figure 2.5 Structure of the outer cell wall of (a) gram negative bacteria; (b) gram positive bacteria and; (c) mycobacteria. Adapted from Maillard and Russell (2000).

Cytoplasmic membrane composed essentially of a phospholipid bilayer with embedded proteins. It is semipermeable and regulates the transfer of solutes and

metabolites in and out of the cell cytoplasm. It is also associated with several important enzymes involved in various cell metabolic functions (Salton and Owen, 1976; Singer and Nicholson, 1972). The cytoplasmic membrane is often considered as the major target site for antimicrobial agents. Damage to the membrane can take several forms: (i) physical disruption of the membrane; (ii) dissipation of the proton motive force (PMF) and (iii) inhibition of membrane-associated enzyme activity.

Bacterial cytoplasm contains the cell nucleic acid, ribosomes and various enzymes with different functions. These cell components are probably not primary target sites, since an antimicrobial agent has to penetrate within the cell to reach cytoplasmic constituents.

The antimicrobial properties of essential oils and their components have been reviewed in the past and the mechanism of action has not been studied in great detail (Lambert, Skandamis, Coote, and Nychas, 2001). The action of food preservatives and/ or antimicrobial agents on the cells of spoilage microorganisms is based on a multiplicity of individual influences. These include not only physical and physicochemical mechanisms but also biochemical reactions. Mode of actions of food preservatives such as salts, inorganic acids, and antibiotics, can differently inhibit growth or destroy microbial cell. The initial reaction of an antimicrobial agent with a microbial cell involves an initial binding to the surface, although target sites might be found within the cell. Subsequently, changes to the outer cell layer may occur to allow an antimicrobial agent to penetrate the cell and reach its target sites. Alteration of the bacterial structure at the outer layer, cytoplasmic membrane or within the cell cytoplasm may contribute to the bactericidal or bacteriostatic effect of the chemical agent, depending upon the extent of damage and the nature of the target sites.

Plant oils and extracts, primarily from clove, oregano, rosemary, thyme, tea tree and sage have shown significant inhibitory activity, while less potent activity was shown by other spice materials. Expression of antimicrobial activities is often very clear, but the mechanism of antimicrobial actions is incompletely understood. The antimicrobial action of essential oils may be due to impairment of a variety of enzyme systems including involved in energy production and structural component synthesis. In general, studies on the mechanism of action of essential oils have used a common methodology that attempts to illustrate deleterious effects on cellular membranes, for example: permeability and proton motive force (Cox et al., 1998; Helander et al., 1998; Tassou, Koutsoumanis, and Nychas 2000; Ultee, Kets, and Smid. 1999;). The large number of different groups of chemical compounds present in essential oils, it is most likely that their antibacterial activity is not attributable to one specific mechanism but that there are several targets in the cell (Carson, Mee, and Riley, 2002). The locations or mechanisms in the bacterial cell can be classified into one or more of the following groups (Figure 2.6): (a) degradation of the cell wall (Helander et al., 1998), (b) reaction with the cytoplamic membrane, damage to cytoplasmic membrane (Knobloch et al., 1989; Lambert, et al. 2001; Sikkema, De Bont, and Poolman, 1994) and damage to membrane proteins (Juven, Kanner, Schved, and Weisslowicz, 1994; Ultee et al., 1999), (c) inactivation of essential enzymes (Thoroski, Blank, and Biliaderies, 1989; Wendakoon and Sakaguchi, 1995), (d) leakage of cell contents or inorganic ions (Cox et al., 1998; Inoue, Shiraishi, Hada, Hirose, Hamashima, and Shimada, 2004; Lambert et al., 2001) and also loss of pH homeostasis (Fitzgerald, Stratford, Gasson, Ueckert, Bos, and Narbad, 2004), (e) coagulation of cytoplasm (Gustafson et al., 1998), (f) destruction or inactivation of genetic materials (Davidson, 1993). Not all of these mechanisms are separate targets; some are affected as a

consequence of another mechanism being targeted.

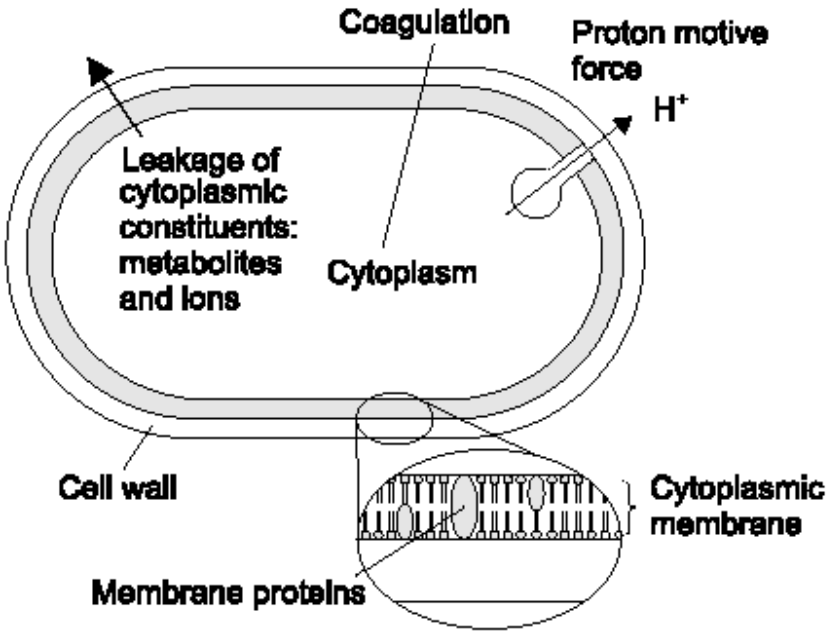

Figure 2.6 Locations and mechanisms in the bacterial cell thought to be sites of action for essential oil components: degradation of the cell wall; damage to cytoplasmic membrane; damage to membrane proteins; leakage of cell contents; coagulation of cytoplasm and depletion of the proton motive force (Burt, 2004).

Overall mechanisms of action of an antimicrobial agent which it has its main activity. Thus, three levels of interaction can be classified (i) interaction with outer cellular components; (ii) interaction with the cytoplasmic membrane and; (iii) interaction with cytoplasmic constituents (Maillard, 2002).

2.4.1. Interactions with outer cell components.

Several antimicrobial agents can interact with outer cellular components, although cell viability might not be affected. One of the noticeable effects of antimicrobial agent interaction with the bacterial cell is a change in cell hydrophobicity.

Gram negative bacteria are generally less sensitive to antimicrobial agents than gram positive bacteria because of their outer membrane (Figure 2.5). One of the more dramatic effects of inhibitory action appears in two separate reports where outer membrane of *E. coli* and *S.* Typhimurium disintegrated following exposure to carvacrol and thymol (Helander et al., 1998). Similar studies were reported by Lucchini, Core, and Cremieux (1990) with these agents using a different strain of *E. coli* and *P. aeruginosa*. Yeast and gram positive bacteria showed no such changes in cell wall morphology. This was probably due to the solubility of lipopolysaccharides (LPS) in the outer membrane in phenolic-based solvents.

2.4.2 Interaction at the cell cytoplasmic membrane level.

2.4.2.1 Disruption of the cytoplasmic membrane.

Disruption of the cytoplasmic membrane is often exemplified by the leakage of intracellular components, potassium (K^+) followed by inorganic phosphates, pool of amino acids and materials absorbing at 260 nm, nucleic acids and proteins (Lambert and Hammond, 1973). Leakage is best considered as a measure of the disruption of the cell permeability barrier and it might reflect a bacteriostaic effect rather than cell death. The rate and extent of leakage may depend upon the microorganisms, type of membrane active agents and the factors affecting the efficacy of the antimicrobial agents. Many of the antimicrobial agents act as the cytoplasmic membrane, altering its function and in some

instances structure, causing swelling and increasing its permeability (Holley and Patel, 2005). The study of spheroplasts and protoplasts can provide useful information since these bacteria are more sensitive to anitmicrobial agents and lyse after exposure to compounds that cause gross membrane damage (Hugo and Longworth, 1964; Davies, Bentley, and Field, 1968). Propidium iodide (Cox et al., 2000; Fitzgerald et al., 2004), the nucleic acid dye ethidium bromide (Lambert et al., 2001) as well as the self-quenching probe rhodamine B (Ultee, Bennik, and Moezelaar, 2002) were used to monitor cell membrane integrity and uptake of essential oil components.

Oregano essential oil, thymol and carvacrol inhibited against *Pseudomonas aeruginosa* and *S. aureus* but oregano essential oil was the main active agents, able to increasing permeability of cells to the nuclear stain ethidium bromide, and causing leakage of inorganic ions (Lambert et al., 2001). Fluorescent probes have been used to study membrane changes. Carvacrol and thymol are able to disintegrate the outer membrane of gram negative bacteria, releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane to ATP (Helander et al., 1998). Studies with *B. cereus* have shown that carvacrol interacts with cell membrane, where it dissolves in the phospholipid bilayer and is assumed to align between the fatty acid chains (Ultee, Kets, Alberda, Hoekstra, and Smid, 2000a). This distortion of the physical structure would cause expansion and destabilization of the membrane, increasing membrane fluidity, which in turn would increase passive permeability (Ultee et al., 2002). Measurment of the average phase transition temperature of the bacterial lipids confirmed that membranes instantaneously became more fluid in the presence of carvacrol (Ultee et al., 2000a). The passage of *B. cereus* cell metabolites across the cell membrane on exposure to carvacrol has also been investigated. Measurements of the membrane potential of exponentially

growing cells revealed a sharp decrease on the addition of carvacrol and indicated a weakening of the proton motive force. The pH gradient across the cell membrane was weakened by the presence of carvacrol and was completely dissipated. Furthermore, intracellular levels of potassium ions dropped whilst extracellular amounts increased proportionately, the total amount remaining constant (Ultee et al., 1999), It was concluded that carvacrol forms channels through the membrane by pushing apart the fatty acid chains of the phospholipids, allowing ions to leave the cytoplasm (Ultee, 2000). Oregano essential oils, containing carvacrol as a major component, cause leakage of phosphate ions from *S. aureus* and *P. aeruginosa* (Lambert et al., 2001).

2.4.2.2 Dissipation of the proton motive force.

The proton motive force is involved in active transport, oxidative phosphorylation and adenosine triphosphate (ATP) synthesis in bacteria (Hugo,1978). The PMF is expressed as a proton gradient across the cytoplasmic membrane from the interior of the cell to the outside. A carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) was used by Lambert et al. (2001) to follow changes in cytoplasmic pH following essential oil challenge.

Action of oregano essential oil against bacterial cells not only disrupted cell membrane but also dissipated pH gradients as indicated by the CFDA-SE fluorescent probe irrespective of glucose availability (Lambert et al., 2001). Gill and Holley (2003) reported that antimicrobial activity of eugenol and cinnamaldehyde against *Listeria monocytogenes* and *Lb. sakei* were not consistent with a protonphore uncoupler mechanism. Eugenol, like carvacrol, is a substitued phenolic compound and cinnamaldehyde is a substitued aromatic compound. Treatment of un-energized cells of

L. monocytogenes with eugenol and cinnamaldehyde prevented cellular ATP pools from increasing following addition of glucose.

Aromatic and phenolic compounds exert their antimicrobial effects at the cytoplasmic membrane by altering its structure and function. Efflux of K^+ is usually an early sign damage (Walsh, Maillard, Russell, Catrenich, Char-bonneau, Bartolo, 2003) and is often followed by efflux of cytoplasmic constituents (Cowan, 1999; Ultee, 2001). The loss of the differential permeability character of the cytoplasmic membrane is frequently identified as the cause of cell death. Some researchers have explored this further, reasoning that loss of membrane function is only part of the explanation for antimicrobial activity (Walsh et al., 2003). Other events which could lead to membrane dysfunction and subsequently disruption include dissipation of the two components of the proton motive force in cells (pH gradient and the electrical potential) either by changes in ion transport of depolarization through structural changes in the membrane; interference with the energy production (Helander et al., 1998; Lambert et al., 2001; Ultee et al., 2001).

2.4.2.3 Interactions with other enzymatic systems.

Many proteins embedded in the cytoplamic membrane are enzymes. Cinnamon oil and its components have been shown to reduce/ inhibit amino acid decarboxylases in *Enterobacter aerogenes* possibly due to the hydroxyl group on eugenol and carbonyl group on cinnamaldehyde bind to proteins, preventing enzyme action (Wendakoon and Sakaguchi, 1995). Eugenol, the major essential oil of clove, have been found to inhibit the production of α-amylase, protease and subtilisin by *B. subtilis* (Thoroski et al., 1989).

2.4.3 Interactions with cytoplasmic constituents including cell nucleic acids and other cell constituents

Ribosomes are responsible for the translation of messenger RNA into a peptide chain. They can be damaged by antimicrobial agents e.g. hydrogen peroxide, p-chloromercuribenzoate and proflavine, although they might not be primary target sites (Nakamura and Tamaoki, 1968). A number of biocides will undoubtedly react with cytoplasmic constituents other than nucleic acids and ribosomes. Alkylating and oxidizing agents are highly reactive chemicals that strongly interact with the bacterial cell. Ethylene oxide, propylene oxide and β-propiolactone act as alkylating agents. They react with amino, carboxyl, sulfhydryl and hydroxyl groups in bacterial protein as well as nucleic acids (Hoffman, 1971; Adams, Burdon, Campbell, Leader, and Smellie, 1981). Aldehydes, especially glutaraldehyde, ortho-phthaladehyde and formaldehyde, are highly reactive chemical compounds that combine with proteins (to give intermolecular cross-links) and nucleic acids (Gorman, Scott, and Russell, 1980). Oxidizing agents such as halogens, hydrogen peroxide, paracetic acid and ozone may also interact with a number of cellular constituents. Hydrogen peroxide activity results in the formation of hydroxyl radicals, which oxidize thiol groups in proteins and enzymes (Turner, 1983). Cationic compounds and especially chlorhexidine have been shown to cause cytoplasmic coagulation (Fitzgerald, Fitzgerald, Davies, and Russell, 1989).

Because essential oils contain a variety of compounds from different chemical classes, it is not possible to isolate a single mechanism by which these compounds act on microorganisms. The major antimicrobial components of cloves and cinnamon are eugenol [2-methoxy-4-(2-propenyl)-phenol] and cinnamic aldehyde (3-phenyl-2-propenal), respectively. Fabian *et al*. (Davidson, 2001) tested 10% extracts of cinnamon

and clove against *B. subtilis* and *S. aureus* and found cinnamon only slightly inhibitory while clove had high inhibitory properties. Antimicrobial activity of oregano and thyme has been attributed to their essential oils, which contain the terpenes, carvacrol [2-methyl-5-(1-methylethyl) phenol] and thymol [5-methyl-2-(1-methylethyl) phenol], respectively. Firouzi et al. (Davidson, 2001) found that thyme essential oil was the most effective antimicrobial agent against *L. monocytogenes*.

2.5 D, L-1'- acetoxychavicol acetate

1'- Acetoxychavicol acetate is a white crystal powder ($C_{13}H_{14}O_4$), molecular weight 234.25 which present in seeds, leaves and rhizomes of *A. galanga* and some species of the Zingiberaceae; *A. officinarum* rhizomes, *Z. officinale* rhizomes, *Kaempferia galanga* (*K. galanga*) rhizomes (LKT Laboratories, Inc., USA; Janssen and Scheffer, 1985). This compound has been reported to suppress tumor promoter-induced Epstein-Barr virus activation *in vitro* (Kondo, Ohigashi, Murakami, Suratwadee and Koshimizu, 1993) and was subsequently shown to inhibit skin tumor promotion in mice (Murakami, Ohura, Nakamura, Koshimizu and Ohigashi, 1996), suppress chemically induced oral carcinogenesis (Ohnishi et al., 1996) and both colonic aberrant crypt foci and adenocarcinoma formation in rats (Tanaka et al., 1997a; Tanaka et al., 1997b).

ACA was reported as an antiulcer (Mitsui, Kobayashi, Nagahori and Ogiso, 1976) and antitumor agent (Itokawa, Morita, Sumitomo, Totsuka, and Takeya, 1987). There have been a considerable number of reports indicating the effectiveness of ACA on the inhibition of chemically induced carcinogenesis or the expression of tumor markers in the initiation and postinitiation (promotion) phases in multistage carcinogenesis models

(Murakami et al., 1996; Ohnishi, et al., 1996). To address the action mechanisms by which ACA suppresses oncogenic processes, it is important to note the biochemical and biological activities of ACA thus far found: (1) inhibition of xanthine oxidase activity (Noro et al., 1988); (2) suppression of superoxide (O_2^-) generation in leukocytes (Murakami et al., 1996); (3) antilipid peroxidation (Murakami et al., 1996); (4) inhibition of induction of proliferation markers such as silver-stained nucleolar organizer region's protein and 5-bromo-2'-deoxyuridine labeling (Onishi et al., 1996); (5) inhibition of polyamine synthesis (Ohnishi et al., 1996); (6) inhibition of ornithine decarboxylase activity (Ohnishi et al., 1996); and induction of both glutathione *S*-transferase (GST) and quinine reductase (QR) activities (Tanaka et al., 1997b). The ability of xenobiotic enzymes to be induced may be important as the action mechanism in the initiation phase. On the other hand, we have recently found that ACA inhibits inducible nitric oxide synthase expression (Ohata, Fukada, Murakami, Ohigashi, Sugimura, and Wakabayashi, 1998). Furthermore, ACA can suppress double-12-*O*-tetradecanolyphorbol-13-acetate (TPA) application-induced hydrogen peroxide formation and other biochemical parameters related to inflammation in mouse skin (Nakamura, Murakami, Ohto, Torikai, Tanaka, and Ohigashi 1998).

ACA was also identified as the pungent principal of galangal rhizomes. In comparison with other known pungent compounds, it imparts a delayed spicy and pungent sensation and a clean taste without a lingering effect. It can be used as an alcohol enhancer or replacement in beverages (Yang and Eilerman, 1999).

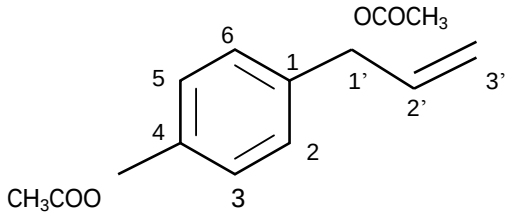

Figure 2.7 D, L-1'-acetoxychavicol acetate (ACA)

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CHAPTER III

ANTIMICROBIAL EFFECTS OF SPICE EXTRACTS IN THE ZINGIBERACEAE FAMILY ON SELECTED FOODBORNE PATHOGENS

3.1 Abstract

Crude extracts of spices in the Zingiberaceae Family (galangal, ginger, turmeric and krachai) were prepared by lyophilized distilled water, methanol, ethanol or ethyl acetate. By using a broth dilution method, the lyophilized water-extracted ginger at a concentration of 5.00 mg/ml was found to have strong inhibitory effects on the growth of representative foodborne pathogens—*Staphylococcus aureus* and *Escherichia coli*, whereas the same spice extracts at concentration of 5.00 mg/ml marginally inhibited the growth of both bacteria. Water-extracted krachai at a concentration of 5.00 mg/ml strongly inhibited the growth of *S. aureus*. Turmeric at 5.00 mg/ml inhibited the growth of both bacteria. The same extracts of ginger showed inhibitory effects to *S. aureus* at 1.25-5.00 mg/ml and *E. coli* at 5.00 mg/ml. The dried methanol, ethanol and ethyl acetate extracts of spices were tested antimicrobial activities using an agar disc diffusion assay. All the extracts of spices were able to inhibit the growth of *S. aureus* especially the ginger extract showed the strongest effect. Ethyl acetate soluble extract of galangal and methanol soluble extract of ginger were able to inhibit the growth of both *S. aureus* and *E. coli*. The extracts of krachai

showed inhibitory effects only *S. aureus*. They had no effect against *E. coli.* Ethanol and ethyl acetate soluble extract of turmeric had inhibitory effects against both test bacteria but its methanol extract had effected to *S. aureus.* Minimum inhibitory concentration (MIC) of galangal methanol extract (GMet) and galangal ethanol extract (GEt) against *S. aureus* were 0.800 mg/ml and 0.325 mg/ml, respectively. Minimum bactericidal concentration (MBC) of GMet and GEt against *S. aureus* were 1.600 mg/ml and 1.300 mg/ml, respectively. The GMet and GEt were also inhibited *Staphylococcus epidermidis* and *Saccharomyces cerevisiae*. The extracts were less antimicrobial activity against *Bacillus cereus* and *Bacillus megaterium* and there were no antimicrobial activity against some gram negative bacteria e.g. *Salmonella* sp., *Enterobacter aerogenes* and *Pseudomonas aeruginosa*. The major compound of the galangal ethanol extract and methanol extract was D, L-1'-acetoxychavicol acetate (ACA) which was identified by TLC, GC-MS and NMR.

