Effect of *Eleutherine americana* Merr. Bulb Extracts on Food Poisoning *Staphylococcus aureus* and its Application in Food Systems

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Abstract

Bulbs of *Eleutherine americana* Merr. were examined for their antibacterial activities against *Staphylococcus aureus* isolated from foods. Ready-to-eat foods were purchased over a period of 3 months out of which 76 (71.69%) were contaminated with *Staphylococcus aureus*. The isolates were characterized phenotypically using traditional biochemical methods. Ninety-four percent of the isolates were mannitol fermenters, 86% positive for coagulase test, while 80% produced lipase enzyme. Antibiotic susceptibility test revealed that 21% and 63% of the food isolates were resistant to oxacillin and penicillin, respectively. The results showed that 22 (20.75%) food samples were contaminated with methicillin-resistant *S. aureus* (MRSA).

The antibacterial activity of the acetone, ethanol, ethanol+hexane, and hexane extracts from the bulbs of *E. americana* were investigated by paper disc agar containing 2.5 mg of the crude extract. The various solvents produced similar inhibition zones, ranging from 14.5 and 15.7 mm for the food isolates, and 12.2-17.0 mm for the enterotoxin-producing reference strains. The minimal inhibitory concentration (MIC) values ranged from 0.06 to 1.00 mg/ml for food isolates and 0.25 mg/ml for the three reference strains. Growth curve in the presence of the crude ethanol extract at 4MIC showed bacteriostatic effect by 5 log reduction relative to the control.

The ethanolic extract from the bulb of *E. americana* was investigated for its ability to inhibit enzymes and enterotoxin production by *S. aureus*. Preliminary

screening of the isolates for enzyme synthesis revealed that higher percentage of the isolates produced lipase more than protease enzymes. About 15% of the 106 isolates were positive for enterotoxin production with staphylococcal enterotoxin A (11.3%), enterotoxin B (3.7%), enterotoxin C (10.3%), and no enterotoxin D was produced. The production of staphylococcal enterotoxins A–D in the presence or absence of the crude extract was carried out. In the broth system, the extract reduced enterotoxin production at subminimal inhibitory concentrations compared with the control. At MIC, total enterotoxin inhibition was observed for enterotoxin C production, whereas synthesis of enterotoxins A, B, and D were totally eliminated at 2MIC. The food system study revealed that the extract could delay production of enterotoxins A, B, and C compared with the control. The extract at 2 mg/ml delayed production of toxins A and C for 8 and 4 h, while toxin B was not detected in the pork at 48 h. The ability of *E. americana* extract to inhibit lipase and protease enzymes and to delay enterotoxin production in food could present it as a novel food additive to combat the growth of *S. aureus* in food.

The mechanisms of action of ethanolic extract from *E. americana* against *S. aureus* was investigated. Treatment of *S. aureus* ATCC 27664 with crude extract at 2MIC reduced the inoculum size by 5 log at 24 h compared with the control. The combined effect of the extract and 7.5% NaCl on the enterotoxin-producing ATCC strain resulted to total elimination within 24 h, compared to the control. The release of cell materials after extract treatment was determined by measuring optical density of the suspensions at 260 nm. It was observed that the treatment resulted in cytoplasmic leakage. Optical density determination at 620 nm showed that the extract did not cause gross cell wall damage. However, observation of staphylococcal cells treated with 2MIC and 4MIC of the crude extract under electron microscope revealed that *E. americana* caused damages to membrane integrity with some dark spots in the cytoplasm. A knowledge and understanding of the mechanism of action of *E. americana* extract could offer useful hints in the search for novel antibacterial both in clinical and food system.

The ethanolic extract was investigated for its antistaphylococcal activity both *in vitro* and in different food systems. The extract activity against *S.*

aureus was better at 35ºC than at 10ºC and 4ºC, respectively. The extract exhibited excellent stability to heat and pH treatments. The scavenging activities of crude ethanolic extract from *E. americana* was investigated. The results revealed that the extract produced IC_{50} values of 8.4 μg/ml and 0.78mg/ml on 2,2-diphenyl-1-picrylhydrazyl and hydroxyl free radicals, respectively. Total phenolic content of the extract was determined using the Folin–Ciocalteu reagent and the crude extract yielded high phenolic content of 4.56 μmol gallic acid equivalent/mg dried extract.

The crude extract was incorporated into home made salad dressing and examined for its antibacterial, physical, chemical, and sensory properties during storage at 4ºC for 16 days. A reduction of more than one log in *S. aureus* count was observed, compared to the control. Similarly, the extract at different concentrations were incorporated into pork, cooked in the microwave, and stored at 4ºC for 9 days. Antibacterial activity of the extract against *S. aureus* in the cooked pork revealed that addition of 10.8 mg/100 g of the extract reduced the bacterial population by 0.57 log compared to control at 9 day of storage. The crude extract demonstrated antioxidant activity which increases with increased extract concentration and retarded lipid oxidation in the salad dressing and cooked pork. Furthermore, addition of the extract led to increase in the redness values of the pork and this was acceptable from the sensory point of view. The sensory evaluations for both salad dressing and cooked pork revealed that the products at all extract concentrations were acceptable. The extract from *E. americana* can be a promising novel additive to improve the quality and safety of home made salad dressing, as an antioxidant to prevent lipid oxidation and a potential natural colour enhancer of red meat and meat products.

Antimicrobial activity of the crude ethanolic extract was further examined using fourteen strains of bacteria, six fungi, and two yeasts. The extract demonstrated good antibacterial activities and produced inhibition zones ranging from 13.0-20.0 mm against all the Gram-positive bacteria tested, and only one out of the Gram-negative bacteria. In addition, the extract exhibited antifungal activity against *Aspergillus niger, Rhizopus* spp., and *Penicillium* spp. while all the dermatophytic fungi and yeast strains were resistant to the extract treatment. Growth curve in the presence of the crude ethanol extract at 4MIC showed bacteriostatic and fungistatic

effects by 5 and 3 log reduction, respectively relative to the control. Data from this study revealed that *E. americana* bulb may be a good antimicrobial agent against foodborne pathogen and food spoilage organisms.