Key words: Zingiberaceae, *Staphylococcus aureus*; *E. coli*; antimicrobial activity; D, L-1'-Acetoxychavicol acetate

3.2 Introduction

At present, food safety is a fundamental concern to both consumers and food industries in particular as there are an increasing number of reported cases of food associated infections. Most consumers prefer high quality, nutritious and long shelf life food products with no preservative agents. In fact, they generally tend to avoid all processed foods. Food preservation, however, are the basis of some of the largest and the

most modern food industries in the world. Spices and their essential oils have been widely used as natural food preservatives. This type of processed foodstuff is therefore, safe for consumers.

Rhizomes of some members of the Zingiberaceae Family such as galangal [*Alpinia galanga* (Linn.) Stuntz], ginger (*Zingiber officinalis* Roscoe), turmeric (*Curcuma longa* Linn) and krachai [*Boesenbergia pandurata* (Roxb.)Schltr] have been extensively used as condiment for flavoring and local medicines for the stomachache, carminative and treating diarrhea. They are known to contain various antimicrobial agents. Galangal has characteristic fragrance as well as pungency; hence, its rhizomes are widely used as a condiment for foods in Thailand. Galangal is also used as a medicine for curing stomachache in China and Thailand (Yang and Eilerman, 1999). It has been shown that essential oils from both fresh and dried rhizomes of galangal have antimicrobial activities against bacteria, fungi, yeast and parasite (Fransworth and Bunyapraphatsara, 1992). Janssen and Scheffer (1985) have reported that terpinen-4-ol, one of the monoterpenes in the essential oil from fresh galangal rhizomes, contains an antimicrobial activity against *Trichophyton mentagrophytes*. Acetoxychavicol acetate (ACA), a compound isolated from an n-pentane/ diethyl ether-soluble extract of dried rhizomes, is active against some bacteria and many dermatophyte species (Janssen and Scheffer, 1985). In addition, the ability of ACA to act as an anti-ulcer (Mitsui, Kobayashi, Nagahori and Ogiso, 1976) and anti-tumor agents (Itokawa, Morita, Sumitomo, Totsuka and Takeya, 1987) as well as an inhibitor of chemically induced carcinogenesis is an event (Murakami, Ohura, Nakamura, Koshimizu and Ohigashi, 1996; Ohnishi et al., 1996).

The staphylococcal food poisoning or food intoxication syndrome was first studied in 1894 (Jay, 2000). Staphylococcal gastroenteritis is caused by ingestion of

enterotoxins produced by some staphylococcal species and strains. *Staphylococcus aureus* is non-spore forming gram positive cocci, which in some strains, are able to produce an enterotoxin (Vanderzant and Splittstoesser, 1992). In processed foods in which *S. aureus* should have been destroyed by processing, the reappearance of this particular bacterium can cause damages to food industries as it is a vector of food poisoning. It may be inferred that sanitation or temperature control or both were inadequate. There is no guarantee that foodstuff is safe enough for consumption, although only a trace amount of *S. aureus* is present. Natural preservatives such as spices and plant essential oils are used as additives, instead of chemical preservatives because food remains safe for consumers while *S. aureus* is eliminated. The purposes of this study were (1) to compare the antimicrobial effects of aqueous-soluble and organic solvent-soluble extracts of four selected spice members in the Zingiberaceae Family against the representative foodborne pathogens—*S. aureus* and *E. coli,* (2) to determine the minimum inhibitory concentration and minimum bactericidal concentration of galangal extracts against *S. aureus* and (3) to identify the main component of the GMet and GEt.

3.3 Materials and methods

3.3.1 Preparation of spices

Rhizomes of galangal [*Alpinia galanga* (Linn.) Stuntz], ginger (*Zingiber officinalis* Roscoe), turmeric (*Curcuma longa* Linn) and root of krachai [*Boesenbergia pandurata* (Roxb.) Schltr] were purchased from a local market in Nakhon Ratchasima, Thailand. The fresh rhizomes were cleaned thoroughly with water to remove dirts and infected rhizomes before being sliced and dried in a tray-dryer oven at 50°C for 24 h and

ground in a blender (National, MX-T2GN, Taiwan) to make powder.

3.3.2 Preparation of test microbes

Stock cultures of *Staphylococcus aureus* 209P and *Escherichia coli* NIHJ JC-2, used throughout this study, were kindly provided by the Laboratory of Ecological Circulation, Faculty of Bioresources, Mie University, Japan. They were maintained on nutrient agar (NA, Difco, USA) slants at 4°C. The bacteria were cultured separately in 5 ml of nutrient broth (NB, Daigo, Japan Pharmaceutical Co., Japan) and incubated at 37°C for 18-24 h. Each bacterial suspension was subsequently streaked on NA plates and incubated at 37°C for 48 h. A single colony was transferred to 10 ml of NB and incubated at 37°C for 18-24 h. This culture was used for the antibacterial assay.

3.3.3 Bacterial density adjustment based on optical density of the bacterial suspensions

A single colony on the above mentioned plate was transferred into 10 ml of nutrient broth, followed by incubating at 37°C for 18-24 h. This culture was diluted to 1:4, 1:24, 1:124, 1:624, 1:3124, and 1:15,624 with NB and this series of diluted bacterial suspensions was subjected to the dilution method. Aliquot 0.1 ml of each diluted suspension was spread on a NA plate and incubated at 37°C for 24-48 h. The number of colonies on each plate was determined as colonies forming unit/ml (CFU/ml). The absorbance reading of the original and diluted cultures was determined at 600 nm using a spectrophotometer (Beckman, DU®530, Live Science, UV/Vis Spectrophotometer) for estimation of the bacterial population based on the relationship between the absorbance

values and CFU/ml.

3.3.4 Preparation of the spice extracts

Chemical components were extracted from the ground powders of test spices by the methods of Achararit, Panyayong, and Rachatagomut (1983); Caichompoo (1999) and Harborne, Mabry, and Mabry (1975) with some modifications as described below.

3.3.4.1 Aqueous extraction from test spices

Ten grams of spice powder was added into 100 ml of distilled water and heated at 80°C in a water-bath (Yamato BM42, Tokyo, Japan) for 1 h, then left at room temperature to cool down. The powder suspensions were filtered through Whatman filter paper No.1 (Struers Kebo Lab, Denmark). The filtrates were lyophilized at -140˚C (Yamato, Tokyo, Japan), and kept at 4°C until use.

3.3.4.2 Organic extraction from test spices

Ten grams of spice powders were extracted with 100 ml of organic solvents; ethyl acetate (Carlo erba, France), methanol (Carlo erba, France) and ethanol (Carlo erba, France) and left at room temperature overnight. The corresponding suspensions were filtered through Whatman filter paper No.1 (Struers Kebo Lab, Denmark). The filtrates were dried by using a rotary evaporator (BUCHI Rotavapor R-114, Switzerland) at 40°C for 6 h and kept at 4°C until used (Achararit et al., 1983; Caichompoo, 1999; Harborne, et al. 1975 with some modifications).

3.3.5 Test for antimicrobial activities of extract components

Antimicrobial activities of the aqueous extracts against test bacteria were examined by the broth dilution method, whereas those of the ethyl acetate, methanol and ethanol extracts were tested by the agar disc diffusion assay (modified from Caichompoo, 1999; Shimizu et al., 2000).

3.3.5.1 Broth dilution method

The lyophilized components from the aqueous extracts of selected spices were dissolved in sterile distilled water to a final concentration of 5 mg/ml for galangal, ginger, turmeric, and krachai, then sterilized by filtration through a membrane filter (MILLEX®-GV, Millipore, S.A., 67120, Molsheim, France, pore size = 0.22 μm). Each original solution was serially diluted in sterilized NB to 1, 1:1, 1:3, 1:7, 1:15, 1:31 and 1:63. One ml of the diluted solution was mixed with 1 ml of NB culture of test bacteria of which absorbance was adjusted to 0.03 at 600 nm by using spectrophotometer (Beckman, DU®530, Live Science, UV/Vis Spectrophotometer) that corresponds to ca. 10^8 CFU per ml (original suspension). The mixture was incubated at 37°C for 24 h. One hundred microliters of the mixture were subjected to the absorbance measurement at 600 nm by using a spectrophotometer (Beckman, DU®530, Live Science, UV/Vis Spectrophotometer). Growth of test bacteria in each mixture was determined at absorbance of 600 nm by using spectrophotometer and compared to normal cells and mixture of NB and spice extracts (Figures 3.1 and 3.2). The inactivation effect was assessed by comparing absorbance of normal cell suspension (control) and bacteria treated spice extracts suspension (Marino, Bersani, and Comi, 2001; Sagdic,

Kuscu, Ozcan, and Ozcelik, 2002). The percent inactivation was calculated as

$$\frac{(\text{control population} - \text{treated population}) \times 100}{(\text{control population})}$$

3.3.5.2 Agar disc diffusion assay

Single colony of the respective test bacteria was transferred into 5 ml of NB and incubated at 35°C for 18-24 h. Three milliliter of each culture was mixed with 100 ml of melted NA at about 45°C and poured onto the surface of an agar plate containing 2% agar.

Organic extracts (80 µl) was loaded onto sterile filter paper discs (∅ 8 mm, Advantec, Tokyo, Japan) twice with air-drying in between. Control disc was similarly prepared using distilled water, ethyl acetate, 100% methanol or 100% ethanol. Antibiotics disc: Chloramphenicol (30 µg), vancomycin (30 µg), Nalidixic acid (30 µg) and Sulfamethoxazole (23.75 µg) - trimethoprim (1.25 µg) were used as controls. Each of the discs was placed on the surface of bacterial culture plates and incubated at 35°C for 18-24 h. Inhibition zones (including the diameter of disc) were measured and recorded.

3.3.5.3 Determination of Minimum Inhibitory Concentration (MIC)

S. aureus was cultured in 5 ml of Trypticase Soy Broth (TSB, Difco, USA), and incubated in a shaker at 35°C for 18-24 h. The turbidity of the bacteria was adjusted to the standard of McFarland No.0.5 with 0.85-0.9% w/v sterile sodium chloride solution to achieve a concentration of approximately 10^8 CFU/ml (Appendix A). The final

concentration of the cell number of approximately 10^6 CFU/ml was obtained by being diluted 100 times with sterile sodium chloride solution. One milliliter of the bacterial suspension at 10^6 CFU/ml was transferred into 1 ml of Mueller Hinton Broth (MHB, Difco, USA).

The GMet and GEt were diluted in 100% methanol and ethanol, respectively to 1, 1:1, 1:3, 1:7, 1:15 (v/v), and 80 µl was loaded onto sterile filter paper discs (∅ 8 mm, Advantec, Tokyo, Japan) twice with air-drying in between. Infiltrated paper disc (160 µl) was submerged into 1 ml of MHB. Then, 1 ml of test bacteria (ca. 10^6 CFU/ml) was added and incubated with shaking at 35°C for 18-24 h. The minimum inhibitory concentration (MIC) of the GMet and GEt were regarded as the lowest concentration of dilutions that were not permit any turbidity of the tested microorganism compared to fresh MHB (modified from Caichompoo, 1999; Lorian, 1995).

3.3.5.4 Determination of Minimum Bactericidal Concentration (MBC)

All the tubes were used in the MIC studies from 3.2.5.3 that did not show any turbidity of the bacteria and the last tube with turbidity were determined for MBC. Aliquot 0.1 ml of the suspensions was spread onto Tripticase soy agar (TSA, Difco, USA) and incubated at 35°C, 18-24 h. The MBC was the lowest concentration which the initial inoculum was killed as 99.9% or more which was followed by the NCCLS method (Lennette et al., 1991; Lorian, 1995).

3.3.6 Effects of antimicrobial galangal extracts on growth of *S. aureus*

3.3.6.1 Antimicrobial activities of galangal ethanol extract against other microorganisms

GMet and GEt were selected for determining antimicrobial activity against other microorganisms e.g. *Staphylococcus epidermidis* TISTR No. 518, *Bacillus cereus* TISTR No. 687, *Bacillus megaterium*, *Streptococcus lactis*, *Salmonella* sp., *Enterobacter aerogenes*, *Pseudomonas aeruginosa* and *Saccharomyces cerevisiae* by using modified agar disc diffusion method as previously described.

3.3.6.2 Establishment of growth curve of test bacteria

A single colony of *S. aureus* on NA plate was transferred into 2 ml of LB broth, incubated in a water-bath shaker (Taitec, Personal-11, Japan) at 35°C for 18-24 h to prepare the original bacterial suspension. Twenty microliters of the original suspension was transferred into 2 ml of fresh LB broths, incubated in a water-bath shaker at 35°C. Growth of this bacterium was estimated from absorbance reading at 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 24 h by using a spectrophotometer (Beckman, DU®530, Live Science, UV/Vis Spectrophotometer) at 600 nm. From the growth curve, the log phase of the growth was determined for further experiments.

3.3.6.3 Effects of antimicrobial galangal extract on growth of *S. aureus*

S. aureus was cultured into 2 ml of LB broth at 35°C overnight in water-bath shaker (Taitec, Personal-11, Japan). Twenty microliters of the bacterial suspension was transferred to 2 ml of fresh LB broth. The absorbance reading at 600 nm of the suspension

by spectrophotometry (Beckman, DU®530, Live Science, UV/Vis Spectrophotometer) showed that log phase of this bacterium at 4-9 h after initiation of culture. Based on this result, it was decided that this bacteria should be treated by the GMet and GEt at 5 h (mid log phase of bacterial growth) after initiation of incubation which corresponds to the intermediate of the log phase of growth. GMet (1.20 mg/ml) and GEt (0.40 mg/ml) at 160 µl were loaded onto sterile filter paper discs. Methanol, ethanol extract and sterile deionized water were used as controls. One disc was submerged into one test tube of the above bacterial suspension at 5 h after initial culture incubation. This suspension was shake-cultured at 35°C continuously at 0, 2, 6, 12 and 24 h for GMet and at 0, 2, 6, 16 and 24 h for GEt, respectively. Survival of bacteria in each test tube was examined by spread plate method.

3.3.7 Determination of compounds of galangal methanol and ethanol extract

The chemical compounds of galangal methanol and ethanol extract were analyzed by Thin Layer Chromatography (TLC), Gas Chromatography-Mass Spectrophotometry (GC-MS) (Network GC System, 6890N, USA) and Nuclear Magnetic Resonance (^{1}H-NMR and ^{13}C-NMR) were used to determined chemical structures.

3.3.7.1 Thin Layer Chromatogram (TLC) compared with D, L-1'-acetoxychavicol acetate standard

The major components of methanol and ethanol extracts were isolated by preparative TLC. The concentrated galangal methanol and ethanol extract solutions and D, L-1'-acetoxychavicol acetate standard (LKT Laboratory, USA) were spotted on a silica gel_{60} GF_{254} (Merck, Darmstadt, Germany). TLC plate and chromatographed using

toluene: ethyl acetate (9:1) as a mobile phase were then, sprayed with vanillin-H_3PO_4 reagent prepared by dissolving 1 g vanillin in 25 ml 100% EtOH, 25 ml water and 35 ml 85% H_3PO_4. The immersed plate was warmed at 105°C for 2-3 min in order to determine of essential oil compounds (Jork, Funk, Fischer and Wimmer, 1990). The major galangal methanol and ethanol extract compounds were confirmed by TLC comparing to D, L-1'-acetoxychavicol acetate standard and reference R_f values.

3.3.7.2 Gas Chromatography-Mass Spectrometry (GC-MS)

The compounds and structures of major GMet and GEt were analyzed by GC-MS (Network GC System, 6890N, USA). Chromatographic column used was 30.0 m Agilent 19091S-433, capillary column with a diameter 250.00 μm and a film thickness of 0.25 μm. Chromatographic conditions were as follows: split ratio; 10:1 carrier gas: helium; initial oven temperature 100°C; initial time 3 min; equilibrium time 2 min; injection volume 1 μl. Mass spectrometer condition (Agilent 5973 Network Mass selective detector): ion source 230, threshold 100 eV.

Percentage of each compound was calculated as the ratio of the peak area to the total chromatographic area. The identification of the major peak was compared to the standard reference D, L-1'-acetoxychavicol acetate (LKT Laboratory, USA) and minor compounds to software chemstation, library search: Wiley (Appendix B).

3.3.7.3 Nuclear Magnetic Resonance Spectrometer (NMR)

The structures of major galangal methanol extract and ethanol extract were confirmed by ^{1}H-NMR, ^{13}C-NMR (Nuclear Magnetic Resonance Spectrometer; Inova 300) using Deuterate ($CDCl_3$) as a solvent, compared with standard reference D, L-1'-

acetoxychavicol acetate (LKT Laboratory, USA) (Mitsui et al., 1976; Noro et al., 1988).

3.3.8 Statistics

The triplicate data were statistically analyzed by the variance analysis and the mean separated according to LSD test with significance level (p) of 0.05 using SPSS 11.2 programs.

3.4 Results and discussion

3.4.1 Growth of test bacteria

The prime objective was to examine growth of test bacteria. There are two major methods to obtain growth curves of bacteria: reading optical density of bacterial suspension and estimating by colony forming units per milliliter (CFU/ml). The former method is simple but absorbance reading of the suspension reflects the population of both living and dead bacteria, while the latter is time- and labor-consuming but reflects only the population of living bacteria only. Therefore, in this section the relationships of absorbance readings at 600 nm and CFU/ml counts for *S. aureus* and *E. coli* were performed.

The bacterial suspension of *S. aureus* and *E. coli* were determined the absorbance of cell by measuring spectrometry and enumerated the viable cell number by the agar plate count. It was indicated that relative coefficients of these factors were 0.758 for *S. aureus* (Figure 3.1) and 0.720 for *E. coli* (Figure 3.2). For the former bacterium, less than 0.02 absorbance reading was not reliable for this correlation as indicated in Figure 3.1.

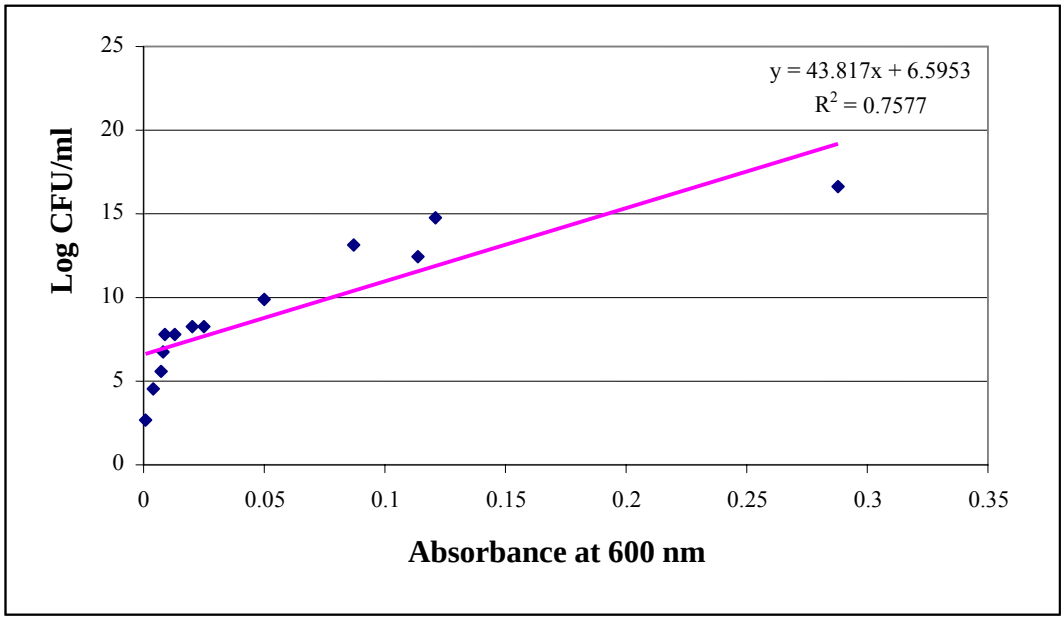

Figure 3.1 Correlation between absorbance reading and CFU/ml of *S. aureus*.

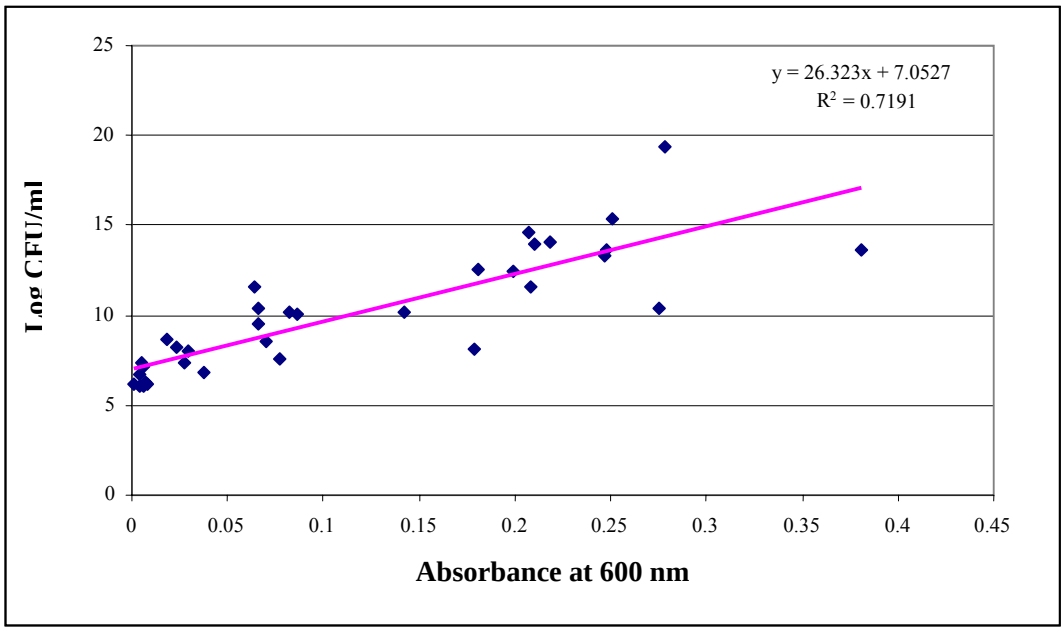

Figure 3.2 Correlation between absorbance reading and CFU/ml of *E.coli*

3.4.2 Inhibitory effects of water extracts of test spices on test bacteria

The percent inactivation of *S. aureus* and *E. coli* by the different concentrations of galangal, ginger, turmeric and krachai water extracts were reported as shown in Tables 3.1 and 3.2.

It indicated that the presence of the spice extracts at different concentrations tested produced increase or decrease in percent inactivation of growth of bacteria. Percent inactivation of the spice extracts against microorganisms appeared to depend on the type and concentration of the extracts and the type of microorganisms tested (Marino, Bersani, and Comi, 1999).

Percent inactivation of galangal, ginger, turmeric and krachai extracts at 5.00 mg/ml against *S. aureus* and *E. coli* were higher than other concentrations of the extracts. All spice extracts at 5.00 mg/ ml had good inhibitory effect against both selected bacteria. Ginger and krachai extracts had strong antimicrobial effect against *E. coli*. All concentrations (except 0.63 mg/ml) of ginger water extracts were able to inhibit the growth of *S. aureus*. *E. coli* was sensitive to ginger water extracts at 1.25 mg/ml to 5.00 mg/ml. Concentration of Krachai at 1.25 mg/ml to 5.00 mg/ml showed good inhibitory against the growth of *E. coli*.

Table 3.1 Inactivation (%) of various concentrations of extracts against *S. aureus*

Concentration of extracts (mg/ml)	% Inactivation			
	galangal	ginger	turmeric	krachai
0.63	-35.55± 29.47	-10.57±10.52[a]	-79.68±31.96	-137.63±66.07[b]
1.25[ns]	-51.99±61.05	0.08±1.69	-17.58±52.48	-40.95±23.18
2.50[ns]	-48.84±41.20	5.08±3.75	-49.72±29.46	-36.15±4.11
5.00[ns]	24.61±1.95	45.66±3.73	46.42±19.13	25.48 ±28.36

Mean in the row followed by different letters are significantly different at $p< 0.05$ according to LSD test

[ns] in the same row are nonsignificant different $p< 0.05$

The percent stimulation = negative activation (percent)

Table 3.2 Inactivation (%) of various concentrations of extracts against *E. coli*

Concentration of extracts (mg/ml)	% Inactivation			
	galangal	ginger	turmeric	krachai
0.63	-83.33±36.61[b]	1.88±15.15[a]	-2.42±10.50[a]	-26.34±49.64[ab]
1.25	-103.55±50.15[b]	1.05±6.07[a]	-3.67±12.58[a]	10.31±16.17[a]
2.50	-69.86±50.65[b]	0.50±12.67[a]	-2.18±13.59[a]	14.77±14.26[a]
5.00	10.28±4.51[b]	38.68±18.46[a]	61.12±5.85[a]	53.02±13.40[a]

Mean in the row followed by different letters are significantly different at $p< 0.05$ according to LSD test

The percent stimulation = negative inactivation (percent)

At concentration less than 5.00 mg/ml of galangal, turmeric and krachai extracts stimulated the growth of *S. aureus*. All concentrations (except at 5.00 mg/ml) of galangal and turmeric extracts had a stimulatory effect on growth of *E. coli*. At low concentration of spice extracts both *S. aureus* and *E. coli* showed an increase in percent stimulation possibly due to crude water extracts contained polysaccharides, polypeptides and others (Cowan, 1999) which were necessary nutrients to supporting the metabolism of the cells.

3.3.2 Effects of methanol, ethanol and ethyl acetate extracts of test spices on growth of test bacteria

Table 3.3 Width of growth inhibition zone on *S. aureus* and *E. coli* caused by methanol, ethanol and ethyl acetate extracts of spices.

Spices	Extracts	Width of growth inhibition zone (mm)	
		S. aureus 209P	*E. coli* NIHJ JC-2
Galangal	Methanol	22.33 ± 1.15 (S^a)	0 (R^a)
	Ethanol	22.33 ± 0.58 (S^a)	0 (R^a)
	Ethyl acetate	24.00 ± 0.00 (S^a)	0 (I^a)
Ginger	Methanol	14.50 ± 0.00 (S^a)	10.00 ± 0.00 (I^a)
	Ethanol	11.00 ± 0.00 (I^a)	0 (R^a)
	Ethyl acetate	14.50 ± 0.00 (S^a)	0 (R^a)
Turmeric	Methanol	10.00 ± 0.00 (I^a)	0 (R^a)
	Ethanol	10.00 ± 0.00 (I^a)	10.00 ± 0.00 (I^a)
	Ethyl acetate	12.00 ± 0.00 (I^a)	10.00 ± 0.00 (I^a)
Krachai	Methanol	12.50 ± 0.00 (I^a)	0 (R^a)
	Ethanol	11.00 ± 0.00 (I^a)	0 (R^a)
	Ethyl acetate	12.00 ± 0.00 (I^a)	0 (R^a)
Control	Methanol	0 (R^a)	0 (R^a)
	Ethanol	0 (R^a)	0 (R^a)
	Ethyl acetate	0 (R^a)	0 (R^a)
	Deionized water	0 (R^a)	0 (R^a)
Antibiotics	Chloramphenicol	14.00 ± 0.00 (S^a)	0 (R^a)
	Nalidixic acid	0 (R^a)	21.75 ± 2.47 (S^a)
	Vancomycin	18.00 ± 0.00 (S^a)	0 (R^a)
	Sulfamethoxasole-Trimethoprim (SXT)	0 (R^a)	23.00 ± 0.00 (R^a)

(Lorian, 1995)

[a] Interpretation into categories of susceptibility is achieved by comparing zone sizes of test organisms with appropriate control organisms on the same test plate.

R, resistant: ≤ 9 mm; I, intermediate: ≥ 10-13 mm; S, susceptible: ≥ 14 mm.

Table 3.3 summarized widths of growth inhibition zones appeared around the paper discs. Gram positive *S. aureus* was more sensitive to all tested spice extracts than the gram negative *E. coli*. Among the extracts, GMet and Get showed the same result to both bacteria. Galangal ethyl acetate extract had inhibitory effect against *S. aureus* than *E. coli.* The ginger extracts had more inhibitory effect against *S. aureus* than *E. coli*. *E. coli* was not shown sensitivity to all of the spice extracts. All of krachai extracts were effective to only *S. aureus*. The ethanol and ethyl acetate extracts of turmeric were similarly inhibitory to both bacteria but its methanol extract had an effect against only *S. aureus*. In this study, Antibiotics disc: chloramphenicol and vancomycin were used for positive control for gram positive bacteria sensitivities and Nalidixic acid and sulfamethoxazole-trimethoprim were used to test susceptibility of gram negative bacteria (Walker, 1998). The results of spice extracts and antibiotics showed that the inhibitory effects against bacteria depend on solvent used for extraction from plant material, type and concentration of spice extracts, which relevant to many publications documented that antimicrobial activity of the essential oils and plant extracts appeared to depend on the type and concentration of the oil, the type of microorganisms tested (Burt, 2004; Marino, Bersani, and Comi, 1999). These reasons can explain that galangal ethanol extract had the most an inhibitory effect against *S. aureus* probably due to the higher concentration of active compounds than another extracts.

Agar and broth dilution methods are commonly used to test antimicrobial effect from plant extracts (Hammer, Carson, and Riley, 1999). The method frequently used for screening plant extracts for antimicrobial activity is the agar disc diffusion technique (Smith-Palmer, Stewart, and Fyfe, 1998). Therefore, the organic galangal, ginger, turmeric and krachai extracts were screened their antimicrobial activities against selected

foodborne pathogens by using agar disc diffusion assay. Due to these results (Table 3.3), the GMet and GEt were selected to determine MIC and MBC (Table 3.4). The figures 3.3-3.7 showed growth inhibition zones of *S. aureus* caused by the Zingiberaceae extracts.

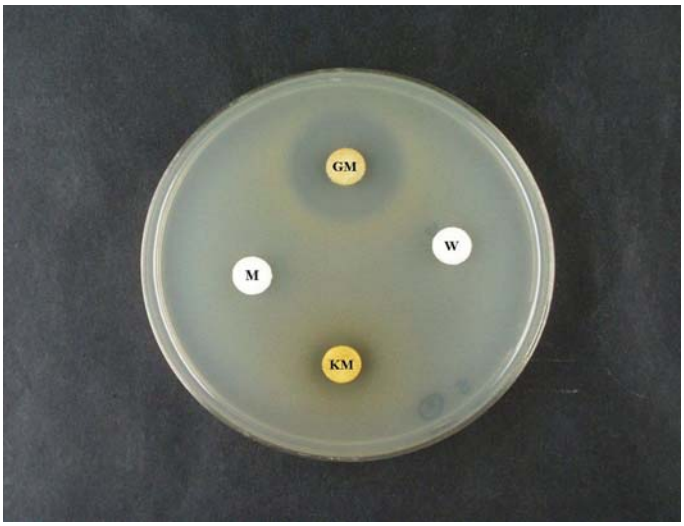

Figure 3.3 Growth inhibition zones of *S. aureus* caused by the air-dried galangal methanol extract (GM), air-dried krachai methanol extract (KM). M: air-dried methanol only, W: air-dried deionized water only.

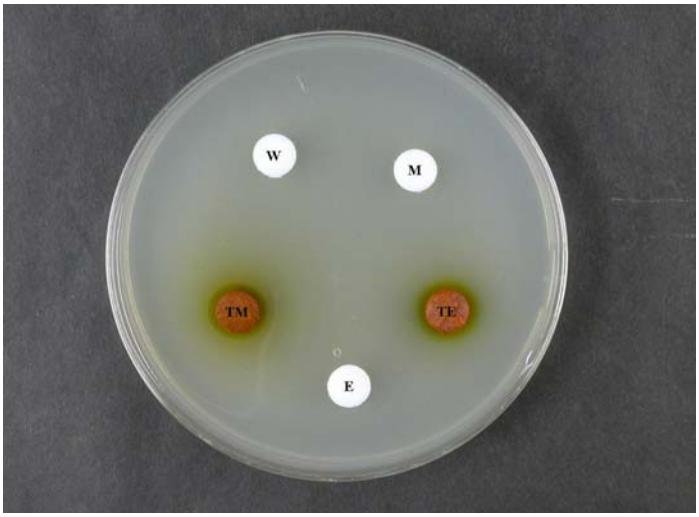

Figure 3.4 Narrow growth inhibition zones of *S. aureus* caused by the air-dried turmeric methanol extract (TM), air-dried turmeric ethyl acetate extract (TE), M: air-dried methanol only, W: air-dried deionized water only, E: air-dried ethyl acetate only.

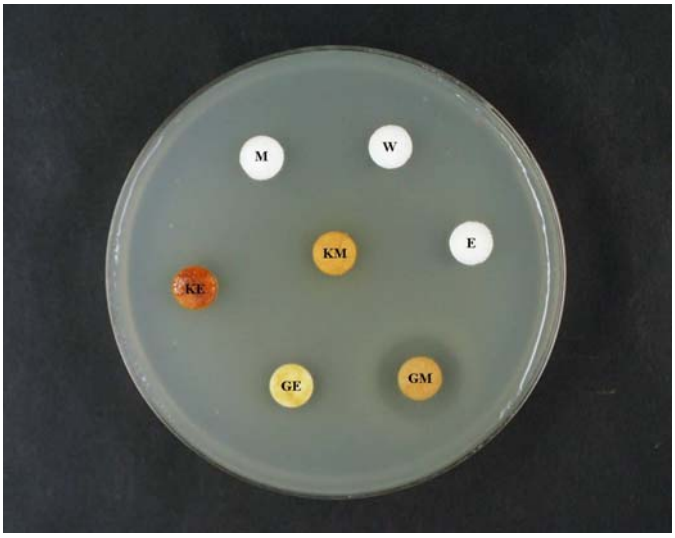

Figure 3.5 Growth inhibition zones of *E. coli* surrounding the air-dried galangal methanol extract (GM), air-dried galangal ethyl acetate extract (GE), air-dried krachai methanol extract (KM), air-dried krachai ethyl acetate extract (KE), M: air-dried methanol only, W: air-dried deionized water only, E: air-dried Ethyl acetate only.

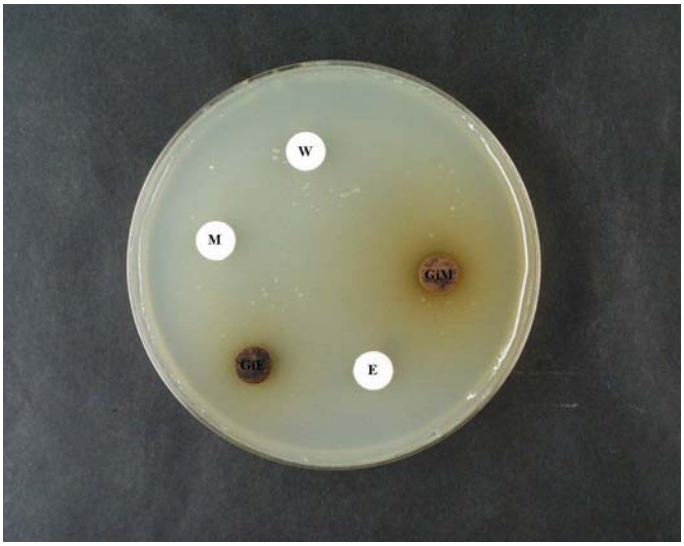

Figure 3.6 Growth inhibition zones of *E. coli* around the air-dried ginger methanol extract (GM), air-dried ginger ethyl acetate extract (GiE). M: air-dried methanol only, W: air-dried deionized water only, E: air-dried ethyl acetate only.

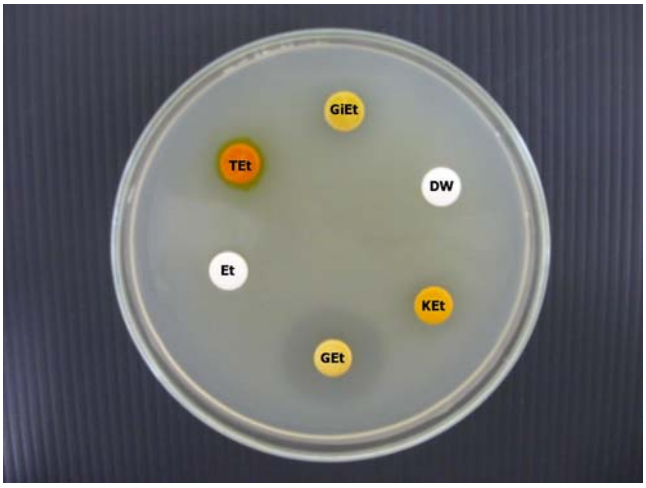

Figure 3.7 Growth inhibition zones of *S. aureus* caused by the air-dried galangal ethanol (GEt), air-dried ginger ethanol (GiEt), air-dried turmeric ethanol (TEt), air-dried krachai ethanol (KEt). Et: air-dried ethanol only, DW: air-dried deionized water only.

3.4.3 Minimum Inhibitory Concentration (MIC)

GMet and GEt were selected to study MIC and MBC against *S. aureus.* MIC of GMet was 0.800 mg/ml and MBC was 1.600 mg/ml. MIC and MBC of GEt were lower than GMet (Table 3.4) which shown at 0.325 mg/ml and 1.300 mg/ml for MIC and MBC subsequently.

Table 3.4 Mininum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against *S. aureus* caused by galangal methanol and ethanol extracts.

Extracts	Mininum inhibitory concentration (MIC) (mg/ ml)	minimum bactericidal concentration (MBC) (mg/ ml)
Galangal methanol (GMet)	0.800 ± 0.02^{a}	1.600 ± 0.02^{a}
Galangal ethanol (GEt)	0.325 ± 0.018^{b}	1.303 ± 0.0004^{b}

Mean in the column followed by different letters are significantly different at $p < 0.05$ according to t-test

Because of agar disc diffusion technique is limited to the generation of preliminary, qualitative data only, as the hydrophobic nature of most essential oils and plant extracts prevents the uniform diffusion of these substances through agar medium (Hammer et al., 1999). Gel matrix of agar medium dramatically reduced the inhibitory effect of the oil. This was presumed to be due to the limitation of diffusion by the structure of the gel matrix (Skandamis, Tsigarida, and Nychas, 2000). Therefore, broth dilution method was selected to determine MIC and MBC of galangal extracts against *S. aureus.*

The results obtained from agar disc diffusion assay and broth dilution method differ as many factors between assays (Janssen, Scheffer, and Svendsen, 1987). These

factors were differences in microbial growth, exposure of microorganisms to plant oil, the solubility of oil or oil components (Hammer et al., 1999). Many publications reported that polysaccharides, polypeptides and lecthins commonly contained in crude aqueous extracts (Cowan, 1999), polyacetylenes, terpenoids, sterol, and propolis were obtained in crude ethanol plant extract (Cowan, 1999; Habtemariam, Gray, and Waterman, 1993; De Pasquale, Germano, Keita, Sanoga, and Iauk, 1995), and the methanol plant extracts commonly contained terpenoids, xanthoxyllines, totarol, quassinoids, and phenones (Cowan, 1999; Peres, Monache, Cruz, Pizzolatti, and Yunes, 1997; Taylor, Edel, Manandhar, and Towers, 1996). In previous studies, galangal, ginger, turmeric and krachai extracts contained various and different active compounds (Chrubasik, Pittler, and Roufogalis, in press; Fransworth and Bunyapraphatsara, 1992), therefore, spice water extracts had different antimicrobial effects, against *S. aureus* and *E. coli*. Chen, M. Chang, and T. Chang (1985) indicated that treatment of ginger root with boiling water abolished the antibacterial effect against various microorganisms including *E. coli* and *S. aureus* due to the antibacterial principle was heat labile. Therefore, GMet and GEt were selected as candidates for chemical compounds analysis and mode of action studying.

3.4.4 Antimicrobial activities of galangal extracts

Antimicrobial activities of GMet and GEt are present in Table 3.5. The results indicate that *S. epidermidis* and *Saccharomyces cerevisiae* were susceptible to the extract. The extracts had less antimicrobial activity against *Bacillus cereus* and *Bacillus megaterium* and there was no antimicrobial activity against some gram negative bacteria: *Salmonella* sp., *Enterobacter aerogenes* and *Pseudomonas aeruginosa*. *Saccharomyces cerevisiae* was a primary representative of eukaryotic microorganisms that showed the

susceptibility to the galangal extracts the same as the previous study of Janssen and Scheffer (1985) which indicated that acetoxychavicol acetate had antifungal effect against *Trichophyton mentagrophytes*.

Table 3.5 Width of growth inhibition zone on microorganisms caused by GMet and GEt.