Partially-purified fractions from *E. americana* extract were identified by column chromatography. The fractions were examined for their antibacterial activity on MRSA isolates obtained from foods. Fraction Ea6.3 produced MIC values of 125-500 µg/ml and minimal bactericidal concentration (MBC) of 250->1,000 µg/ml, whereas fraction Ea9 yielded MIC/MBC of 125-250/500->1,000 µg/ml against all the MRSA isolates. The MIC/MBC values for the enterotoxin-producing reference strains were 250/500 µg/ml for Ea6.3 while Ea9 produced MIC/MBC values of 125/>1,000 µg/ml. Growth curves in the presence of fraction Ea6.3 at 4MIC resulted in total elimination of all the test strains between 20 and 24 h, while fraction Ea9 reduced bacterial population by at least 6 log relative to the control. The partially-purified fractions were further purified to obtain pure compounds which produced MICs ranging from 31.25 to 1,000 µg/ml against *S. aureus* reference strains.

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Ifesan, B.O.T

CONTENTS

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF TABLES (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

ABBREVIATIONS

cm	=	centimeter
CFU	=	colony forming unit
°C	=	degree celcius
DMSO	=	dimethylsulfoxide
DPPH	=	2,2-diphenyl-1-picrylhydrazyl
g	=	gram
h	=	hour
HCI	=	hydrochloric acid
L	=	liter
pH	=	-log hydrogen ion concentration
µg	=	microgram
µl	=	microliter
mg	=	milligram
ml	=	milliliter
mm	=	millimeter
min	=	minute
m	=	molarity
NPRC	=	natural product research centre
%	=	percent
NaCl	=	sodium chloride
NaOH	=	sodium hydroxide
sd	=	standard deviation
SE	=	staphylococcal enterotoxin
SFP	=	staphylococcal food poisoning
TBARs	=	thiobarbituric acid reactive substances

LIST OF APPENDIX

CHAPTER 1

INTRODUCTION

Staphylococcus aureus is a versatile pathogen of human and animals causing a wide variety of diseases ranging from superficial infections to more severe diseases such as soft-tissue, respiratory tract, urinary tract, joints and bones, pneumonia, endocarditis, bacteremia with metastatic abscess formation, septicaemia, staphylococcal scalded skin syndrome, impetigo, meningitis, brain abscesses, and a variety of toxin-mediated diseases including gastroenteritis, and toxic shock syndrome (Ing *et al*., 1997; Archer, 1998; Lowry, 1998; Fidalgo *et al.,* 1990; Roberts *et al.,* 1991). One of the key factors enabling *S. aureus* to survive, colonize, proliferate, and cause human infections is the expression of virulence factors. For the majority of diseases caused by *S. aureus*, pathogenesis depends on the ability of the strain to survive, multiply under a number of extreme environments and produce various extracellular substances such as haemolysins, nuclease, protease, coagulase, lipase and enterotoxin. In several of these infections, research has shown that the bacterial strain causing severe infection is indistinguishable from that colonizing the nares (Noble, 1997).

The normal microbial habitat of *S. aureus* is warm-blooded mammals, with approximately 20% of humans permanently colonized with this organism, and as much as 50% of the population transiently colonized (Tenover and Gayness, 2000). *Staphylococcus aureus* is present on the skin and mucosa of human, animals, and the environment (Jay, 2000). This bacterium is also present on the skin and mucosa of food-producing animals including ruminants. It is frequently associated to subclinical mastitis leading to contamination of milk and dairy products (Jablonski and Bohach, 1997). As a consequence, food products may originally become contaminated during or after processing either from the hands and body of processors, equipments, and processing plants.

Staphylococcal food poisoning (SFP) is a prevalent cause of foodborne disease worldwide (Jablonski and Bohach, 2001). It is an important foodborne pathogen due to its ability to produce enterotoxins. Staphylococcal food poisoning is caused by ingestion of food containing *S. aureus* cells or preformed staphylococcal enterotoxin (SE), produced by some strains of *S. aureus* and occasionally by other staphylococci (Genigeorgis, 1989; Jablonski and Bohach, 1997). When large numbers of enterotoxigenic staphylococci grow in foods, they elaborate enough toxin to cause food poisoning after the foods are ingested. Staphylococcal food poisoning caused by ingestion of enterotoxins is characterized by emesis and in many cases is accompanied by gastroenteritis (Dinges *et al.,* 2000).

Inspite of modern improvements in food production techniques, food safety is an increasing important public health issue documented by World Health Organization (WHO, 2003). The increasing demand for natural food additives has made herbs and spices to emerge as popular food ingredients. Due to the awareness on chemical preservatives the food industry is now reflected by the consumer opinions for safer additives and thus focusing on natural GRAS (Generally Recognized As Safe) preservatives (Dillion and Board, 1994). Most consumers now prefer nutritious food with extended shelf life and without synthetic preservatives. Furthermore, interest in plants with antibacterial properties has revived as a consequence of current problems associated with the use of antibiotics and the presence of antibiotic resistance in potentially harmful bacteria in foods of animal origin. A number of medicinal plant extracts and their essential oils have been reported to possess antimicrobial activities. The antibacterial activity of certain medicinal plants against a wide range of pathogenic bacteria (Voravuthikunchai *et al.,* 2007) such as methicillin resistant *S. aureus* (MRSA) (Voravuthikunchai and Kitpipit, 2005), and *Escherichia coli* O157:H7 (Voravuthikunchai and Limsuwan, 2006) have been previously reported. In addition, naturally-sourced substances are becoming more widely used in the food industry both as flavouring and tenderizing agents (Garg and Mendiratta, 2006; Jayathilakan *et al.,* 2007) as well as antimicrobial agents (Ahn *et al.,* 2007; Solomakos *et al*., 2008).

It is of advantage to develop a potential food additive that can inhibit the growth of *S. aureus* in foods in case of poor handling and temperature abuse.