Microorganisms	Width of growth inhibition zone (mm)				
	Galangal methanol	Galangal ethanol	Deionized water	Methanol	Ethanol
Staphylococcus epidermidis	22.33± 1.53 (S^a)	19.67± 0.58 (S^a)	0 (R^a)	0 (R^a)	0 (R^a)
Streptococcus lactis	9.00± 0.00 (R^a)	0 (R^a)	0 (R^a)	0 (R^a)	0 (R^a)
Saccharomyces cerevisiae	22.33± 2.31(S^a)	21.67± 1.16 (S^a)	0 (R^a)	0 (R^a)	0 (R^a)
Bacillus sp.	0 (R^a)	0 (R^a)	0 (R^a)	0 (R^a)	0 (R^a)
Bacillus subtilis	0 (R^a)	0 (R^a)	0 (R^a)	0 (R^a)	0 (R^a)
Bacillus cereus	11.00± 0.00 (I^a)	11.00± 0.00 (I^a)	0 (R^a)	0 (R^a)	0 (R^a)
Bacillus megaterium	11.50± 0.00 (I^a)	11.00± 0.00 (I^a)	0 (R^a)	0 (R^a)	0 (R^a)
Pseudomonas aeruginosa	0 (R^a)	0 (R^a)	0 (R^a)	0 (R^a)	0 (R^a)
Enterobacter aerogenes	0 (R^a)	0 (R^a)	0 (R^a)	0 (R^a)	0 (R^a)
Salmonella sp.	0 (R^a)	0 (R^a)	0 (R^a)	0 (R^a)	0 (R^a)
Methicillin Resistant *Staphylococcus aureus*	0 (R^a)	0 (R^a)	0 (R^a)	0 (R^a)	0 (R^a)

(Lorian, 1995)

[a] Interpretation into categories of susceptibility is achieved by comparing zone sizes of test organisms with appropriate control organisms on the same test plate.

R, resistant: ≤ 9 mm; I, intermediate: ≥ 10-13 mm; S, susceptible: ≥ 14 mm.

These results (Tables 3.3 and 3.5) showed the spice extracts had more inhibitory effect against gram positive than gram negative bacteria as similar to results from most studies investigating on the action of spice and essential oils against food spoilage organisms and foodborne pathogens (Delaquis, Stanich, Girard, and Mazza, 2002; Shelef, 1983; Sivropoulou, Kokkini, Lanaras, and Arsenakis, 1995). The essential oils

from sage, mint, hyssop, and chamomile were marked against gram positive bacteria than gram negative bacteria (Marino et al., 2001). Gram negative organisms are less susceptible to the action of antibacterial agents, perhaps to be expected that they possess an outer membrane surrounding the cell wall (Ratledge, and Wilkinson, 1988). It was different in cell surface hydrophobicity between two groups of bacteria, gram negative bacteria having more hydrophobic surfaces can be offset by the presence of its porin proteins in the outer membrane (Annuk, Hirmo, Turi, Mikelsaar, Arak, and Wadstrom, 1999; Zita, and Hermannson, 1997). The extracts were not able to penetrate through the outer membrane, which was composed of a lipopolysaccharide monolayer surrounding the cell wall that restricts diffusion of hydrophobic compounds (Vaara, 1992; Ratledge and Wilkinson, 1988). The compounds of galangal extracts affected the phospholipids layer, which was a cell wall components of *S. aureus* (gram positive bacteria). The extracts might attach to the lipids partition of cell membrane, and hydrophilic parts of extract act on cell proteins embedded in the cell membrane and interfered with the enzyme located in the cytoplasmic membrane as observed in previous studies of essential oils (Juven, Kanner, Schved and Weisslowicz, 1994; Marquis et al., 2003).

3.4.5 Effects of antimicrobial galangal extracts on growth of *S. aureus*

3.4.5.1 Establishment of growth curve of test bacteria

Figure 3.8 shows the duration time of the log or exponential phase started at the forth to the tenth hours. The mid log phase of *S. aureus* was generally used to study the antimicrobial effects against microorganisms (Simpson, Whittington, Earnshaw, Russell, 1999; Abid, Maalej, and Rouis, 2004). Therefore the fifth hour was selected for further cytological effects of GMet and GEt on growth of *S. aureus* studies.

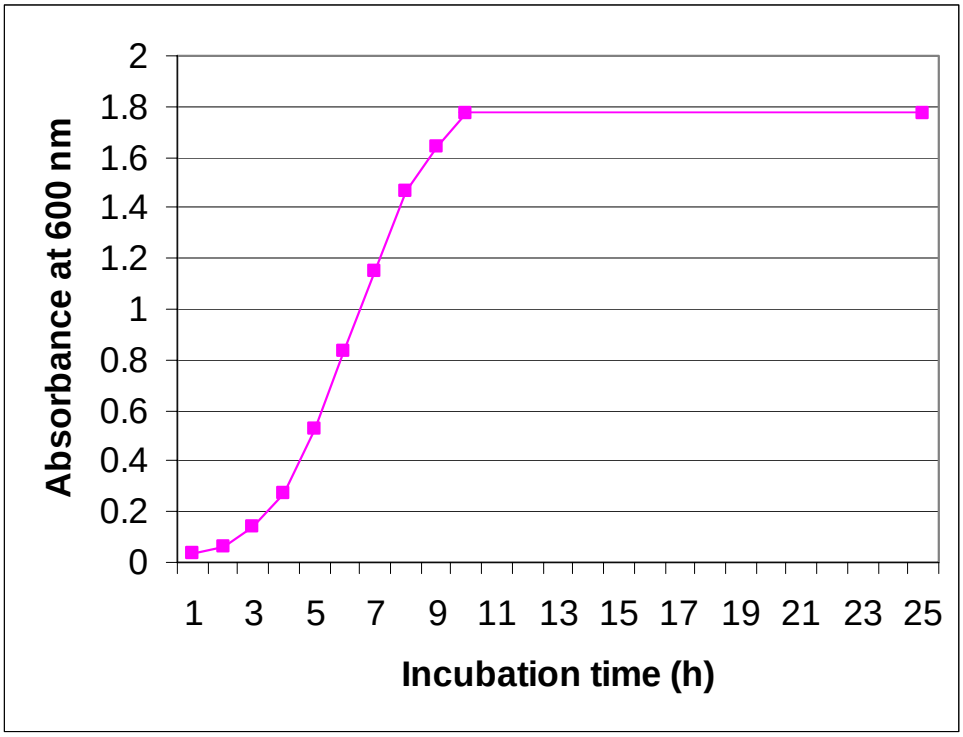

Figure 3.8 The growth curve of *S. aureus* in LB.

3.4.5.2 Effects of antimicrobial galangal extract on growth of *S. aureus*

The exposure time required for causing the bacterial death with the GMet at sublethal concentration were examined at 2, 6, 12 and 24 h, GEt at sublethal concentration were examined at 2, 6, 16 and 24 h, respectively (Figures 3.9 and 3.10). Survival of the bacterium depended on the exposure time with the extracts. As the figures 3.11 and 3.12, the presence of the extracts showed an increase in the exposure times and a reduction in the rate of growth of the selected microorganisms. The extracts caused the delay in the growth of *S. aureus* after exposing at 2 h compared to the control (deionized water or no extracts added). Increasing of exposure time lead to decreasing of cell viability due to the

fact that active compounds in the extracts can attribute to bacterial cells for a long period (Fitzgerald, Stratford, Gasson, Ueckert, Bos, and Narbad, 2004; Marino et al., 2001).

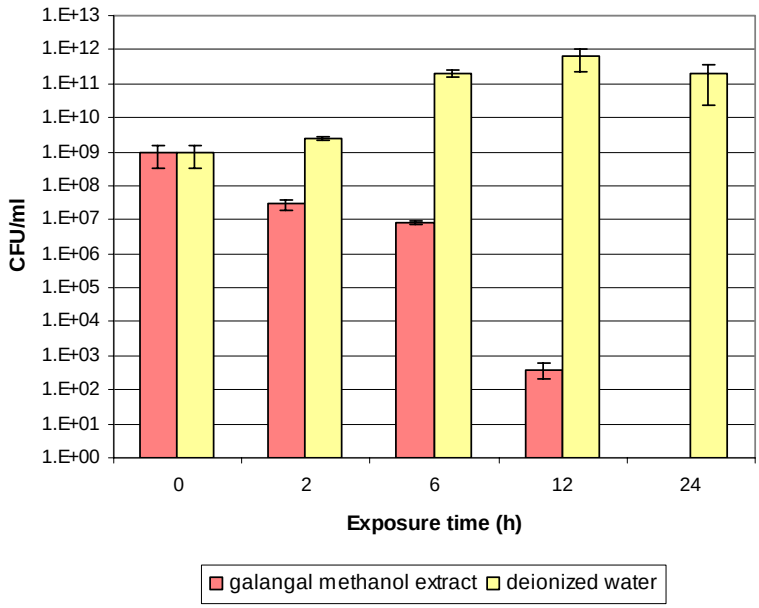

Figure 3.9 Effect of galangal methanol extract on growth of *Staphylococcus aureus* in broth at various exposure times; 0, 2, 6, 12, and 24 h.

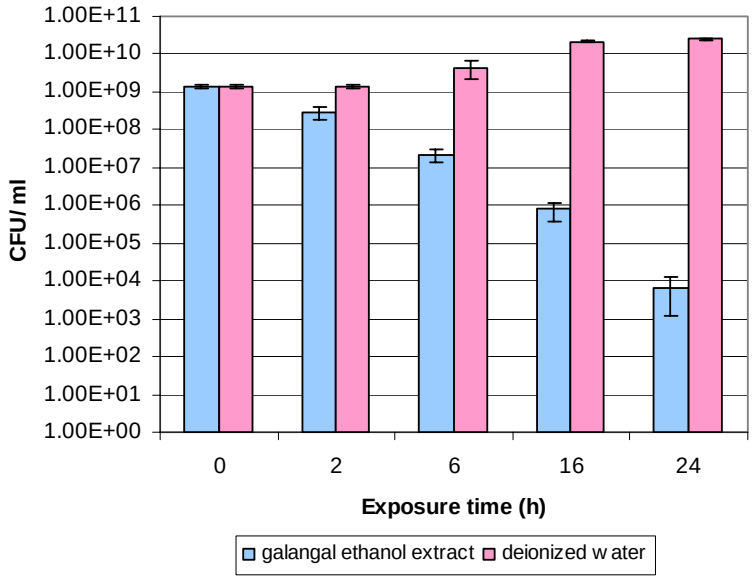

Figure 3.10 Effect of galangal ethanol extract on growth of *Staphylococcus aureus* in broth at various exposure times; 0, 2, 6, 16, and 24 h.

3.4.6 Determination of GMet and GEt compounds

The major components of GMet and GEt showed the same patterns when determined by TLC using silica gel_{60} GF_{254} absorbent (Merck, Darmstadt, Germany) and the solvent system composing of toluene: ethyl acetate (9:1) and sprayed the plate with vanillin-H_3PO_4 reagent and heated at 105˚C for 1-3 minutes (Figure 3.11). In GMet and GEt, D, L-1'- Acetoxychavicol acetate (ACA) was found to be the main constituent which showed purple-red bands. R_f value of GEt is 0.7 and R_f valve of GMet is 0.48 which corresponded to ACA is 0.69 [Figure 3.11 (a)] and 0.45 [Figure 3.11 (b)].

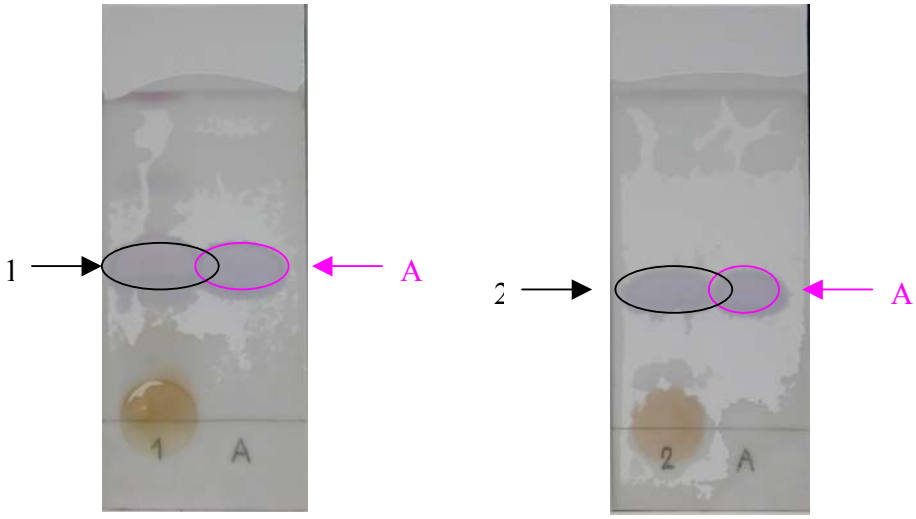

Figure 3.11 Thin layer chromatography of galangal ethanol and methanol extracts

absorbent : silica gel_{60} GF_{254}

solvent system: toluene : ethyl acetate (9:1)

detection : vanillin-H_3PO_4 reagent and heat at 105˚C for 1-3 min

1 = galangal ethanol extract

2 = galangal methanol extract

A = D, L- 1'- acetoxychavicol acetate

3.4.6.1 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS analysis of GMet and GEt showed various components of phenolic compound derivatives, fatty acids and sesquiterpenes. The major and minor components of GMet and GEt were identified by using software chemstation, library search: Wiley (Figure 3.12, 3.13 and Appendix B). The major compound for GMet was shown at peak number 11, 13, and 14 which are identical to D, L- 1'- acetoxychavicol acetate [78.41 % (v/v)] as similar to standard ACA in Figure 3.14, *p*-coumaryl diacetate [6.77 % (v/v)], and 1'-acetoxyeugenol acetate [2.40 % (v/v)], respectively and peak number 2, 4, 6, 7, 8 and 9 for GEt which are identical to D, L- 1'- acetoxychavicol acetate [76.49 % (v/v)], *p*-coumaryl diacetate [7.96 % (v/v)], palmitic acid [3.19 % (v/v), 1'-acetoxyeugenol acetate [3.06 % (v/v)], β-bisabolene [2.31 % (v/v)], and 9-octadecenoic acid [2.28 % (v/v)], respectively (Appendix B).

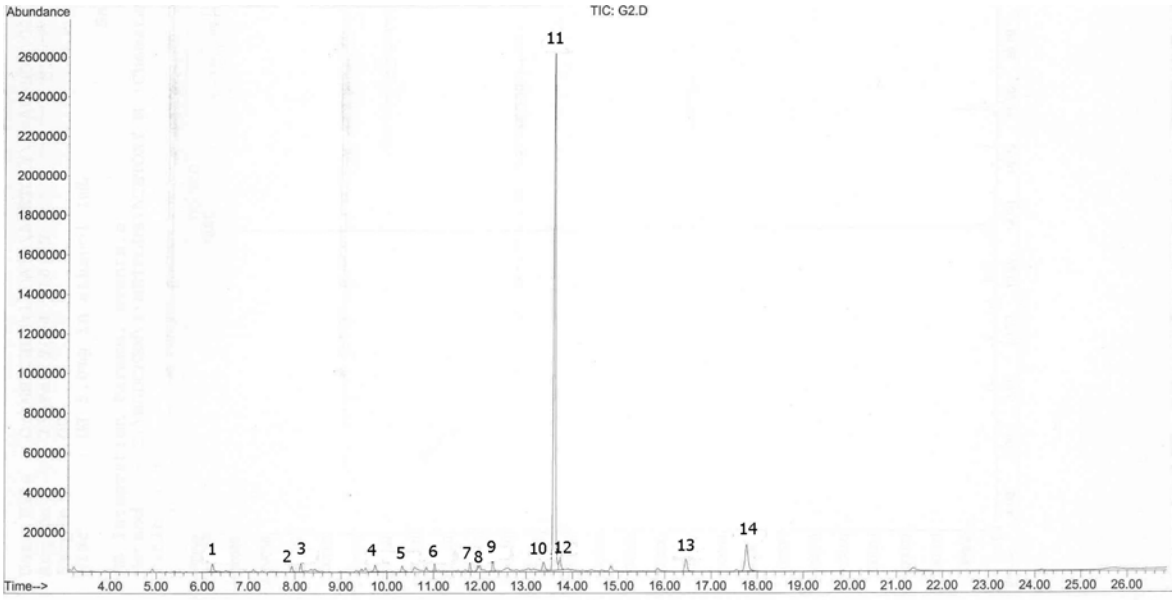

Figure 3.12 Mass chromatogram of GMet.

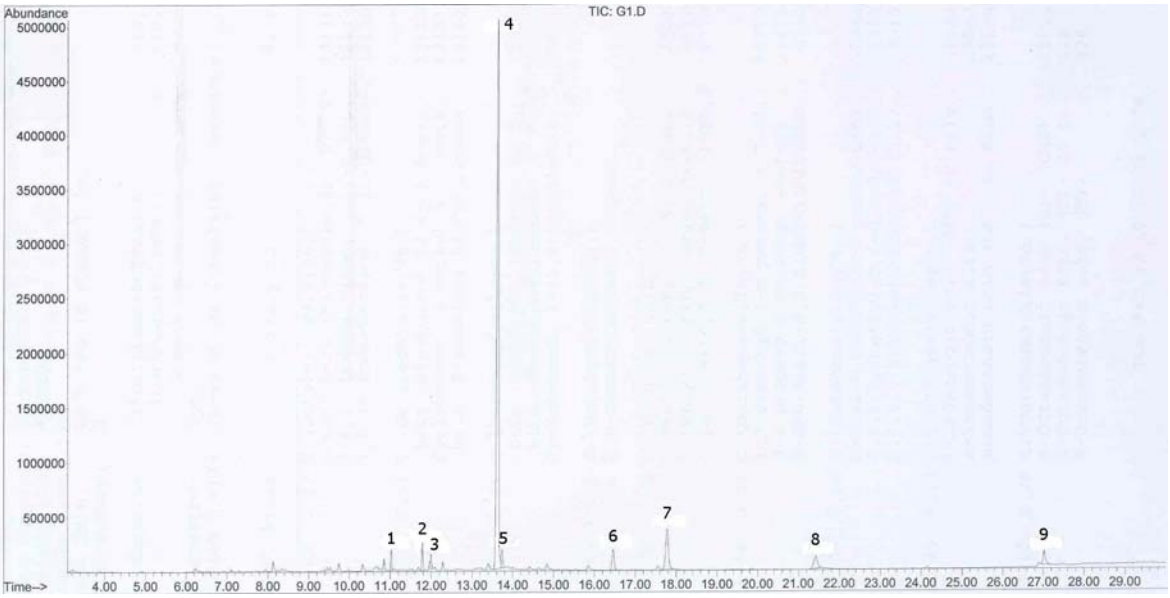

Figure 3.13 Mass chromatogram of GEt.

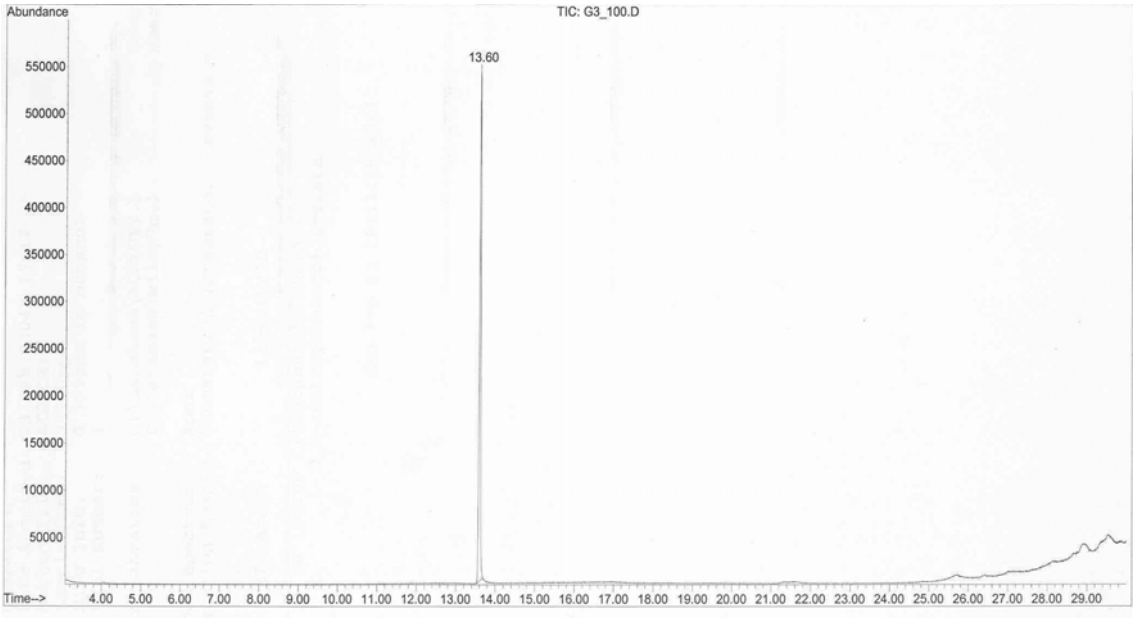

Figure 3.14 Mass chromatogram of ACA.