Eleutherine americana is an herbal plant whose red bulb was used as a folk medicine for the treatment of cardiac diseases, especially coronary disorders (Ding and Huang, 1983). In addition, the bulb is used in Asian cuisine. Several compounds including eleutherin, eleutherol, isoeleutherin as well as anthraquinones and their glycosides have already been isolated from the bulbs of this plant (Hara *et al*., 1997; Qui *et al*., 2005; Xu *et al.,* 2006). Preliminary data by our research group suggested that the ethanolic extract of the bulb of this plant exhibited broad spectrum antibacterial activity against Gram-positive pathogenic bacteria. There is a need for more detailed investigation on the activity of this plant against *S. aureus* isolated from foods, some of its extracellular enzymes and the applications of the extract into food systems.

OBJECTIVES OF THE RESEARCH

1. To investigate incidence of food contamination by *Staphylococcus aureus*
2. To determine the minimal inhibitory concentration (MIC) and minimal bactericidal concentrations (MBC) of crude extract, partially-purified fractions, and pure compounds from *Eleutherine americana* against *Staphylococcus aureus* isolated from foods
3. To investigate the inhibitory effect of extract on some extracellular enzymes secreted by *Staphylococcus aureus*
4. To study the effect of crude extract on staphylococcal enterotoxins in food
5. To examine the possible mechanism of action of crude extract on *Staphylococcus aureus*
6. To screen the antimicrobial activity of *Eleutherine americana* crude extract
7. To apply the crude extract in food systems

CHAPTER 2

LITERATURE REVIEW

2.1 Food Poisoning

Foodborne disease has been defined by WHO as a disease of an infectious or toxic nature caused by the consumption of food or water (Adams and Moses, 1995). Food poisoning is a common, usually mild, but sometimes deadly illness. The Centers for Disease Control and Prevention (CDC, 2000) estimates that in the United States alone, food poisoning causes about 76 million illnesses, 325,000 hospitalizations, and up to 5,000 deaths each year. Food poisoning can be caused by foodborne bacterial, viral, or protozoal pathogens that produce toxins and, or invade tissue of the gastrointestinal tract. Bacteria are the leading cause of foodborne diseases and appear to be the causative agents of more than two thirds of the recorded outbreaks. Korea Food and Drug Administration reported that *Salmonella* and *S. aureus* especially were major bacterial agents causing foodborne diseases (KFDA, 2006). Of these foodborne outbreaks, mass catering food services such as school and company accounted for 72%. In most cases of foodborne illnesses considered, the pathogenic effects occur in the alimentary tract giving rise to symptoms including diarrhoea and vomiting. Bacteria causing foodborne infections have a pathogenesis centered on their ability to penetrate, survive, and multiply in host cells. The pathogenesis of bacteria causing foodborne poisoning depends on their capacity to produce toxins before the food is consumed or after ingestion in the digestive tract. The most important foodborne pathogenic bacteria are listed in Table 1. Food poisoning can be divided into infections and intoxications, where *Salmonella* spp. and *Shigella* spp. are typical examples of infections and *Clostridium botulinum* and *S. aureus* for intoxications (Table 2).

Table 1. Important food poisoning organisms

Organisms	Infective dose	Incubation time	Symptoms[a]	Reference (s)
Aeromonas spp.	10^6-10^8	6-48 h	A, D,DH	Kirov, 1993
Bacillus cereus (diarrhoeal)	10^5-10^7	6-12 h	A, D, DH	Kramer & Gilbert, 1989
Bacillus cereus (emetic)	$\geq 10^5$	1-6 h	A, V	Kramer & Gilbert, 1989
Campylobacter spp.	10^3-10^5	2-5 day	BD, F	Butzle & Oosterom, 1991
Clostridium botulinum	Toxins	12-36 h	A, D, ND, V	Hauschild, 1989
Clostridium perfringens	10^8	6-16 h	A, D, DH	Granum, 1990
Escherichia coli (LT)[b]	10^5-10^8	16-18 h	A, BD, DH	Doyle & Padhye, 1989
Escherichia coli (ST)[c]	10^5-10^8	4-6 h	D, F	Doyle & Padhye, 1989
Listeria monocytogenes	10^7-10^8	1-90 days	I, M	Schuchat *et al*., 1991
Salmonella spp.	10-10^6	7 h-21 day	A, D, F, DH, V	D'Aoust, 1989
Shigella spp.	10^2-10^5	1-7 day	A, BD, D, DH, F	Wachsmuth & Morris, 1989
Staphylococcus aureus	Toxins	1-6 h	A, D, V	Bergdoll, 1989
Vibrio cholerae	10^8	2-5 day	A, D, DH	Wachsmuth *et al*., 1994
Vibrio parahaemolyticus	10^3-10^4	9-24 h	A, F, V,	Adams & Moses, 1995
Yersinia enterocolitica	10^7	2-7 day	A, D, DH, V	Kapperud, 1991

[a]A: Abdominal pain, BD: Bloody diarrhoea, DH: Dehydration, D: Diarrhoea, F: Fever, I: Influenza-like, M: Meningitis, ND: Neurological disturbances; V: Vomiting.

[b]LT: Heat-labile toxin, [c]ST: Heat-stable toxin

Table 2. The four different types of food poisoning

Food poisoning type	Description of type	Bacteria belonging to the groups
1.	Infections	*Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Yersinia enterocolitica, Listeria monocytogenes*, some *Escherichia coli,* and some *Aeromonas* spp.
2.	Interaction	*Vibrio cholerae*, *E. coli* (ETEC) and some *Aeromonas* spp.
3	Enterotoxin production in the intestine of the host without any interaction	*Bacillus cereus, Clostridium perfringens*
4	Intoxication	*Clostridium botulinum*, *Bacillus cereus* (emetic type), *S. aureus*

Source: Granum *et al*., 1995

Bacteria in groups two and three do not harm the host directly, the bacterium in this location is like an 'enterotoxin factory'. The bacteria belonging to group three do not interact with the epithelial cells in the intestine, while the bacteria of group two must colonize the epithelial cells prior to enterotoxin production. Thus organisms that cause food poisoning can be divided into three groups based on their effects on the person or the symptoms produced. Invasive pathogens, infilterate the gastrointestinal tract and cause fever and dysentery, due to the inflammatory response of the body. The noninvasive pathogens are those which impair function of the gastrointestinal tract to produce malabsorption and fluid loss (Johnson, 1999). Toxigenic pathogens are organisms that affect the lining of the gastrointestinal tract and produce direct irritation or hypermotility and hypersecretion. *Staphylococcus aureus* is one of the

pathogen that causes acute bacterial food poisoning and over half of documented food poisoning cases are caused by this bacterium. The symptoms produced result from ingestion of a preformed enterotoxin which is a result of direct effect of the toxin on the chemoreceptor trigger site in the medulla.

2.2 *Staphylococcus aureus*

Staphylococci are Gram-positive, catalase positive cocci, which colonize human or animal skin and mucous membrane. They are spherical cells, 0.5-1.0 μm in diameter, occurring singly, in pairs and irregular clusters. They are non motile, non sporing and facultative anaerobes (Holt, 1994). The staphylococcal cell wall is resistant to lysozyme and sensitive to lysostaphin, which specifically cleaves the pentaglycin brigdes of *Staphylococcus* spp. They produce numerous virulence factors that promote their survival and subsequent dissemination. Staphylococcal infections cause a variety of cutaneous and systemic infections, including impetigo, subcutaneous abscess, staphylococcal scalded syndrome, toxic shock syndrome (TSS) and neonatal toxic shock syndrome (Iwatsuki *et al*., 2006).

Although *S. aureus* is an important pathogen, many healthy people carry it as part of the normal population of microorganism associated with the nose, throat, perineum or skin. Among staphylococci, *S. aureus* is usually regarded as a transient, pathogenic organism in the skin, and approximately 20% of the general population always harbours it on the mucosa without any pathogenic event (Peacock *et al*., 2001). However, it is known to cause several diseases syndrome in humans including foodborne gastroenteritis.

While a range of staphylococci can produce enterotoxin, the vast majority of outbreaks of SFP are due to the presence of *S. aureus* (Jay, 2000; Bremer *et al.,* 2004). *Staphylococcus aureus* has been reported to be present in foods (Aycicek *et al*., 2005; Normanno *et al*., 2005; Normanno *et al*., 2006). In a study of food poisoning in England, the most prevalent contaminated foods (75%) were meat (ham), poultry or their products. Other contaminated food products included fish and shell-fish (7%) and milk products

(8%) while most contamination took place in the home followed by restaurants and food stores (Wieneke *et al*., 1993). The largest 2005 outbreak was reported from luncheon and meat when 138 people became ill from *S. aureus* in sausage at a Kansas work place (Dewaal and Bhuiya, 2007). An outbreak of acute gastroenteritis in an Australian boarding school was reported where *S. aureus* was implicated. About 101 out of 113 cases were hospitalized, while the causative agent was isolated from stool specimens and swab of palmar skin lesion of one of the healthy kitchen worker (Schmid *et al.,* 2007). During 2004 to 2006, *S. aureus* was recovered from 8.8%, 11.3%, and 4.3% of pork carcass samples, respectively, collected at 53 slaughterhouses in Taiwan (Lin *et al*., 2009). Table 3 shows some of the reported outbreaks. Minor outbreaks of SFP are not usually reported and true incidence is probably underestimated (Dinges *et al*., 2000). Nevertheless, SFP represents a considerable social burden in terms of hospital expenses, loss of patients' working days and productivity, together with the cost of disposing the contaminated food (Normanno *et al*., 2005).

Table 3. Reported staphylococcal food poisoning outbreaks from restaurants

Toxin	Source	Establishment	Country	Reference
SEA	Pork and chicken	Roadside restaurant	USA	De Saxe *et al*., 1982
SEA	Cooked chicken	School lunch	USA	De Saxe *et al*., 1982
SEC	Turkey	Buffet	USA	MMWR weekly, 1986
SEC	Ham salad sandwich	School girl restaurant	USA	Bergdoll, 1989
SEA	Prepacked chicken	Restaurant	UK	Robinson *et al*., 1989
SEA	Chicken salad	School restaurant	USA	US Food & Drug Administration, 1992
SEA	Ham	School restaurant	USA	Richards *et al*., 1993
SEA, SEC	Eclairs	College restaurant	Thailand	Thaikruea *et al*., 1995
SEA	Imported canned mushroom	Restaurant	USA	Levine *et al*., 1996
SEA	Scrambled egg	Cafetaria	Japan	Miwa *et al*., 2001
SEA	Chicken	School restaurant	Australia	Schmid *et al*., 2007

Modified from Soriano *et al*., 2002.

Antimicrobial resistance has been considered as a major problem in Public Health. Methicillin-resistant *S. aureus* human isolates unlike animal isolates, are frequently resistant to the penicillinase-resistant penicillins (Kloos and Bannerman, 1995; Tenover and Gaynes, 2000). *Staphylococcus aureus* becomes methicillin resistant by the acquisition of the *mec*A gene which encodes a penicillin binding protein (PBP2a) with a low affinity for ß-lactams. The strains producing PBP2a are resistant to all ß-lactams (Chambers, 1997). Such organisms are also frequently resistant to most of the commonly used antimicrobial agents, including the aminoglycosides, macrolides, chloramphenicol, tetracycline, and fluoroquinolones (Mandell *et al.*, 1995). Methicillin-resistant *S. aureus* strains have been reported in major food animals, and this was attributed to the extended use and misuse of antibiotics in animal husbandry (Lee, 2003; Kitai *et al*., 2005; Pesavento *et al*., 2007). In the analysis of 1913 specimens from milk, beef, pork and chicken meat, 15 strains of *S. aureus* harboured the *mec*A gene (Lee 2003). A community-acquired MRSA infection was reported when a family was involved in an outbreak after eating baked pork meat, contaminated from the food handler (Jones *et al*., 2002). Furthermore, Normanno *et al*., (2007) reported the presence of *mec A* in 6 out of 160 *S. aureus* strains analyzed and all the MRSA strains were able to synthesize SEs. When a few cells of *S. aureus* enter an immunocompetent individual, they are destroyed by gastric juices, but when an immunocompromised patient's food contains cells of *S. aureus*, these can reach the circulatory system and cause infections that may evolve to septicaemia (Pesavento *et al*., 2007).