There was only one major peak detected from the GC-MS analysis of the GMet and GEt, and its corresponding mass spectrum is showed in Figures 3.15 and 3.16. The major peak of the galangal extracts showed molecular ions of m/z 234 which is a molecular ion of the standard ACA. Mass spectra consisted of a series of even mass ions separated by 42 mass units (mu) due to losses of one alkoxyl group (CH_2=C=O), m/z 192/234 and two alkoxyl groups, m/z 150/192, respectively, as shown in Figures 3.15 and 3.16. The mass spectrum of the major compound of galangal extracts shows m/z 132/192, m/z 133/192, m/z 150/192 and m/z 192/234.

1

2

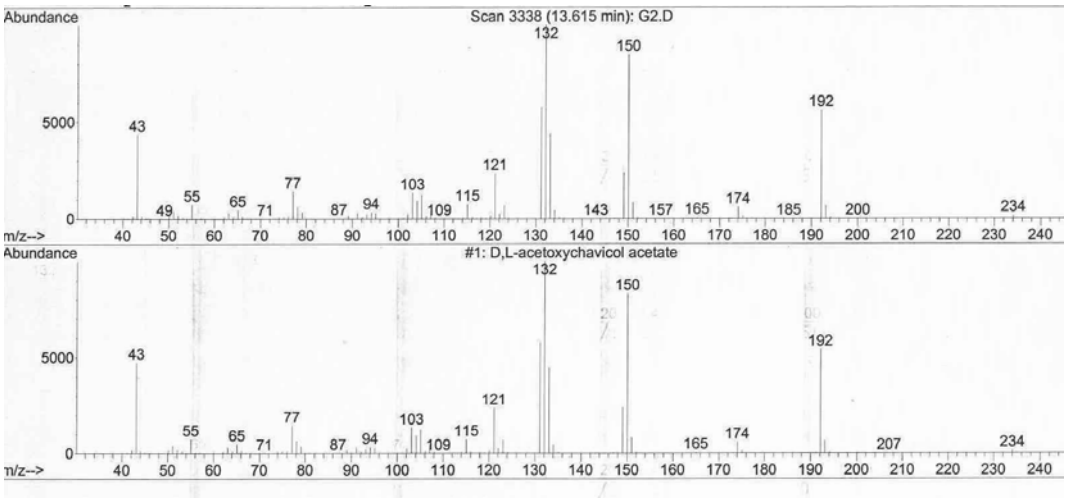

Figure 3.15 Mass spectrum of GMet, GMet (1); ACA (2)

1

2

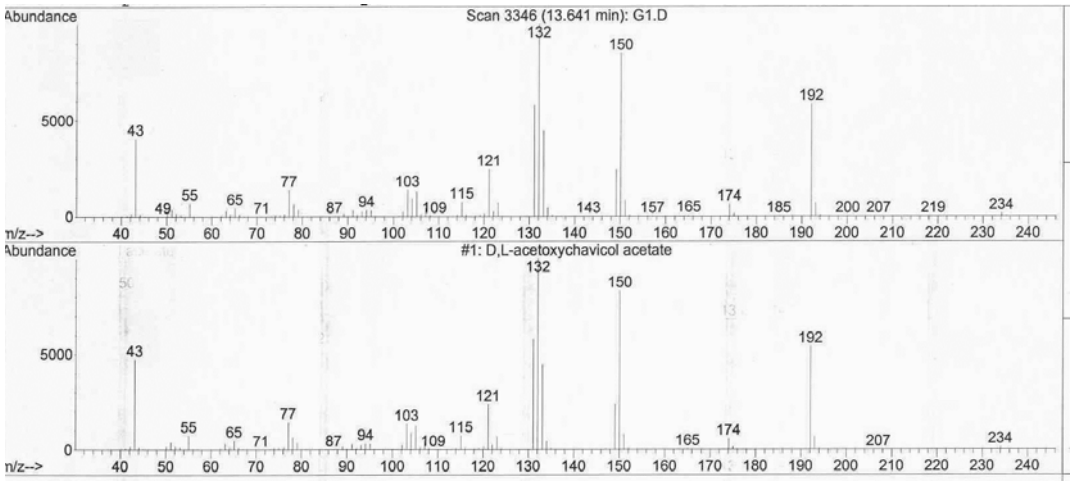

Figure 3.16 Mass spectrum of GEt, GEt (1); ACA (2)

3.4.6.2 Nuclear magnetic resonance spectroscopy (NMR)

GMet and GEt were identified the structure by ^{1}H-NMR, ^{13}C-NMR using $CDCl_3$ as a solvent, comparing to standard references ACA (LKT Laboratory, USA) (Figure 3.20). It showed chemical shift in Figures 3.17, 3.18, 3.19 and 3.20).

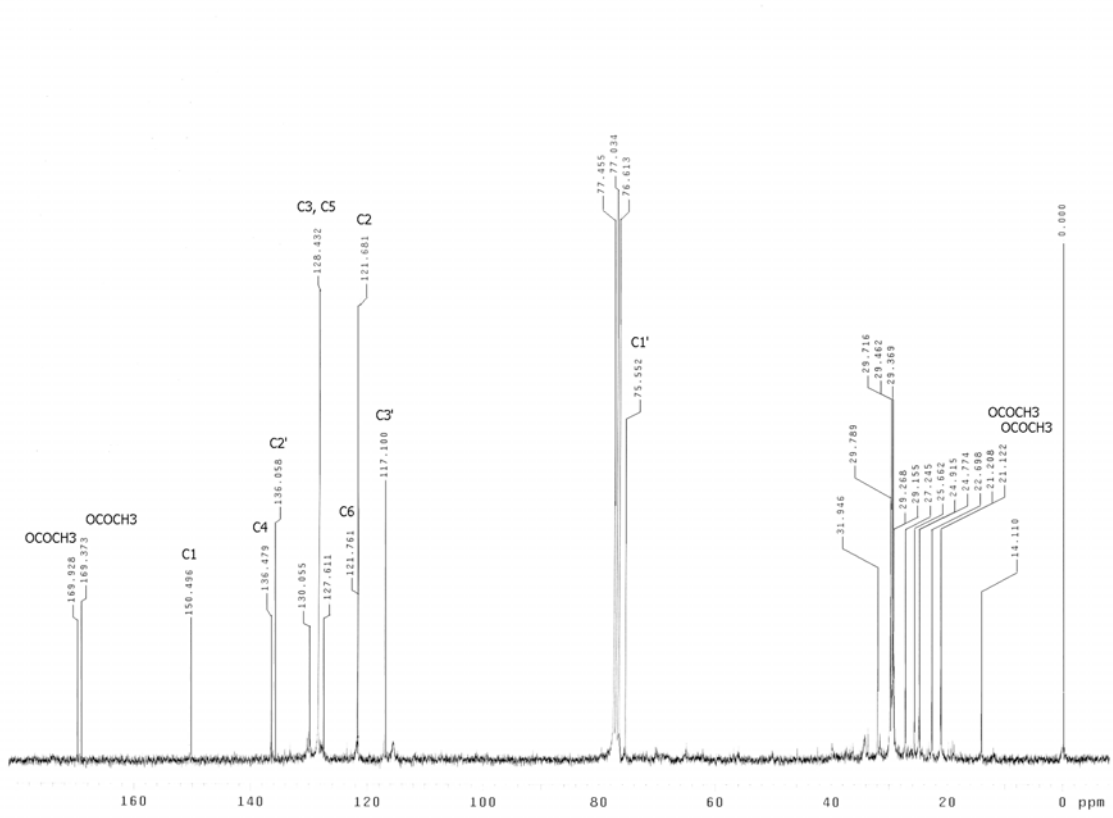

Figure 3.17 ^{13}C-NMR (300 MHz) spectrum of major GEt compound

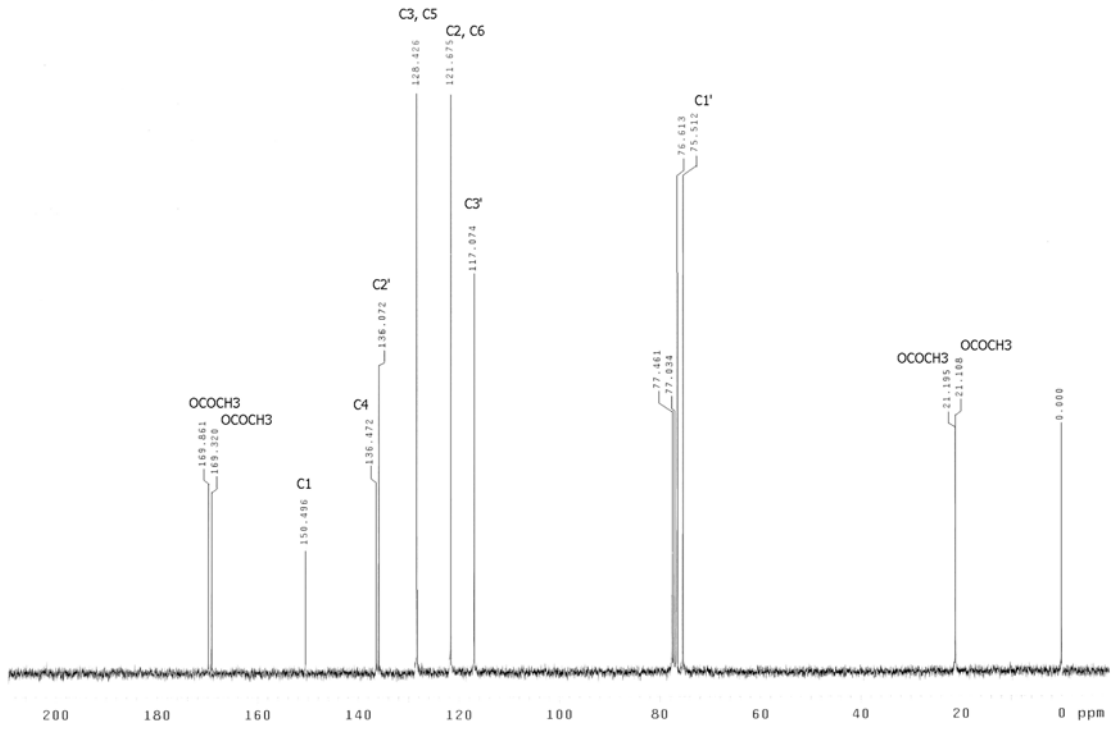

Figure 3.18 ^{13}C-NMR (300 MHz) spectrum of ACA standard.

Comparison of ^{1}H-NMR, ^{13}C-NMR, chemical shifts of each GMet and GEt are shown in Figures 3.17-3.20. Figures 3.19-3.21 showed signal ^{1}H-NMR (300 MHz, $CDCl_3$) δ2.09 (3H, s, 1'-$OCOCH_3$), 2.28 (3H, s, 4-$OCOCH_3$), 5.23 (1H, dd, H-3'a), 5.27 (1H, dd, H-3'b), 5.99 (1H, ddd, H-2'), 6.26 (1H, d, H-1'), 7.06 (2H, d, H-3, 5), 7.36 (2H, d, H-2, 6). Figures 3.19-3.20 showed signal ^{13}C-NMR (300MHz, $CDCl_3$) δ21.108, 21.195 (2s, 2x methyl carbon of the acetyl groups), 75.512 (s, C-1'), 117.074 (s, C-3'), 121.675 (s, C-2 and C-6), 128.426 (s, C-3 and C-5), 136.072 (s, C-2'), 136.472 (s, C-1), 150.496 (s, C-4), 169.320, 169.861 (2s, 2x carbonyl carbon of the acetyl groups).

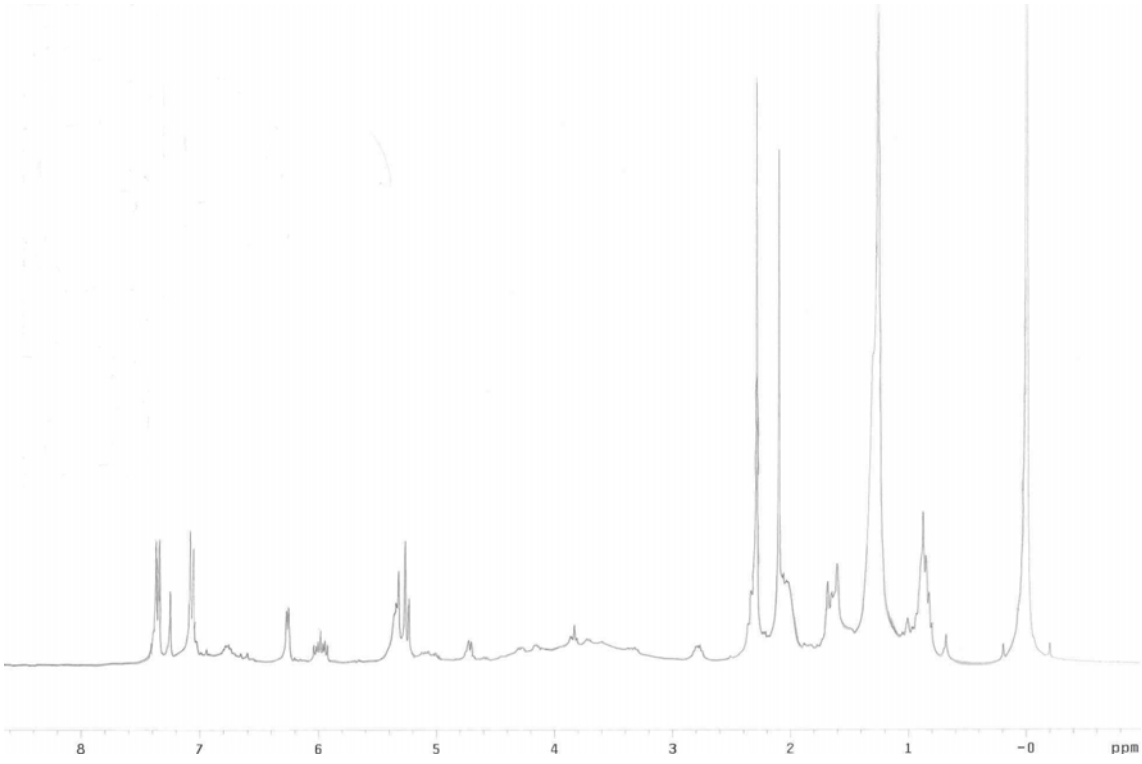

Figure 3.19 ^{1}H-NMR (300 MHz) spectrum of major GEt compound

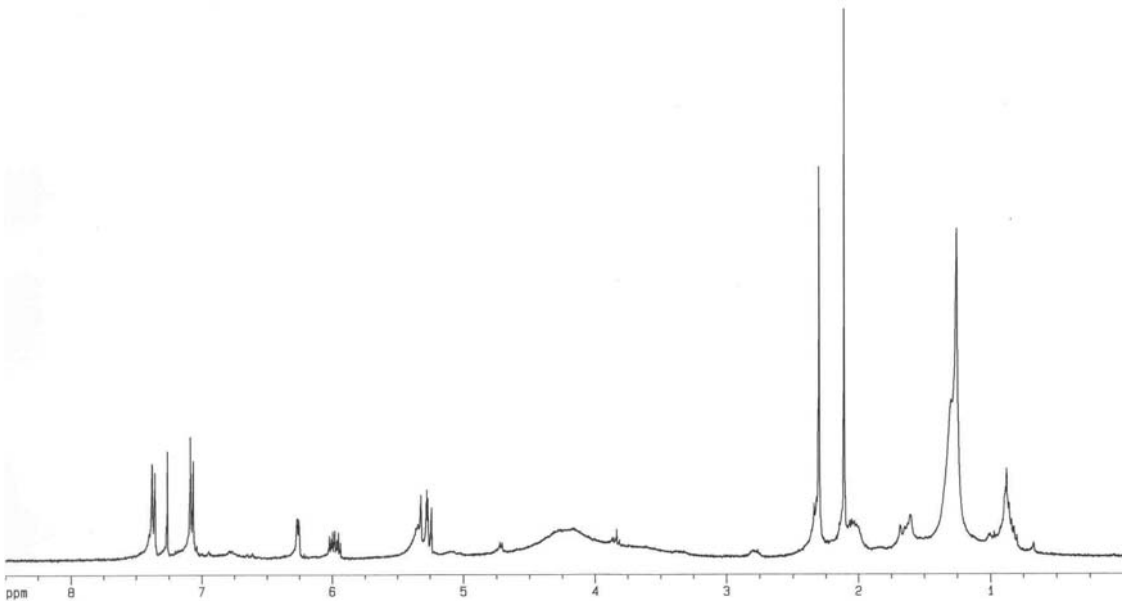

Figure 3.20 ^{1}H-NMR (300 MHz) spectrum of major GMet compound

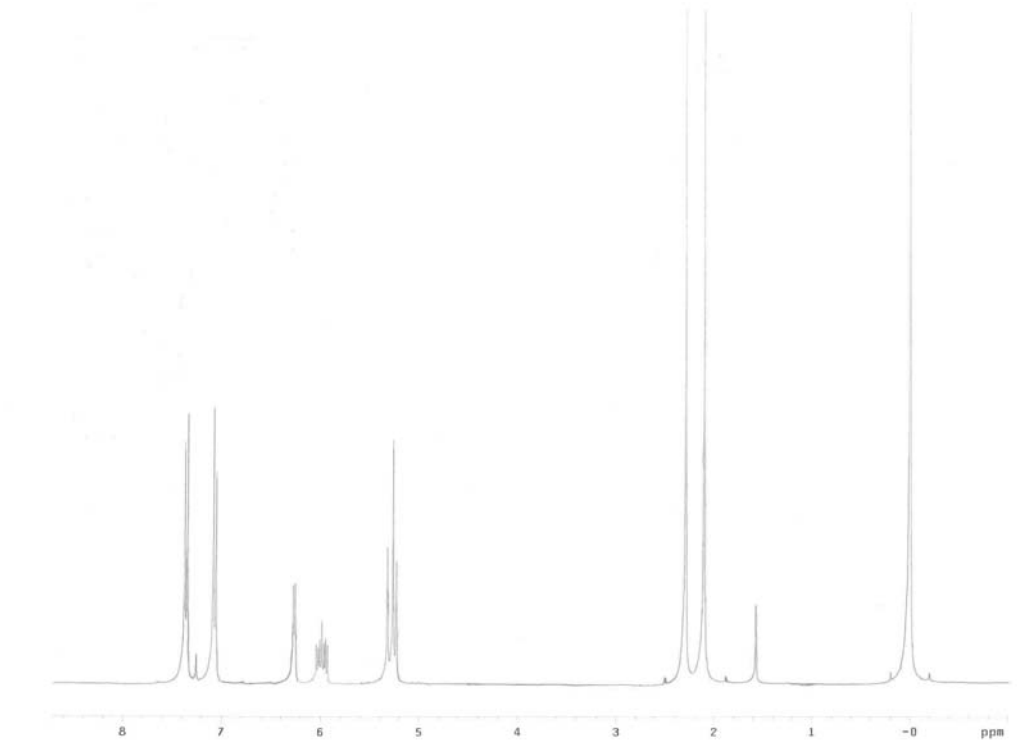

Figure 3.21 ^{1}H-NMR (300 MHz) spectrum of ACA standard

The structure of major compound in galangal ethanol extract was analyzed by the results from GC-MS chromatogram and NMR spectrum as follow below:

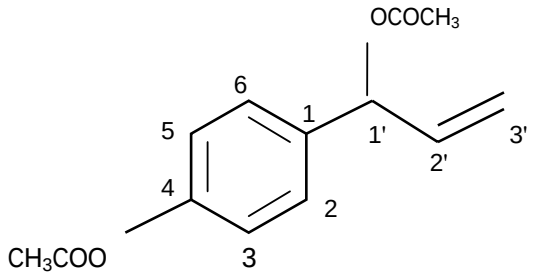

Figure 3.22 D, L-1'-acetoxychavicol acetate (ACA)

3.5 Conclusions

Percent inactivation of galangal, ginger, turmeric and krachai extracts at 5.00 mg/ml against both *S. aureus* and *E. coli* had good inhibitory effects. Ginger and krachai extracts had strong antimicrobial effect against *E. coli*. All concentrations (except 0.625 mg/ml) of ginger water extracts were able to inhibit the growth of *S. aureus*. GMet and GEt had a greater inhibitory effect against *S. aureus* than ginger, turmeric or krachai. Minimum inhibitory concentration (MIC) of GMet and GEt were 0.800 mg/ml and 0.325 mg/ml, respectively. Minimum bactericidal concentration (MBC) of GMet and GEt were 1.600 mg/ml and 1.300 mg/ml against *S. aureus*, respectively. Some gram positive bacteria, particularly Staphylococcal species and yeast are susceptible the effects of these extracts. The galangal extracts caused the delay of the growth of *S. aureus* after exposing at 2 h. The chemical constituents of GMet and GEt are D, L-1'-acetoxychavicol acetate (ACA), *p*-coumaryl diacetate, palmitic acid, 1'-acetoxyeugenol acetate, β-bisabolene, 9-octadecenoic acid and other trace compounds.