2.2.1 Identification of *Staphylococcus aureus*

Staphylococcus aureus is an important pathogen due to a combination of toxin-mediated virulence, invasiveness, and antibiotic resistance. On Baird-Parker medium, colonies are circular, smooth, convex, moist, 2-3 mm in diameter on uncrowded plates, grey to jet-black, frequently with light-coloured (off-white) margin, surrounded by opaque zone and frequently with an outer clear zone. Colonies have buttery to gummy consistency when touched with inoculating loop (Bennet and Lancette, 1998). It is differentiated from other species on the basis of the gold pigmentation of colonies and positive results of coagulase, mannitol fermentation, and

deoxyribonuclease test (Betley *et al.,* 1992; Wilkinson, 1997). Different media used for detection and quantification of *S. aureus* have been reviewed by Baird and Lee (1995). Most pathogenic strains ferment mannitol in addition to producing yellow pigment, haemolysin, and coagulase. *Staphylococcus aureus* strains can be classified into different biotypes according to their human or animal origin. A biotype schema including six different biotypes (human, non-ß-hemolytic human, avian, bovine, ovine, and nonspecific), based on biochemical characteristics was developed (Devriese, 1984).

2.2.2 Growth requirement

The nutritional requirements of *S. aureus* are complex and vary from strain to strain. The conditions under which this bacterium grows also depend on the composition of the food. In general, *S. aureus* grows between 7ºC and 47ºC, with an optimal temperature of 30 to 37ºC. The range of environmental parameters over which *S. aureus* will produce enterotoxin can be narrower than the range over which it will grow. Enterotoxins are produced between 10ºC and 46ºC, with an optimum temperature of 35-45ºC. Enterotoxin production is substantially reduced at 20-25ºC. It is generally accepted that the enterotoxin production is unlikely to occur at temperatures below 10ºC. Optimal enterotoxin production occurs at pH 6-7 and it is influenced by atmospheric conditions, carbon and nitrogen and salts level. Reduced levels of water activity may also inhibit toxin synthesis more than growth. Optimal growth and toxin production occur at water activity level less than 0.99 and toxin production has been reported to occur at as low as water activity 0.86 (Nottermans and Heuvelman, 1983; Miller *et al.,* 1997; Jay, 2000).

2.2.3 Resistance of the organism

Staphylococcus aureus is a hardy organism that can withstand dessication and can survive in dust and on dry metal, glass or porcelain surfaces for a long period of time. In aerobic cultures, hydrogen peroxide does not accumulate because the organisms elaborate catalase. Staphylococci possess lipolytic enzymes which render them resistant to bactericidal lipids of human skin. This organism has a high heat resistance for nonsporing bacteria with D-value of 60ºC being reported to

range from 2 to 50 min depending on the types of food. Most chemical sanitizers used routinely in food industry including chlorine, other halogens, and quartenary ammonium compounds will destroy *S. aureus* on surfaces only when applied correctly. Unfortunately, cells that recovered after exposure to sanitizers among populations established on poultry processing equipments were subsequently more resistant to them (Bolton *et al.,* 1988; Kusumaningrum *et al.,* 2003).

2.2.4 Staphylococcal enterotoxin

Staphylococcal enterotoxins function both as potent gastrointestinal toxins as well as superantigens that stimulate non-specific T-cell proliferation (Balaban and Rasooly, 2000). When grown in artificial media, pathogenic staphylococci release a number of different exotoxins whose production is stimulated in an atmosphere of 30% carbon dioxide. Staphylococcal enterotoxins are a family of thermostable protein, resistant to proteolytic enzymes such as rennin, papain, and chymotrypsin but staphylococcal enterotoxin B is sensitive to pepsin at pH 2 (Balaban and Rasooly, 2000; Jay, 2000), forming a single chain with a molecular weight ranging from 26,000 to 29,600 Da. They are produced during the phases of growth, but mainly during the middle and at the end of the exponential phase. Enterotoxins are emetic toxins and are classified as members of pyrogenic toxin superantigen family because of their biological activities and structural relatedness. Staphylococcal enterotoxins are identified as separate proteins due to their differences in antigenicity and are sequentially assigned a letter of the alphabets in order of their discovery. The primary sequences of all the classical SEs have been studied with protein sequencing and recombinant DNA methods (Genigeorgis, 1989; Jablonski and Bohach, 1997; Balaban and Rasooly, 2000; Dinges *et al.,* 2000; Blaiotta *et al.,* 2004). The amount of enterotoxin necessary to cause intoxication is very small. The emetic dose in a monkey assay was approximately 5-20 μg/animal (Bergdoll, 1965). Ingestion of 100-200 ng of enterotoxin can induce symptoms of food poisoning (Bergdoll, 1988; Evenson *et al*., 1988). In an outbreak of gastroenteritis in United States due to chocolate milk containing staphylococcal enterotoxin A (SEA), the mean amount of SEA in the 400 ml container was 0.5 ng/ml (Evenson *et al*., 1988).

Eighteen types of SEs are currently known (that is SEA to SEE, SEG to SEQ, SER and SEU) (Su and Wong., 1995; Munson *et al.,* 1998; Fitzgerald *et al.,* 2001; Letertre *et al.,* 2003; Blaiotta *et al.,* 2004; Normanno *et al*., 2007). Five major antigenic types of SE (SEA to SEE) (Dinges *et al.,* 2000) and four additional SEs (SEG to SEJ) have been reported, and their corresponding genes have been described (Ren *et al.,* 1994; Munson *et al.,* 1998; Rosec and Gigaud, 2002). The list of the SE family was expanded by the detection of further genes (SEK, SEL, SEM, SEN, SEO, SEP, SEQ, SER, SEU) encoding homologous enterotoxins (Jarraud *et al.,* 1999; Letertre *et al.,* 2003; Omoe *et al.,* 2003). However, the relationship between these new SEs and human food poisoning is not fully understood at present (Blaiotta *et al.,* 2004; Jorgensen *et al.,* 2005). Nevertheless, only three of the novel SEs, SEG to SEI have been shown like SEA to SEE, to cause vomiting after oral administration to a primate. The other SEs either lack emetic properties (SEL, SEQ) or are yet to be tested (SEJ, SEK, SEM to SEP, SER, SEU) and it has been suggested that they be designated SE-like proteins (Lina *et al.,* 2004; Kerounanton, *et al.,* 2007). Staphylococcal enterotoxin C represent several antigenic subvariants (SEC1, SEC2, SEC3, SEC_{ovine}, and SEC_{bovine}) that feature minor epitope differences (Monday and Bohach, 1999; Chen *et al.,* 2004; Normanno *et al.,* 2007).

Many studies have been carried out on the detection and evaluation of the occurrence of the five classical SEA to SEE in foodborne strains and or directly in the food sample (De Luca *et al.,* 1997; Akinedon *et al.,* 2001; Rosec and Giguad, 2002; Ercolini *et al.,* 2004). The ability of *S. aureus* to grow and produce SEs under a wide range of conditions is evident by the variety of foods that have been implicated in SFP (Atanassova *et al.,* 2001; Le Loir *et al.,* 2003; Blaiotta *et al.,* 2004). Staphylococcal enterotoxin A is the most common enterotoxin recovered from food-poisoning outbreaks (Casman, 1965; Holmberg and Blake, 1984; Bergdoll, 1990), with SED being the second most frequent, while fewest number of outbreaks are associated with SEE. Staphylococcal enterotoxin B has been reported as a potential microbiological weapon of warfare (Greenfield *et al.,* 2002). The amount of SEA needed to cause symptoms in humans is about 100 ng (Balaban and Rasooly, 2000). For SEB, SEC, and SEE, 11%, 10%, and 3% were found among 3367, 1581 and 1072 strains, respectively (Jay, 2000).

Some enterotoxin strains also produce toxic Shock Sydrome Toxin 1 (TSST-1) and some of the symptoms of TSST-1 appear to be caused by SEA, SEB and SEC. The genes for SEA, SEB, SEC, SEE are chromosomal, whereas SED is plasmid borne (Iandolo, 1989; Johnson *et al.*, 1991; Balaban and Rasooly, 2000). The SEs and TSST-1 are considered 'superantigens', a term coined to describe microbial (bacterial or viral) proteins that activate large numbers of specific T-cells against conventional antigens (Choi *et al.,* 1989; Marrack and Kappler, 1990). The emetic effect of enterotoxin is probably the result of central nervous system stimulation after the toxin acts on neural receptors in the gut. Toxic Shock Syndrome Toxin-1 is the prototypical superantigen which promotes the protean manifestations of the TSS. Several methods for the detection of SEs from contaminated foods have been developed. These include biological, immunological, chromatographic, and molecular assays (Balaban and Rasooly, 2000; Blaiotta *et al.,* 2004; Chen *et al.,* 2004; Ercolini *et al.,* 2004; Jorgensen *et al.,* 2005; Jusufagic *et al.,* 2007).

2.2.4.1 Chemical and physical properties of enterotoxin

Enterotoxins are simple proteins and upon hydrolysis yield 18 amino acids with aspartic, glutamine, lysine and tyrosine being the most abundant. The amino acid sequence of SEB was the first to be determined (Jay, 2000). Although various enterotoxin differ in certain physiochemical properties, each has about the same potency. The gene for SEA is carried by a temperate bacteriophage (Betley and Mekalanos, 1985). It is expressed from the mid-exponential phase of growth, but is not regulated by the accessory gene regulator, (*agr*) (Tremaine *et al*., 1993). The coding region of SEB gene contains about 900 nucleotides (Johns and Khan, 1988). The gene is chromosomal in clinical isolates of *S. aureus* from food poisoning cases (Shafer and Iandolo, 1978), however, in other strains the gene is carried by a 750 kb plasmid (Shalita *et al*., 1977). Staphylococcal enterotoxin Cs are a group of highly conserved proteins with significant immunological cross-reactivity (Bergdoll *et al*., 1965). The gene encoding SED is located on a 27.6-kilobase penicillinase plasmid designated pIB485 (Bayles and Iandolo, 1989). Enterotoxins are relatively heat resistant, the biological activity of SEB was retained after heating for 16 h at 60ºC and pH 7.3 (Jay, 2000). Although biological activity and serological reactivity are

generally associated, it has been shown that serologically negative enterotoxin may be biologically active. Enterotoxin production tends to be favoured by optimal growth conditions of pH, temperature, redox potential, and water activity. Factors permitting growth and enterotoxin production by *S. aureus* are shown on Table 4.

2.2.4.2 Modes of action

Staphylococcal enterotoxin along with the toxic shock syndrome are classed as bacterial superantigen that binds directly to class II major histocompatibility complex (MHC) of antigen-presenting cells outside the normal antigen-binding groove and stimulate non-specific T-cell proliferation. With the latter, a CD4 T- cell facilitates contact between T-cell antigen receptors and major histocompatibility complex class II molecules. Staphylococcal superantigens bind directly to T-cell receptor ß chains without processing. Once bound to T-cell receptor, superantigens may activate 20% of the naive T-cell population whereas conventional antigens stimulate only 0.01% (Proft and Fraser, 2003). They bypass the common antigen presentation mechanisms and act by crosslinking the MHC class II molecule on antigen-presenting cells with variable Vß region of the T-cell receptor and are not MHC restricted for presentation to T-cells (Marrack and Kappler, 1990). They produce cytokines such as the interleukins (IL), γ-interferon and tumuor necrosis factor. Superantigens are thus protein that activate many different T cells clones. Among the cytokines, an overabundance of IL-2 is produced, and it appears to be responsible for many or most of the symptoms of staphylococcal gastroenteritis (Johnson *et al.,* 1992; Jay, 2000).