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CHAPTER IV

MODES OF ACTION OF GALANGAL EXTRACTS AGAINST *Staphylococcus aureus*

4.1 Abstract

Crude galangal methanol (GMet) and ethanol extracts (GEt) were found to have inhibitory effects on growth of the representative foodborne pathogen—*Staphylococcus aureus*. Modes of action and target sites of compounds in crude GMet and GEt against *S. aureus* were investigated by using transmission electron microscopy (TEM), determining by biochemical assay for enzymatic activities of *S. aureus* was performed by using an API Staph test kit and measuring as an uptake of fluorescent nuclear stain propidium iodide. In bacterial cells treated with the dried GMet, formation of mesosomes, invagination of cell membrane, the lack of cytoplasm and coagulation of cell contents were observed. The dried GEt showed alteration in outer membrane integrity with cell membranes and cell walls being disrupted and damaged resulting in a release of cell materials in cytoplasm by TEM observation. The compounds in the extracts also delay sugar utilization and inhibited the arginine dihydrolase and urease activity within 48 h and 24 h, respectively. Crude GMet and GEt were bacteriostatic agents that also destabilized the membrane integrity by measuring as an uptake of fluorescent nuclear stain PI.

Keywords: galangal, D, L-1'-Acetoxychavicol acetate, coagulation of cell contents, arginine utilization

4.2 Introduction

Galangal (*Alpinia galanga* or *Languas galanga*), a member of the Zingiberaceae, has been used as one of the most popular food seasonings in Thailand. The rhizomes are used as a Thai traditional medicine for treating upset stomach, parasite infection, skin diseases, toothache and others (Farnsworth and Bunyapraphatsara, 1992). They have also been previously reported to possess antibacterial, antifungal, antiulcer, insecticidal and antioxidant activities (Cheah and Gan, 2000; Janssen and Scheffer, 1985). Galangal comprises of several compounds, including D, L, 1'-acetoxychavicol acetate (ACA); (1'S)-1'-acetoxyeugenol acetate; 1, 8-cineole; galangin and etc. However, ACA, an essential oil, is the major component isolated from the n-pentane/ diethyl ether-soluble extracts of galangal (Janssen and Scheffer, 1985).

At present, a great number of consumers are concerned about synthetic chemical additives in foodstuff; therefore, foods preserved with natural preservatives have become popular. This reason encourages researchers and food industries to look for natural food additives that have an antimicrobial property and thus, are healthy. Spices are one of natural preservatives of interest for use in commercial foods. Compounds in spices including essential oils have been widely used as flavoring agents and also shown to successfully inhibit the growth of foodborne pathogens in foodstuff. In fact, they can extend the shelf-life of both unprocessed or processed foods by retarding growth or reducing viability of microorganisms (Burt, 2004; Hao, Brackett, and Doyle, 1998; Holley

and Patel, 2005). The composition, chemical structure as well as functional groups of the essential oils such as phenolic compounds play an important role in spices antimicrobial activity. Phenolic compounds are the most effective against both gram positive and gram negative bacteria. They interfere with the formation of cytoplasmic membrane, disrupt the proton motive force, electron flow and active transport as well as causing coagulation of cell contents (Burt, 2004; Holley, 2005). Although mechanisms of action of the constituents of many spices against microorganisms have been reported, details on functions of these components remain unknown. As an initial step to address functions of specific compounds, the effects of the GMet and GEt on growth of a foodborne pathogen—*S. aureus* were examined by using Agar disc diffusion assay and/ or broth dilution assay, transmission electron microscopy (TEM) and biochemical assay.

4.3 Materials and methods

4.3.1 Preparation of galangal extracts

Rhizomes of galangal [*Alpinia galanga* (Linn.) Stuntz] were purchased from a local market in Nakhon Ratchasima, Thailand and cleaned thoroughly with water to remove the contaminants, before being sliced and dried in a tray-dryer oven at 50°C for 24 h, after which they were ground in a blender (National, MX-T2GN, Taiwan) to make powder.

4.3.2 Microorganism preparation

Staphylococcus aureus 209P, kindly provided by Laboratory of Ecological Circulation, Faculty of Bioresources, Mie University, Japan was used throughout this

study. It was maintained on Trypticase soy agar (TSA) slants at 4°C before being subcultured in 5 ml of Trpticase soy broth (TSB) and incubated at 37°C for 18-24 h. The bacterial suspension was subsequently streaked on TSA plates and incubated at 37°C for 48 h. A single colony was transferred to 10 ml of TSB and incubated at 37°C for 18-24 h. This culture was used for the antibacterial assay.

4.3.3 Preparation of the GMet and GEt

Ten grams of galangal powders were extracted with 100 ml of methanol (Carlo erba, France) and ethanol (Carlo erba, France) and left at room temperature overnight. The corresponding suspensions were filtered through Whatman filter paper No.1 (Struers Kebo Lab, Denmark). The filtrates were dried by using a rotary evaporator (BUCHI Rotavapor R-114, Switzerland) at 40°C for 6 h and kept at 4°C until use (Achararit et al., 1983; Caichompoo, 1999; Harborne, et al., 1975 with some modifications).

4.3.4 Preparation of *S. aureus* treated with GMet for transmission electron microscopy (TEM)

(modified from Horiuchi et al., 2001).

S. aureus was cultured in 5 ml of LB broth at 35°C for 18-24 h. Each twenty microliters of the bacterial suspension was transferred to each 2 ml of LB broth and further incubated at 35°C. The absorbance using spectrophotometer (Beckman, DU®530, Live Science, UV/Vis Spectrophotometer) at 600 nm of the suspension showed the growth rate of this bacterium reaching the maximum at 9 h after initiation of the culture. The bacterial culture at 5 h incubation, which corresponded to the mid log phase of *S. aureus* growth curve, was treated with the GMet. One hundred and sixty microliters of

the extract were applied onto four filter paper discs (∅ 8 mm, Advantec, Tokyo, Japan) which were then air-dried in a laminar flow hood for 2 h. One paper disc was submerged into one test tube of the above mentioned bacterial suspension at 5 h after initiation of the culture, before further incubation with shaking at 35°C for 2, 6, 12 and 24 h. At each time point, bacterial cells were collected by centrifugation at 3,500 x *g* for 10 min. They were fixed in a mixture of glutaraldehyde and OsO_4 (2.5% glutaraldehyde, 1% OsO_4 and 4.5% sucrose in 0.1M cacodylate buffer pH 7.0-7.4) at 4°C for 1 h. The pellets were embedded in 2% melted agar and then stained with 0.5% uranyl acetate for 30 min, followed by dehydration through an ethanol series (once each in 50, 70, 99% and three times in 100% ethanol for 30 min each). After that, they were submerged in an intermediate solvent (n-butyl glycidyl ether) (QY-1: Nisshin EM, Japan) for 30 min, followed by being immersed in a mixture of QY-1 and resin (Quetol 651 mixture) at 2:1, 1:1 and 1:2 ratios for 2 h each. They were kept in 100% Quetol mixture at room temperature for 48 h and polymerized in a hot air oven at 60°C for 3 days. Ultra thin sections (ca 80 nm thick) prepared with an ultramicrotome were stained with 1% uranyl acetate and counterstained with lead solution (a mixture of 1% lead acetate trihydrate, 1% lead citrate and 1% lead nitrate) and observed with a transmission electron microscope (H-7000FA, Hitachi, Tokyo, Japan) under an acceleration of 70 kV.

4.3.5 Preparation of *S. aureus* treated with GEt for transmission electron microscopy (TEM).

S. aureus was cultured in three for time points containing 5 ml of TSB at 37°C for 18-24 h. Twenty microliters of the bacterial suspension was transferred to 3 ml of TSB

and further incubated at 37°C for 5 h. One hundred and sixty microliters of the GEt were applied onto three filter paper discs (∅ 8 mm, Advantec, Tokyo, Japan) which were then air-dried in a laminar flow hood for 2 h. One paper disc was submerged into one test tube of the above mentioned bacterial suspension at 5 h after initiation of the culture, before further incubation with shaking at 35°C for 2, 6 and 24 h. At each time point, bacterial cells were collected by centrifugation at 10,000 x *g* for 10 min and fixed in 3 % glutaraldehyde at 4°C for 2 h. Cells were washed three times with 0.1 M phosphate buffer, pH 7.2 and fixed with 2% OsO_4 in 0.1 M phosphate buffer at room temperature for 18-24 h. The pellets were embedded in 2% melted agar and then dehydrated through serial concentrations of ethanol (35, 50, 70, 95, 100 % for 15 min each). They were infiltrated twice in propylene oxide for 15 min each, once in a working mixture (propylene oxide: Spurr' resin, 1:1) for 30 min, followed by Spurr' resin for 60 min. They were sat in 100% Spurr' resin and then polymerized in a hot air oven at 60°C for 72 h. The polymerized samples were thinly sliced with an ultramicrotome (MTX 75500, RMC, USA). Ultra thin sections (ca 80 nm thick) prepared with an ultramicrotome were stained with 1% uranyl acetate and counterstained with lead solution and observed under a transmission electron microscope (JEOL, JEM 2010, Japan) at an operating voltage of 80 kV.

4.3.6 Biochemical and enzymatic assays

The API Staph (BioMerieux, France) system was used to determine an interaction between galangal extracts and microbial enzymatic systems. API Staph strips are consisted of a series of 20 microtubes containing dehydrated substrates and/ or nutrient media in which a dye or chromogenic substrate is an indicator of acids or end

products produced by certain microorganisms. To reconstitute a test, about 150 µl of a bacterial suspension is inoculated into microtubes and incubated at 37˚C for 18-24 h after addition of reagents. Production of the end products as a result of microbial metabolism is revealed by changes in reaction colors.

In this study, *S. aureus* was cultured *S. aureus* was cultured in three for time points containing 5 ml of TSB at 37°C for 18-24 h. Twenty microliters of the bacterial suspension was transferred to 3 ml of TSB and further incubated at 37°C for 5 h. One hundred and sixty microliter of the extract were applied onto three filter paper discs (∅ 8 mm, Advantec, Tokyo, Japan) which were then air-dried in a laminar flow hood for 2 h. One paper disc was submerged into one test tube of the above mentioned bacterial suspension at 5 h. After being treated with the galangal extracts for 2, 6, 12 and 24 h, the bacterial cells were harvested by centrifugation at 3500 x g for 5 min and then washed with 0.85 % (w/v) sterile normal saline solution. Cell pellets were resuspended in 3 ml of API Staph Medium. Cell suspension was inoculated for acidification reaction testing for D-glucose, D-fructose, D-mannose, maltose, lactose, D-trehalose, D-manitol, xylitol, D-melibiose, raffinose, xylose, saccharose (sucrose), methyl-α D-glucopyranoside and N-acetyl-glucosamine and for testing the ability of the bacterium to utilize potassium nitrate, β-naphthyl phosphate, sodium pyruvate (Voges Proskauer), L-arginine and urea. To set up the acidification reaction, one hundred and fifty microliters of bacterial suspension were added to microtubes containing dehydrated substrates. The reaction was incubated at 37°C for 18-24 h. Results were determined by observing changes in colors of the reaction mixture, that is, changing in colors from red to yellow was scored as positive, where as no change in colors/ colorless was scored as negative. Reduction of nitrate to nitrite led to

changes in colors from colorless or light pink to red, after adding the NIT1 and NIT2 reagents. Alkaline phosphatase activity converting from β-naphthyl phosphate to free β-naphthyl caused a color change from yellow to violet. Acetyl-methyl-carbinol production was indicated by changes in colors of the reaction mixture from colorless-light pink to violet-pink. As positives for arginine dihydrolase and urease activities determining alkaline end products, phenol red as an indicator changed from yellow to orange-red and from yellow to red-violet, respectively.

4.3.7 Determination of the membrane integrity

Fluorescence probe propidium iodide (PI) was employed in this study to analyze cell membrane integrity by the method of Fitzgerald, Stratford, Gasson, Ueckert, Bos and Narbad (2004) with some modification. Membrane permeability was measured as an uptake of fluorescent nuclear stain PI. PI has an excitation maximum (λ_{ex}) of 536 nm and an emission maximum (λ_{em}) of 617 nm with a fluorescence enhancement upon binding to DNA. A stock solution of 1 mg/ ml was prepared with sterile deionized water and stored at 4°C.

S. aureus was cultured in test tubes containing 50 ml of TSB at 35°C for 18-24 h. Twenty microliters of the bacterial suspension in each tube were transferred to 3 ml of TSB and further incubated at 35°C for 5 h. One hundred and sixty microliters of the GMet and GEt were applied onto filter paper discs (∅ 8 mm, Advantec, Tokyo, Japan) which were then air-dried in a laminar flow hood for 2 h. One paper disc was submerged into one test tube containing the above mentioned bacterial suspension grown in an exponential phase (5 h after initiation of the culture), before further incubation with

shaking at 35°C for 18-24 h. During this period, GMet and GEt treated bacterial cells were analyzed for changes in membrane integrity by PI fluorescent measurements. Bacterial suspensions (40 µl) were added to 1 ml of 50 mM potassium phosphate buffer (pH 6) containing PI at a final concentration of 10 µg/ ml, mixed and incubated at room temperature for 5 min prior to fluorescence analysis. Red fluorescence of PI was detected at an excitation maximum of 535 nm and an emission maximum of 617 nm with Luminescence spectrometer (LS50B, Perkin Elmer, UK). Cells grown in the absence of extracts served as negative controls.

4.3.8 Statistical analysis

The triplicate data were statistically analyzed by the variance analysis and the mean separated according to LSD test with significance level (p) of 0.05 using SPSS 11.2 programs.

4.4 Results and discussions

4.4.1 Cytological effects of GMet on *S. aureus* examined by using transmission electron microscopy

In the GMet assay (Figure 4.1) were illustrated the water controls (negative control), the bacterial cell wall became clear, and plasma membrane and entire cytoplasm were electron densed without a noticeable nuclear region (Figure 4.1 A). The cells treated with air-dried methanol (negative control) were similar to those in air-dried water controls (Figure 4.1 B). However, in the cells treated with the air-dried GMet for 2 h, the nuclear region became apparent due to low electron density and fibrillar morphology, which was

coagulation of cell contents and this has been reported previously for tea tree oil (Gustafson et al, 1998) and microwave radiation (Woo, Rhee, and Park, 2000), was evident as shown in Figure 4.1 C at 100% of all cells/each field. In the cells treated with the air-dried GMet for 6 h, some of the nuclear regions were coagulated into granules as indicated by a red arrow in Figure 4.1 D. Furthermore, plasma membrane was invaginated to form an electron densed coagulated region between the membrane and the cell wall, these appearance *S. aureus* cells contained multilamellar, mesosome like structure (blue arrow in Figures 4.1 D and 4.1 F) at 100% of all cells/each field. These degenerating patterns in the nuclear regions and plasma membrane were also observed in the cells similarly treated for 12 and 24 h (red and blue arrows in Figures 4.1 E and 4.1 F, respectively) at 100% of all cells/each field. Transmission electron microscopy showed that there was frequently an effect on the *S. aureus* membrane, demonstrated by the appearance of mesosome structures similar to those seen with cationic peptides (Friedrich, Moyles, Beveridge, and Hancock, 2000), and defensins (Shimoda, Ohki, Shimamoto, and Kohashi, 1995). Mesosomes, membranous structures were regarded as being indicative of cytoplasmic membrane alteration, in this case induced by the air-dried GMet compared to negative control (untreated cells) did not contain them. Furthermore, since the cytoplasmic membrane is instrumental in cell wall synthesis and turnover, a perturbation of this membrane may also affect cell wall integrity and autolysin regulation (Kemper, Urrutia, Beveridge, Koch, and Doyle, 1993). Mesosome-like structure may appear before the bacteria lose their viability (Shimoda et al., 1995). Transmission electron microscopy clearly demonstrated that the GMet caused morphological changes in bacterial cells, especially at the nuclear regions and plasma membranes where cytoplasm coagulation occurred. Similar results have been reported when *S. aureus*, *E. coli* and other bacteria

were treated with phenolic compounds, cationic compounds especially chlorhexidine or tea tree oil (Gustafson, 1998; Hugo and Longworth, 1964; Sikkema, Bont, and Poolman, 1995). Cell coagulation affected by the GMet possibly due to interference with the enzymatic function associated with the cytoplasmic membrane. Therefore, a biochemical assay for enzymatic activities of *S. aureus* was performed by using an API Staph (BioMerieux, France) test kit. The result showed that utilization of arginine dihydrolase and urease of *S. aureus* were not detected in cells treated with GMet when compared to untreated cells within 48 h and 24 h, respectively. The extracts were also able to delay the sugar utilization after incubation at 16 h.

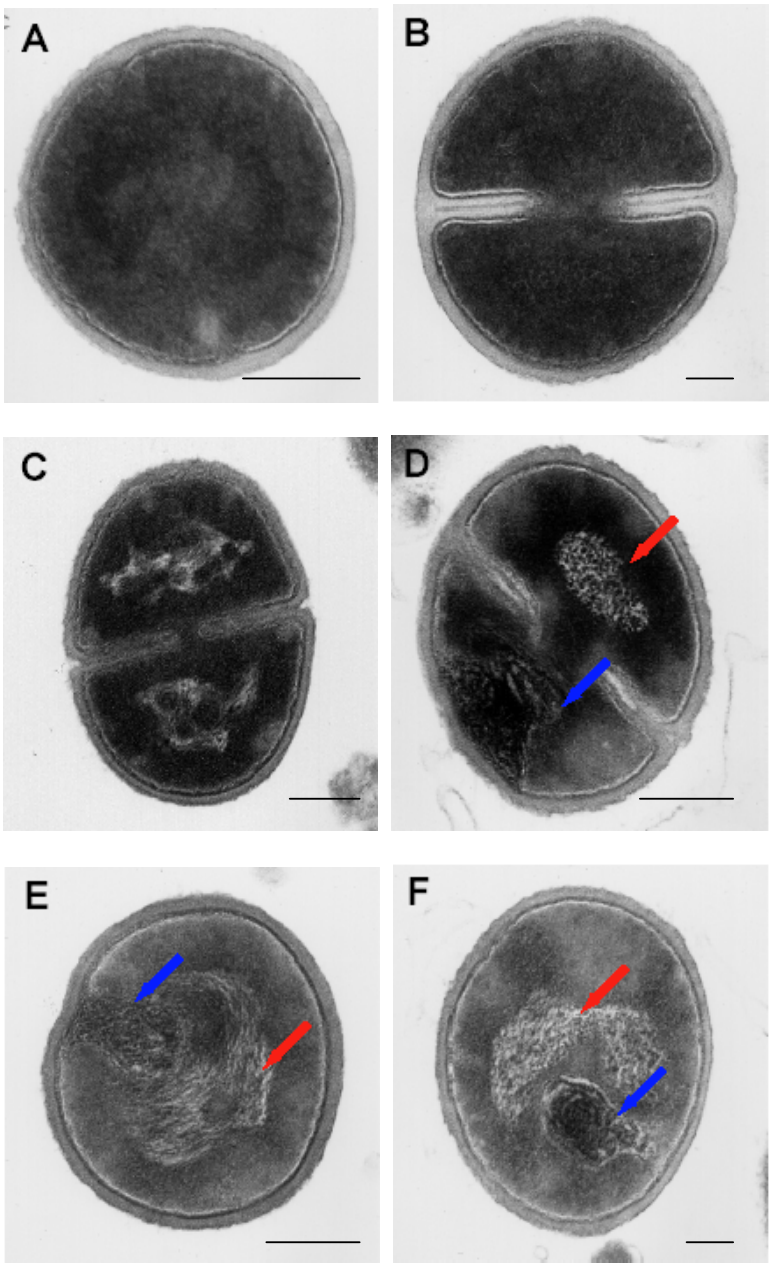

Figure 4.1 Cytological effects of galangal methanol extract (GMet) on *S. aureus* cells. (Bar = 0.1µm, Magnifications: x 40,000), A: *S. aureus* cells after air-dried deionized water treatment; B: *S. aureus* cells after air-dried methanol treatment; C: *S. aureus* cells after air-dried GMet treatment for 2 h; D: *S. aureus* cells after air-dried GMet treatment for 6 h; E: *S. aureus* cells after air-dried GMet treatment for 12 h; F: *S. aureus* cells after air-dried GMet treatment for 24 h.