Table 4. Factors permitting growth and enterotoxin production by *Staphylococcus aureus*

	Growth		Enterotoxin production	
Factor	Optimum	Range	Optimum	Range
Temperature, ºC	35-37	7-48	35-40	10-45
pH	6.0-7.0	4.0-9.8	Enterotoxin A 5.3-6.8, others 6-7	4.8-9.0
NaCl	0.5-4.0%	0-20 %	0.5 %	0-20 %
Water activity	0.98->0.99	0.83->0.99	> 0.99	0.86->0.99
Atmosphere	Aerobic	Aerobic-Anaerobic	5-20% CO_2	Aerobic-Anaerobic
E_h	> +200Mv	< -200 to > +200Mv	> +200mV	not known

Source: Adams and Moses, 1995

2.2.4.3 Factors that affect staphylococcal enterotoxin production

Staphylococcal enterotoxin production has been studied in strains grown in laboratory media. The main regulatory system controlling the gene expression of virulence factors in *S. aureus* is *agr* (Kornblum *et al*., 1990) that acts in combination with the staphylococcal accessory regulator (*sar*) (Cheung *et al*., 1992). Conditions of expression of *agr* are well-documented because it regulates most of the virulence factors in *S. aureus*. Valine is necessary for growth and arginine and cystine are necessary for both growth and SE production in five strains of *S. aureus* that produce SEA, SEB or SEC, while necessities for other amino acids vary with the strains (Onoue and Mori, 1997). Glucose has been demonstrated to have an inhibitory effect on SE production, especially for SEB and SEC (Bergdoll, 1989). This inhibitory effect has been attributed to a drop in pH, as a consequence of glucose metabolism. Glucose and low pH produced inhibitory effect on *agr* expression (Regassa *et al*., 1992; Novick, 2000). Staphylococcal enterotoxin production is optimal at neutral pH and decreases in acidic pH. Usually SE production is inhibited at pH below 5. High concentration of sodium chloride increase the inhibitory effect of acidic pH, with no SE production at salt concentrations above 12%, independent of the pH (Notermans and Heuvelman, 1983). Alkaline pH also decreases the production of SEB, SEC, and SED through decreased expression of *agr* (Regassa and Betley, 1992).

2.2.5 Effects of staphylococcal superantigens

2.2.5.1 *In vivo* effects of staphylococcal enterotoxins

In human and other primates, SE can elicit an emetic response and in rare cases, toxic shock following ingestion of only microgram quantities (Bergdoll, 1988; Dinges *et al.,* 2000). Direct application of SEB to skin elicit local inflammatory response in both healthy subjects and subjects with eczema (Strange *et al.,* 1996). *In vivo* results from mice shows that SEB enters the blood stream more readily than SEA after ingestion, this revealed that these toxins cross the gastric mucosa and subsequently circulate throughout the body (Parsonnet, 1989).

2.2.5.2 Toxic shock syndrome toxin-1

Toxic shock syndrome toxin-1 can also cause systemic toxic shock through *S. aureus* growth on mucosal surfaces (Parsonnet, 1989). Stimulation of mast cells and the release of cysteinyl leukotrienes, which are blocked by a receptor antagonist, can cause emesis and skin reactions in primates (Scheuber *et al.,* 1987).

In human, the toxin is associated with fever, shock and multisystem involvement, including a desquamative skin rash. In rabbit, TSST-1 produces fever enhanced susceptibility to the effects of bacterial lipopolysacchaarides, and other biologic effects similar toTSS but the skin rash and desquamation do not occur.

2.2.6 Extracellular enzymes

In addition to the single chain SEs and TSST-1, which diminish an immune response of a colonized host by knocking out specific subsets of T cells (Smith and Johnson, 1975; Poindexter and Schlievert, 1986; Johnson *et al.,* 1992), *S. aureus* also produces a wide array of potential virulence factors, including wall teichoic acid (TA), protein A, coagulases, haemolysins, leukocidins and surface proteins that promote adherence to damaged tissue (Jawetz *et al.,* 1995; Foster, 2004; Weidenmaier *et al*., 2004). Among the various enzymes released by staphylococci into artificial media, the greatest attention has been given to coagulase. Staphylococci appear to be the only bacteria that produce these enzymes that cause thrombokinase-like action, (citrated or oxalated plasma to coagulate) clotting purified fibrinogen in the presence of a plasma factor which is believed to be a derivative of prothrombin (Jawetz *et al.,* 1995).

A positive coagulase test is generally considered the best laboratory evidence that a given strain of *Staphylococcus* is potentially pathogenic for man. Coagulase positive strains elaborate a number of extracellular enzymes. These include staphylokinase, lipase, hyaluronidase, and DNase. Coagulase is produced by *S. aureus* as an extracellular protein and is thought to have a role to play in cellular attachment. This is important given that attachment is one of the first steps in the pathogenesis of infection (Archer, 1998).

2.2.6.1 Cellular antigen

The immunological specificities of *S. aureus* and *S. albus* are determined by phosphorus-containing polysaccharide antigens. Polysaccharide A extracted from pathogenic strains is a teichoic acid composed of N-acetyl glucosamine residues attached in either alpha or beta linkage to a polyribitol phosphate backbone. The ribitol teichoic acid complexes are covalently linked to the muramic acid mucopeptide of the cell wall (Bohach *et al.,* 1997).

2.2.6.2 Protein A

Most strains of *S. aureus* possess a surface component known as protein or agglutinogen A. It is a relatively small basic protein (MW: 13,000) which has the unique property of reacting with the Fc fragments of the IgG molecules of most mammalian sera. The resulting IgG aggregates, fix complement, and caused hypersensitivity reactions in normal rabbits and guinea pigs. They also generate complement-derived chemotactic factors which may account in part for the characteristic purulence of staphylococcal lesions (Uhlen *et al.,* 1984).

2.2.6.3 Leukocidin

This is a multicomplex protein toxin produced as separate components which act together to damage membrane. This toxin can kill exposed white blood cells of many animals, and are capable of very active intracellular multiplication. Strains of *S aureus*, expressing the virulence factor Panton-Valentine leukocidin (PVL), are emerging worldwide (Holmes *et al*., 2005). They are composed of 2 distinct proteins that are secreted separately but act together, namely LukS-PV and LukF-PV, PVL is a pore-forming toxin that targets and lyses mononuclear and polymorphonuclear cells (Ward and Turner, 1980). Only 2% of all *S. aureus* isolates express leukocidin, but nearly 90% of the strains isolated from severe dermonecrotic lesions express this toxin, which suggests that it is an important factor in necrotizing skin infections.