4.4.2 Cytological effects of GEt on *S. aureus* determined by using transmission electron microscopy

The GEt caused dramatic cytological modifications to *S. aureus* cells as illustrated in Figures 4.2 C, D and E compared to normal cells (Figure 4.2 A and B). *S. aureus* cells treated by GEt for 24 h exhibited the lack of cytoplasm or holes (Figure 4.2 D) about 85-100% of all cells/each field of TEM observation. The lack of cytoplasm can be induced by loss of functionality of the membrane due to the GEt which induce leakage of cellular contents that showed in Figure 4.2 C about 16.67% of all cells/each field. The lack of cytoplasm occurred similar to the synergistic action of pulse electric field and nisin that reported by Calderon-Miranda, Barbosa-Canovas, and Swanson (1999). Besides, *Listeria monocytogenes* treated high sodium chloride (NaCl) level showed the TEM image similar to Figure 4.2 D, caused the collapse of the nucleoid material (Zaika and Fanelli, 2003). GEt treated *S. aureus* showed alteration in outer membrane integrity with cell membranes and cell walls being disrupted and damaged resulting in a release of cell materials in cytoplasm (Figure 4.2 C). The intracellular components of cells aggregated throughout treated cells, which could also cause membrane dysfunction in respect to electron transport, nutrient uptake, nucleic acid synthesis and ATPase activity (Denyer and Hugo, 1991; Nychas, 1995). *S. aureus* cells showed several dark spots in their cytoplasm (Figures 4.2 E and F) about 20% of all cells/each field. However, no dark spots or intracellular components aggregation were observed in the untreated cells (Figures 4.2 A and 4.2 B), suggesting that the dark spots were the result of GEt. These results were similar observed in the result from microwave radiation on *E. coli* and *B. subtilis* (Woo et al., 2000). The dark spots are also thought to be aggregated proteins.

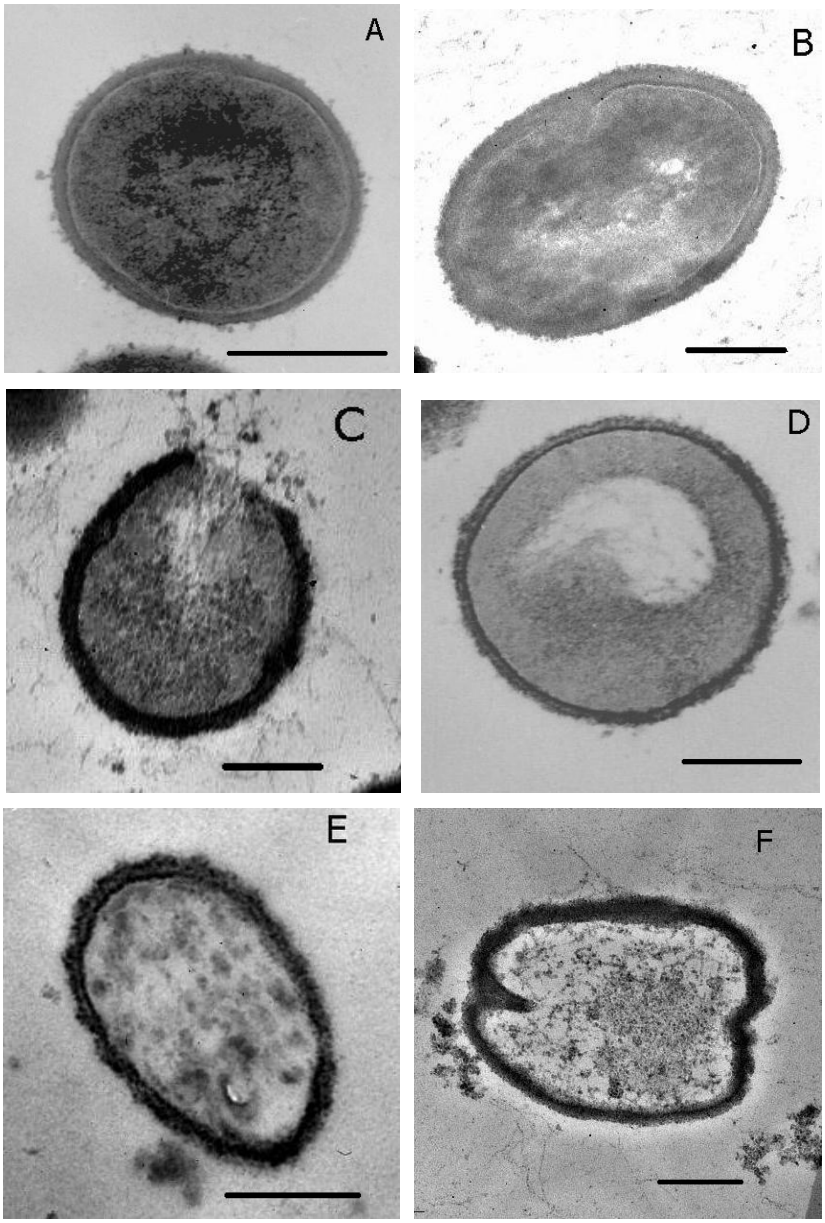

Figure 4.2 Cytological effects of galangal ethanol extract (GEt) on *S. aureus* cells. [Bar = 0.2 μm, Magnifications: x 20,000 (A, D and E), x 15,000 (B and F), x 10,000 (C)], A: *S. aureus* cells after air-dried ethanol treatment; B: *S. aureus* cells after air-dried deionized water treatment; C: *S. aureus* cells after air-dried GEt treatment for 6 h, D and F: *S. aureus* cells after air-dried GEt treatment for 24 h, E: *S. aureus* cells after air-dried GEt treatment for 2h.

4.4.3 Biochemical changes of *S. aureus*

In order to access biochemical changes of *S. aureus* and potential adaptation of cells treated GMet and GEt were analyzed during 2 h, 6 h, 16 h and 24 h of cells exposure to the extracts. Among enzymatic activities followed with the API Staph system, arginine dihydrolase and urease showed significant changes during the stress when compare to control (untreated cells). The results showed that arginine dihydrolase and urease activity were inhibited and /or delayed after 2 h of the extracts exposure and then it was still maintained until 48 h and 24 h incubation in API Staph test kit, respectively. The same result that occurred with cells expose to galangal extracts for 6 h, 16 h, and 24 h. Cells treated galangal extracts were suppressed the alkaline productivity from arginine to citrulline and ammonia by arginine dihydrolase leading to color unchanging as yellow as initial color. Urease activity was repressed the alkaline production from urea to ammonia. Besides, sugar consumption of galangal extracts treated cells were inhibited especially lactose, maltose and sucrose. Acidification reaction of lactose, maltose, and sucrose were retarded within 16 h after incubation, which the results still showed red as initial medium, these related sugar metabolic enzymes were recovered after 16 h. Galangal extracts did not interfered the utilization of *S. aureus* potassium nitrate, β-naphthyl phosphate, sodium pyruvate (Voges Proskauer).

Arginine dihydrolase is one of three arginine deiminase enzymes (ADI) which arginine dihydrolase degrades arginine into citrulline and ammonia; ornithine transcarbamoylase, which cleaves citrulline into carbamoyl phosphate and ornithine; and carbamate kinase, which produces ATP, ammonia and carbon dioxide through dephosphorylation of carbamoyl phosphate (Angelis et al., 2002; Liu, Pritchard, Hardman, and Pilone, 1995). The ADI pathway may fulfill various roles, to provide ATP for

microbial growth under a variety of environmental conditions, especially when carbohydrate is not available or at low concentration; to supply carbomoyl phosphate for biosynthesis of citrulline or pyrimidines; and to protect bacterial damaging caused by acid and /or starvation environmental stresses (Angelis et al., 2002). The arginine deiminase pathway involved in generating adenosine triphosphate (ATP) for cell growth in *Streptococcus faecalis* (Schimke, Berlin, Sweeney, and Carroll, 1966). The galangal extracts were able to inhibit ADI activity, treated *S. aureus* cells might be less acid tolerant, derived less extra energy (ATP), lead to cell survival shorter or increase injured cells or cells death after depletion of the primary energy source (Angelis et al., 2002). ATP for extrusion of cytoplasmic protons was decrease.

4.4.4 Effect of GMet and GEt on membrane integrity

Luminescence spectrometer was used with the nucleic acid stain PI to measure the effect of GMet and GEt on membrane integrity. When cells with damaged or compromised cell membrane are treated with this probe, it enters the cell, binds to double-stranded nucleic acid and becomes highly fluorescent (Fitzgerald, 2004; Shapiro, 2000). PI cannot enter cells with intact membrane resulting in low fluorescence intensity as shown in untreated *S. aureus* cells (Figure 4.3). Cells that have damaged membranes allow the stain to enter the cells and bind to the DNA resulting in high fluorescence intensity. *S. aureus* exposed to GMet and GEt at 18-24 h, had a number of cells with damaged membranes. Fluorescence intensity of GMet and GEt treated *S. aureus* showed at 119.77 and 121.11, respectively. These results, treated cells showed more intensity than untreated cells which showed intensity at 111.06. GMet and GEt treated cells showed more intensity due to the fact that cell membrane integrity of treated cells compromised

the uptake of nucleic acid stain PI. This result indicated that the destabilizing effect of galangal extracts on the membrane was at a sublethal level and might provide an explanation for the extract bacteriostatic effect rather than cell death (Maillard, 2002). The loss of the differential permeability character of the cytoplasmic membrane is frequently identified as the cause of cell death. Some researchers have explored this further, reasoning that loss of membrane function is only part of the explanation for antimicrobial activity (Walsh et al., 2003). This effect on the cell membrane correlated with the result derived from TEM observation (Figure 4.2 C).

Table 4.1 Effect of galangal methanol and ethanol extracts treated *S. aureus* on the propidium iodide uptake.

Treatment	**Fluorescence intensity**
Control	106.11 ± 2.02^{b}
galangal methanol extract-treated cells	127.05 ± 4.95^{a}
galangal ethanol extract-treated cells	125.34 ± 4.09^{a}

Means in the column followed by different letters are significantly different at $p < 0.05$ according to LSD

Regarding modes of action, galangal extracts may affect multiple systems in bacterial cells since both their GMet and GEt are composed of various essential oils and compounds whose functional groups are active against the selected microorganisms (Appendix B). The suggested mechanisms of action of GMet and GEt compounds are (a) a reaction with the cytoplasmic membrane (Figure 4.2 C) resulting in changes in membrane integrity and uptake of extracts measured by the dye-exclusion (fluorescent-probe) of PI intensity in both intact and damaged membranes (Table 4.1). The extract-

treated cells had an increase in the uptake of the double strand nucleic acid stain PI when compared to the intact cells because the galangal extracts were able to destabilize the membrane leading to loss of integrity. This action was similar to several studies such as the antimicrobial action of vanillin against *E. coli*, *Lb. plantarum* and *L. innocua* (Fitzgerald et al., 2004). Membrane damage and loss of cytoplasmic constituents result in the inhibition of cellular growth, loss of viability (Hugo and Bloomfield, 1971). (b) enzymatic inactivation and macromolecules denaturation because of the appearance of mesosomes, lack of cytoplasm, and cell contents and/ or macromolecules coagulation (Figures 4.1D-F and 4.2 D-F) (Denyer and Hugo, 1991). The results from biochemical and enzymatic assays showed that galangal extracts were able to inhibit or delay arginine dihydrolase activity converting arginine to citrulline and ammonia, urease converting to ammonia. Inhibition by the galangal extracts of alkali production from arginine was interpretable mainly as inhibition of membrane transport systems for substrate uptake. This could be an indicator that galangal extracts act on enzymes involved in energy regulation or synthesis of structural components (Conner and Beuchat, 1984).

Detailed compositional analysis is achieved by gas chromatography and mass spectrometry of the essential oils. Essential oils can comprise a large number of individual components (Delaquis, Stanich, Girard, and Mazza, 2002; Marino, Bersani, and Comi, 2001). Major components can constitute up to 80% of the essential, whereas other components are present only as a trace (Senatore, 1996) as similar to the crude galangal extracts which composed 76-79 % of ACA and less other compounds. For GC-MS analysis and some studies (Jannsen and Scheffer, 1985), crude extracts of galangal compose of several essential oils are the lipophilic compounds, soluble in alcohol and to a limited extent in water. The major and minor compounds in GMet have been determined

by GC-MS as ACA, 1'-acetoxyeugenol acetate, p-coumaryl diacetate, eugenol, β-bisabolene, β-farnesene, chavicol, 4-hydroxybenzaldehyde and 1, 2-dimethoxy-4-(2-propenylbenzene). In GEt, ACA, p-coumaryl diacetate, palmitic acid, acetoxyeugenol acetate, eugenol, β-bisabolene, β-farnesene and sesquiphellandrene have been detected. These chemical compounds are phenolic compounds, phenolic derivatives, ester of weak acid, fatty acid, terpenes and others. Beta -bisabolene, β-farnesene and sesquiphellandrene in these crude galangal extracts are terpenes, whereas eugenol and chavicol are phenolic compounds. All of which are constituents of the essential oils in spices whose mechanisms of action should be similar to other terpenes and phenolic compounds, which are interference with functions of the cytoplasmic membrane (Figure 4.2 C) and coagulation of cell contents (Figures 4.2 D-F) (Burt, 2004; Davidson, 2001; Davidson and Naidu, 2000). The phenolic components are chiefly responsible for antibacterial properties of essential oils (Lambert, Skandamis, Coote, and Nychas, 2001; Nguefack, Budde, and Jakobsen, 2004; Thoroski, Blank, and Biliaderis, 1989). There is some evidence that minor component have a critical part to play in antibacterial activity, possibly by producing a synergistic effect between other components (Marino et al., 2001). Eugenol, acetoxyeugenol acetate, and chavicol are phenolic compounds and phenolic derivatives compounds in galangal extracts might shown the result as similar to the above mentioned that they combined the antimicrobial synergistic effect with some terpenes, ACA and others. The phenolic structures, such as eugenol and chavicol, were highly active against the test microorganism that these compounds were either bactericidal or bacteriostatic agents, depending on the concentration used (Pelzar, Chan, and Krieg, 1988). The activity of phenolic compounds are attributed to the characteristic feature of

the phenolic hydroxyl group that is more acidic than that of the aliphatic hydroxyl group and the presence of a system of delocalized electrons (Ultee, Bennik, and Moezelaar,2002). The high activity of the phenolic components may be further explained in terms of the alkyl substitution into the phenol nucleus (Pelzar et al., 1988) that explained similar to the eugenol structure. Furthermore, these compounds might destabilized the cytoplasmic membrane and in addition acts as a proton exchanger, thereby reducing the pH gradient across the cytoplasmic membrane (Ultee et al., 2002). In one study, the addition of an acetate moiety to the molecule appeared to increase the antibacterial activity (Dorman and Deans, 2000). Therefore, mechanisms of action might affect multiple target sites against *S. aureus* cells which Dorman and Deans (2000) reported that the activity of the oils would be expected to relate to the respective composition of the plant volatile oils, the structural configuration of the constituent components of the volatile oils and their functional groups and possible synergistic interactions between components. The essential oils are hydrophobic compound that consists of mixtures of esters, aldehydes, ketones and terpenes (Smid and Gorris, 1999). This enables them to combine with the lipids of the bacterial cell membrane, disturbing the structures and rendering them more permeable. Besides, the essential oils are able to induce the leakage of ions and other cell contents (Carson, Mee and Riley, 2002; Fitzgerald, et al., 2004). The results from TEM and changes in membrane integrity determined by using PI measurement suggested that the extract affected the cytoplasmic membrane of *S. aureus* and induced the loss of nucleic acids and ions (Burt, 2000; Carson, et al., 2002; Woo, et al., 2000).

Acetoxychavicol acetate; the major compound in the crude extracts, is an ester of acetic acid. The methyl ester can penetrate the hydrophobic regions of the membranes

and the carboxyl groups pass through the cell membrane, perturbing internal pH and denaturing intracellular proteins, including enzymes involving in arginine dihydrolase reaction that above mentioned (Figures. 4.1 E-F and 4.2 D-F) (Huang, Forsberg and Gibbins, 1986; Marquis, Clock and Mota-Meira, 2003).

4.5 Conclusions

GMet and GEt affected physiological changes and enzyme function activities of *S. aureus*. GMet induced the lack of cytoplasm or cell coagulation and interfered to the cell membrane of *S. aureus* by TEM observation. *S. aureus* exposed to GEt exhibited the lack of cytoplasm, as a result of the decrease of the membrane functionality, membrane disruption resulting in a loss of cytoplasmic constituents. Both GMet and GEt were able to inhibit the urease and arginine dihydrolase activity and interfered membrane integrity caused cell membrane permeability.

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CHAPTER V

SUMMARY

The crude aqueous, ethanol, methanol and ethyl acetate extracts from root and rhizomes of some members of the Zingiberaceae were selected as natural antimicrobial agents for inhibiting the growth of selected foodborne pathogens. *S. aureus* and *E. coli* was used as representatives of commonly found foodborne pathogens. Galangal methanol (GMet) and ethanol extracts (GEt) have a greater inhibitory effect against *S. aureus* than ginger, turmeric or krachai does. Some gram positive bacteria, especially Staphylococcal species and yeast are susceptible the effects of these extracts. In this study, some spices in the Zingiberaceae namely galangal, ginger, turmeric and krachai were selected as candidates for natural antimicrobial agents. GMet and GEt were selected to study MIC and MBC against *S. aureus.* MIC of GMet was 0.8 mg/ml and MBC was 1.6 mg/ml. MIC and MBC of GEt were lower than GMet. It showed at 0.325 mg/ml and 1.3 mg/ml for MIC and MBC subsequently. The GMet and GEt give the delay of the growth of *S. aureus* after exposing about 2 h. The chemical constituents of GMet and GEt are D, L-1'-acetoxychavicol acetate (ACA), *p*-coumaryl diacetate, palmitic acid, 1'-acetoxyeugenol acetate, β-bisabolene, 9-octadecenoic acid and other trace compounds, determined by using TLC, GC-MS and NMR.

The GMet and GEt were observed the primary and main target site on bacterial cell by using TEM and biochemical assay. A biochemical assay for

enzymatic activities of *S. aureus* was performed by using an API Staph test kit. The result showed that utilization of arginine dihydrolase and urease of *S. aureus* was not detected in cells treated with galangal extracts when compared to untreated cells within 48h and 24h. The extracts might inhibit the arginine dihydrolase and urease activity. Membrane integrity of bacterial cell was measured as an uptake of fluorescent nuclear stain PI. This result indicated that the GMet and GEt were bacteriostatic agents that destabilized the membrane permeability. This effect on the cell membrane correlated with the result derived from TEM observation that the extracts cause internal pH changing, denaturing of intracellular proteins, enzyme inactivating as well as disrupting the cytoplasmic membrane function of the *S. aureus* cells resulting in a loss of cytoplasmic constituents and ions.

This study presents a basic knowledge, which can lead to further investigation on the roles of chemical compounds in galangal extracts in microbial physiology and to gain more information on their mechanisms of action. All information together should eventually lead to effective applications of spice extracts to controlling foodborne pathogens, food spoilage organisms in food industries. There is a need for the use of natural antimicrobial agents instead of chemical preservatives. Combination effects of the compounds in crude galangal extracts and other spices or natural food preservative agents should be studied to maximize the antimicrobial activity and minimize the exposure time and concentrations required to achieve a particular antimicrobial effect. The ability and stability of the extract in foods also remain to be addressed.

APPENDIX

Preparation of McFarland solution

0.048 M $BaCl_2$ (1.175% w/v $BaCl_2$ $2H_2O$)

0.36 N H_2SO_4 (1% v/v)

N0.	0.5	1	2	3	4	5	6	7	8	9	10
Barium chloride (ml)	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
Sulfuric acid (ml)	9.95	9.9	9.8	9.7	9.6	9.5	9.4	9.3	9.2	9.1	9
Approx. cell density ($x10^8$/ ml)	1.5	3	6	9	12	15	18	21	24	27	30

Table 6.1 Qualitative and quantitative composition (expressed as %) of galangal methanol extract separated by gas chromatography and identified by mass spectroscopy

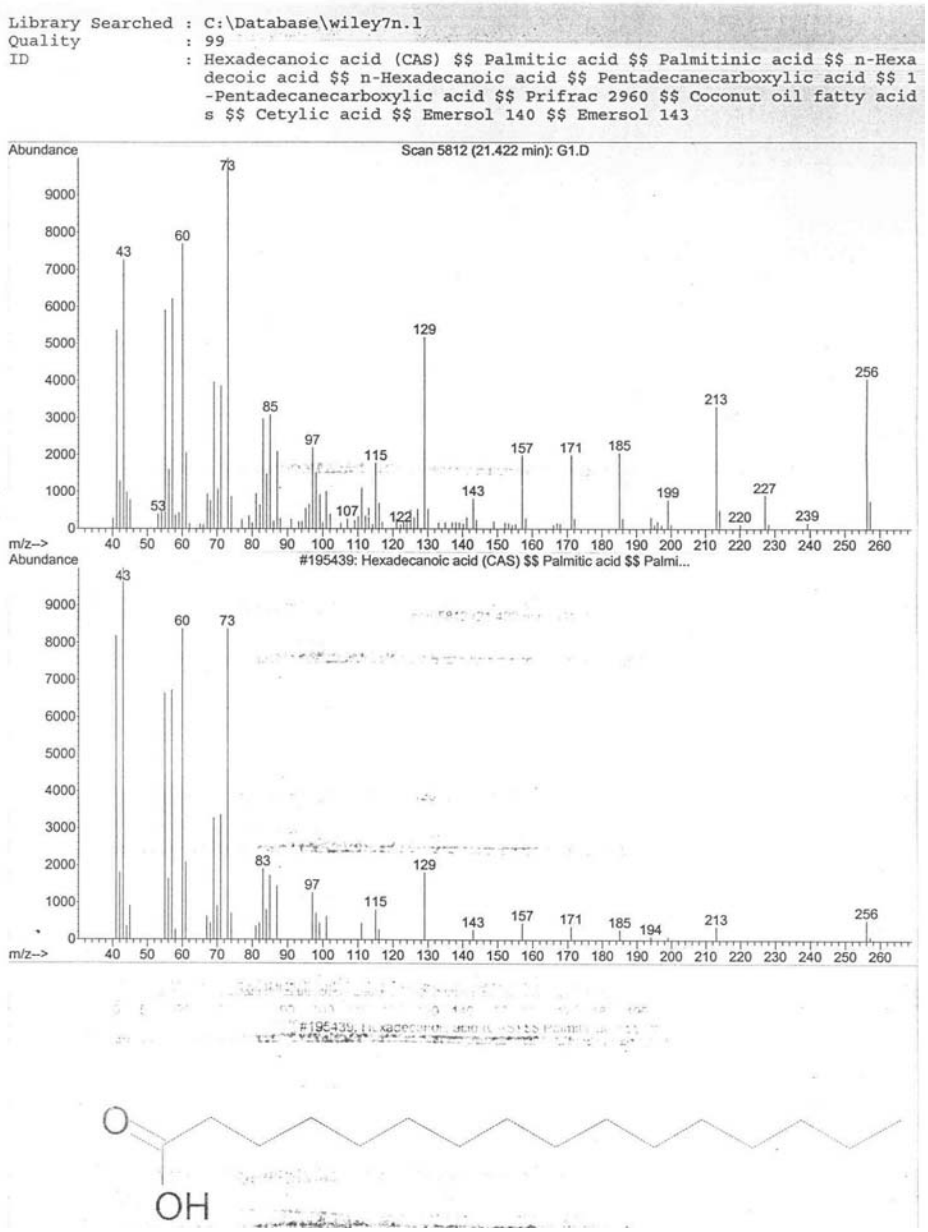
Compounds	Percentage area (v/v)
1. Unidentified	1.34
2. Unidentified	0.79
3. Chavicol	1.07
4. 4-hydroxybenzaldehyde	1.16
5. 1,2-dimethoxy-4-2-propenyl benzene	1.06
6. trans-β-Farnesene	0.76
7. β-bisabolene	1.03
8. Eugenol	0.14
9. Unidentified	1.35
10. 4,3-hydroxy-1-propenyl phenol	1.73
11. D, L-1'- Acetoxychavicol acetate	78.41
12. Unidentified	1.99
13. 1'- Acetoxyeugenol acetate	2.40
14. *p*- Coumaryl diacetate	6.77

Table 6.2 Qualitative and quantitative composition (expressed as %) of galangal ethanol extract separated by gas chromatography and identified by mass spectroscopy

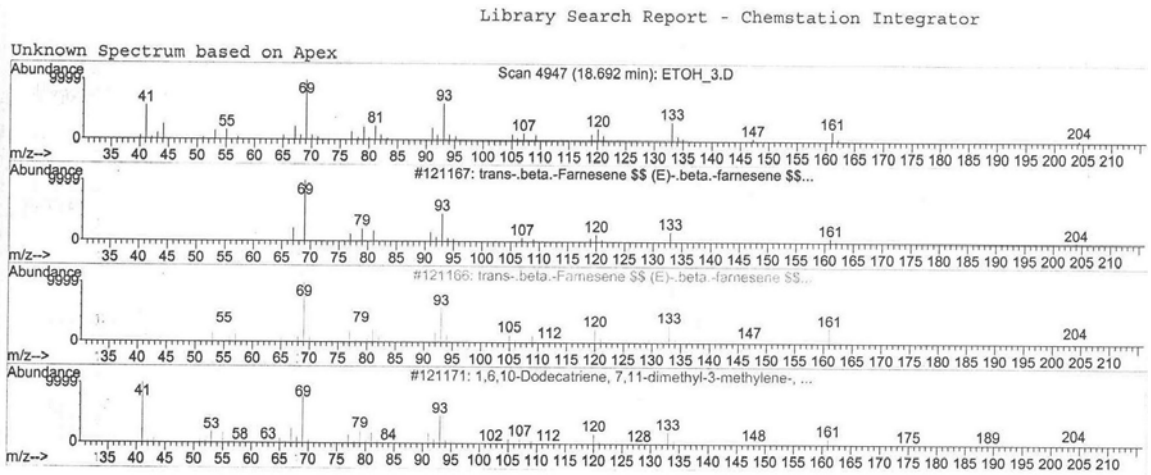
Compounds	Percentage area (v/v)
1. Trans-β-Farnesene	1.59
2. β-bisabolene	2.31
3. β-Sesquiphellandrene	1.18
4. D, L-1'- Acetoxychavicol acetate	76.49
5. 1'- Acetoxyeugenol acetate	1.94
6. *p*- Coumaryl diacetate	3.06
7. Unidentified	7.96
8. Palmitic acid	3.19
9. 9-Octadecenoic acid	2.28

GC-MS spectrum of trace compounds of galangal methanol and ethanol extracts

The mass spectrum of the trace compounds of galangal extracts shows m/z 257, 213, m/z 129, m/z 73, m/z 60 and m/z 43 that match with Palmitic acid or Hexadecanoic acid.

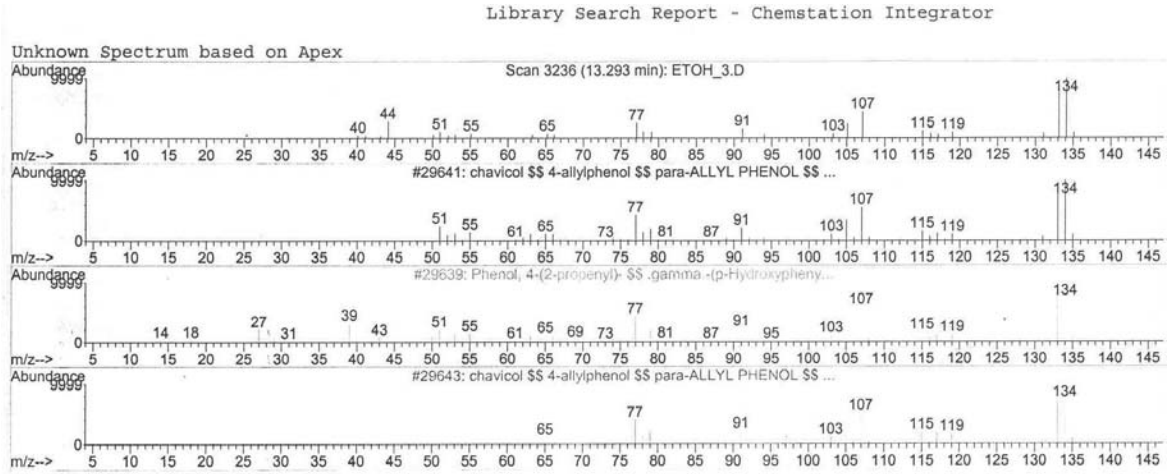


The mass spectrum of the trace compounds of galangal extracts shows m/z 204, 147, m/z 107, m/z 79, m/z 81 and m/z 41 that match with 7,11-dimethyl-3-methylene-1,6,10-dodecatriene.



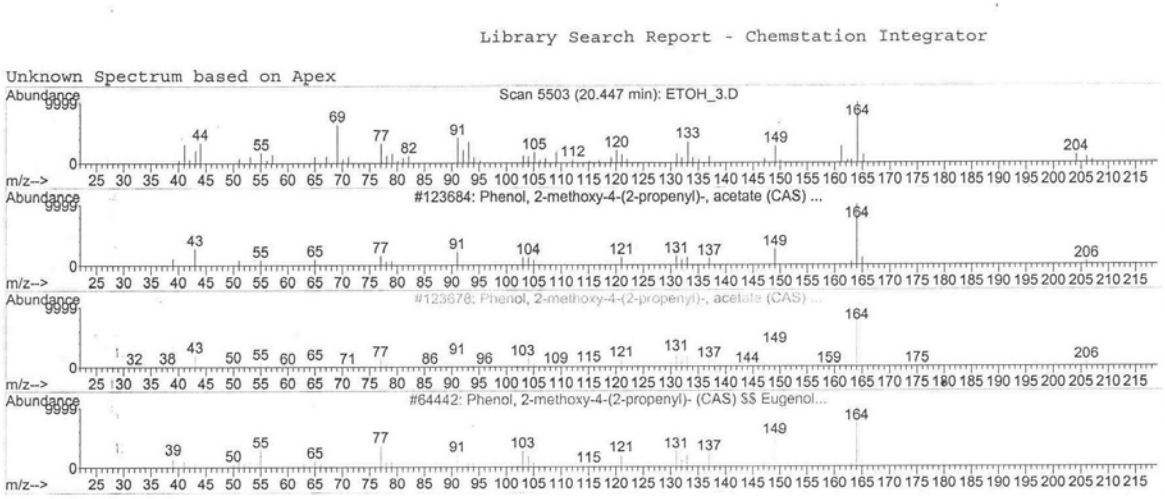
The 3 best hits from each library.	Ref#	CAS#	Qual
C:\Database\wiley7n.l			
1 trans-.beta.-Farnesene $$ (E)-.b...	121167	000502-60-3	96
2 trans-.beta.-Farnesene $$ (E)-.b...	121166	000502-60-3	94
3 1,6,10-Dodecatriene, 7,11-dimeth...	121171	018794-84-8	93

The mass spectrum of the trace compounds of galangal extracts shows m/z 134, m/z 107, m/z 91, m/z 77 and m/z 51 that match with 4-allylphenol (chavicol).



The 3 best hits from each library.	Ref#	CAS#	Qual
C:\Database\wiley7n.l			
1 chavicol $$ 4-allylphenol $$ par...	29641	000501-92-8	94
2 Phenol, 4-(2-propenyl)- $$ .gamm...	29639	000501-92-8	93
3 chavicol $$ 4-allylphenol $$ par...	29643	000501-92-8	87

The mass spectrum of the trace compounds of galangal extracts shows m/z 206, m/z 164, m/z 149, m/z 133, m/z 55 and m/z 43 that match with 2-methoxy-4-(2-propenyl)-, phenol acetate (eugenyl acetate).



The 3 best hits from each library.	Ref#	CAS#	Qual
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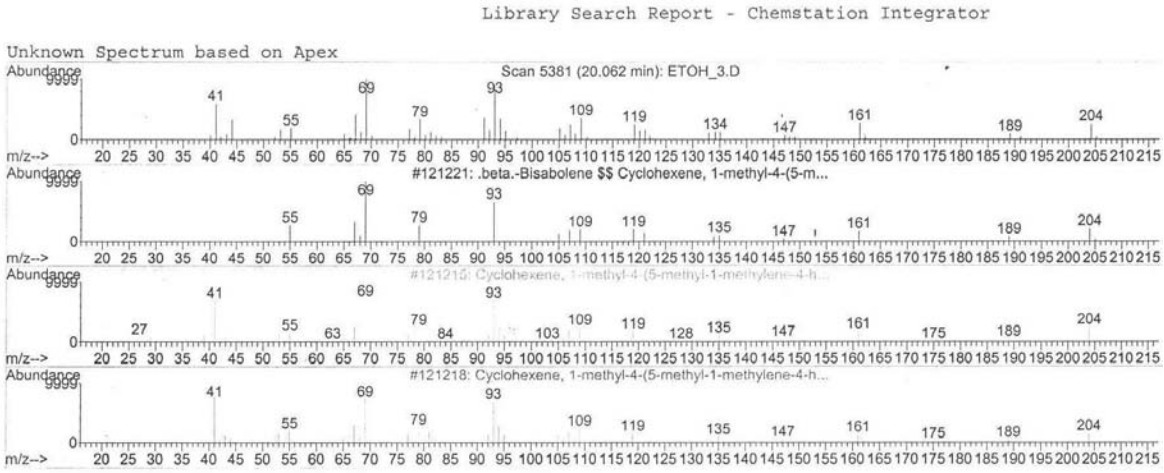
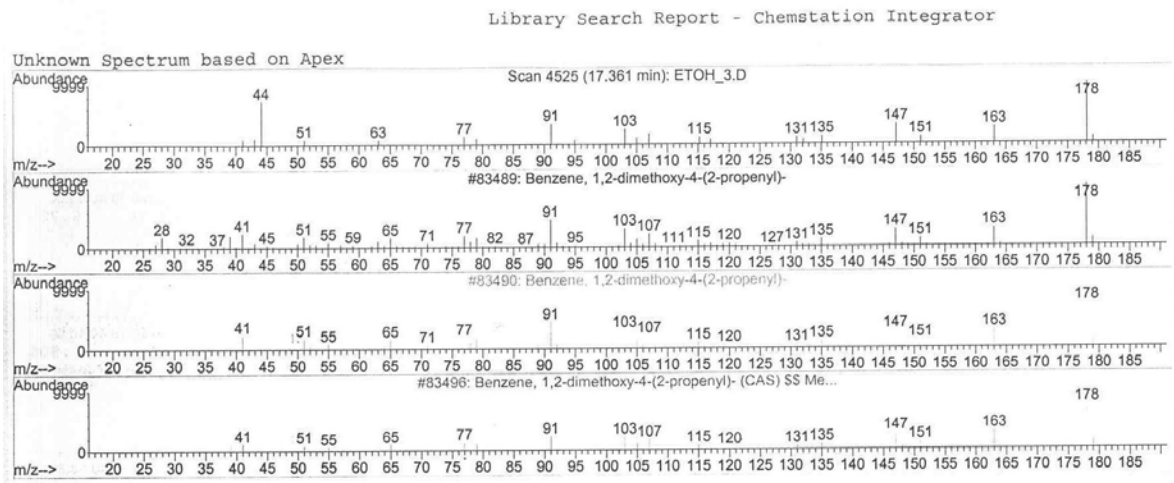
1 Phenol, 2-methoxy-4-(2-propenyl)...	123684	000093-28-7	92
2 Phenol, 2-methoxy-4-(2-propenyl)...	123678	000093-28-7	90
3 Phenol, 2-methoxy-4-(2-propenyl)...	64442	000097-53-0	90

The mass spectrum of the trace compounds of galangal extracts shows m/z 204, m/z 161, m/z 93, m/z 69 and m/z 41 that match with beta-bisabolene.



The 3 best hits from each library.	Ref#	CAS#	Qual
C:\Database\wiley7n.l			
1 .beta.-Bisabolene $$ Cyclohexene...	121221	000495-61-4	98
2 Cyclohexene, 1-methyl-4-(5-methy...	121215	000495-61-4	97
3 Cyclohexene, 1-methyl-4-(5-methy...	121218	000495-61-4	97

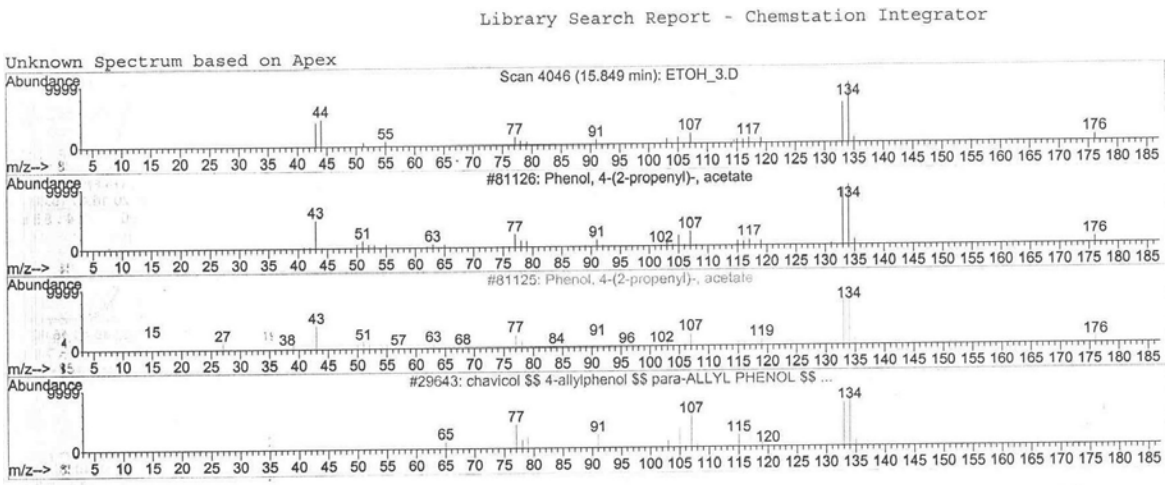
The mass spectrum of the trace compounds of galangal extracts shows m/z 178, m/z 163, m/z 147 and m/z 91 that match with 1,2-dimethoxy-4-(2-propenyl)-benzene.



0.22

The 3 best hits from each library.	Ref#	CAS#	Qual
C:\Database\wiley7n.l			
1 Benzene, 1,2-dimethoxy-4-(2-prop...	83489	000093-15-2	94
2 Benzene, 1,2-dimethoxy-4-(2-prop...	83490	000093-15-2	94
3 Benzene, 1,2-dimethoxy-4-(2-prop...	83496	000093-15-2	93

The mass spectrum of the trace compounds of galangal extracts shows m/z 176, m/z 134, m/z 117 and m/z 43 that match with 4-(2-propenyl)- , phenol acetate.



The 3 best hits from each library.	Ref#	CAS#	Qual
C:\Database\wiley7n.l			
1 Phenol, 4-(2-propenyl)-, acetate	81126	061499-22-7	94
2 Phenol, 4-(2-propenyl)-, acetate	81125	061499-22-7	90
3 chavicol $$ 4-allylphenol $$ par...	29643	000501-92-8	90

BIOGRAPHY

Jirawan Oonmetta-aree was born in Nakhon Ratchasima, Thailand. She attended Srinakharinwirot University, Thailand and received her Bachelor's degree in Biology (1993). In 1997 she received a Master's degree in Pharmacy at Mahidol University, Thailand. In 1999 she attended Suranaree University of Technology, Thailand and in 2000 she was granted a scholarship by Office of the Rajabhat Institute, Ministry of Education.

PRESENTATIONS

Oonmetta-aree, J., Suzuki, T., Eumkeb, G., and Gasaluck, P. (2004). Antimicrobial properties of galangal (*Alpinia galanga* Linn.) on *Staphylococcus aureus*. 10th World Congress on Clinical Nutrition, November 30-December 3, 2004, Pearl Village, Phuket, Thailand.