2.2.6.4 Staphylokinase

Many strains of *S. aureus* express a plasminogen activator called staphylokinase, an enzyme which activates the plasma plasminogen system. This factor lyses fibrin and the genetic determinant is associated with lysogenic bacteriophages. A complex formed between staphylokinase and plasminogen activates plasmin-like proteolytic activity which causes dissolution of fibrin clots. The role of staphylokinase
during bacterial infection is based on its interaction with the host proteins, α-defensins and plasminogen. Binding of staphylokinase to α-defensins abolishes their bactericidal properties, which makes staphylokinase a vital tool for staphylococcal resistance to host innate immunity (Bokarewa *et al*., 2005).

2.2.6.5 Thermostable nuclease

The presence of *S. aureus* in significant numbers in a food can be determined by examining the food for the presence of thermostable nuclease (DNase). This is possible because of the high correlation between the production of coagulase and thermostable nuclease by *S. aureus* strains, especially enterotoxin producers. Victor *et al*. (1969) reported that 232 of 250 (93%) enterotoxigenic strains produced coagulase, and 242 or 95% produced thermostable nuclease. Food examination for this enzyme was first carried out by Chesbro and Auborn (1969), employing a spectrophotometric method for nuclease determination. They showed that as number of cells increased in ham sandwiches, there was an increase in the amount of extractable thermostable nuclease of staphylococcal origin. They suggested that the presence of 0.34 unit of nuclease indicated certain staphylococcal growth and that at this level, it was unlikely that enough enterotoxin was present to cause food poisoning (Jay, 2000). The reliability of the thermostable nuclease assay as an indicator of *S. aureus* growth has been shown by many others (Erickson and Deibel, 1973; Niskanen and Koiranen, 1977; Koupal and Deibel, 1978; Park and ElDerea, 1978; Major, 1994; Yazdankhah *et al.,* 1999). For detectable levels of nuclease, 10^5-10^6 cfu per ml are needed (Niskanen and Koiranen, 1977).

Among the advantages of testing for heat stable nuclease as an indicator of *S. aureus* growth and activity are that because of its heat stable nature, the

enzyme would persist even if the bacterial cells are destroyed by heat, chemicals or bacteriophage or if they are induced to L-forms (Jay. 2000). The nuclease appears to be produced by enterotoxigenic cells before enterotoxin appears, and nuclease is dectectable in unconcentrated cultures of food specimen, whereas enterotoxin detection requires concentrated samples (Jay, 2000). Although *S. epidermidis* and some micrococci produce nuclease, it is not as stable to heat as that produce by *S. aureus* (Victor *et al.,* 1969). Thermostable nuclease would withstand boiling for 15 mins and has been found to have a D value (D130) of 16.6 mins in Brain Heart Infusion (BHI) broth at pH 8.2 (Forrest, 1972).

2.3 Associated foods

Foods that are frequently implicated in SFP include meat and meat products, poultry and egg products, salad such as egg, tuna, chicken, potato and marcaroni. Also implicated are bakery products such as cream-filled pastries, cream pies, chocolate éclairs, sandwich fillings, milk and diary products (Wieneke *et al.,* 1993). Milk has been found to be a good substrate for *S. aureus* growth, and milk products have been the source of many SFP outbreaks (De Buyser *et al.,* 2001; Jorgensen *et al.,* 2005). Also a wide range of foods are involved as sources of SFP in restaurants (Robinson *et al.,* 1989; Miwa *et al.,* 2001; Soriano *et al.,* 2002). In Germany, several outbreaks of SFP in 1988 and 1996, when consumption of contaminated uncooked smoked ham was identified as the source of the intoxication and an outbreak is reported (Atanassova *et al.,* 2001). Soriano *et al*. (2002) reported the presence of staphylococci in 17.3% of Russian salad, and 18.9% of vegetable salad.

Foods that require considerable handling during preparations and that are kept at slightly elevated temperatures after preparation are frequently involved in SFP. The surfaces of equipments and processing rooms can also be source of contaminations (Kusumaningrum *et al.,* 2003). In addition, the hands of food handlers can be pivotal as vectors in the spread of this food poisoning due to poor personal hygiene cross contamination. According to Taylor *et al.* (2000), there is an evidence from the food industry to show that microorganisms are transferred to the hands in the process of handling food and through personal hygiene after visiting the

lavatory, resulting in the hands being heavily contaminated with enteric pathogens (Barza, 2004).

2.4 Gastroenteritis syndrome

The symptoms of SFP usually develop within 4 h of the ingestion of contaminated food, although a range of 1 to 6 h has been reported (Dinges *et al.,* 2000). The symptoms include nausea, vomiting, abdominal cramps (which are usually severe), diarrhoea, sweating, headache, prostration, and sometimes a fall in body temperature. The intoxication is not lethal and the elderly are more susceptible to morbidity and mortality from foodborne-induced gastroenteritis than younger individuals (Balaban and Rasooly, 2000). The mortality rate is very low or nil (Dinges *et al.,* 2000; Jay, 2000; Le Loir *et al.,* 2003).

2.5 Food preservatives

The use of chemicals to enhance the safety of many foods is of great interest to the food industry. Chemical preservatives vary in their ability to kill microorganisms and effectiveness depends on the types of microorganisms and the physical and chemical characteristics of foods (Cherry, 1999). Preservatives can be categorized into three general types: (i) antimicrobials that inhibit growth of bacteria, yeasts, or molds; (ii) antioxidants that slow air oxidation of fats and lipids, which leads to rancidity; and (iii) blocks the natural ripening and enzymatic processes that continue to occur in foodstuffs after harvest.

The objective of chemical food preservation is the prevention or delay of microbiologically induced changes in a food product. In some instances and under certain circumstances, some physical control method of food preservation for example, heat, or chemical compounds such as alkylating agents may result in product sterilization by actually killing the indigenous microbiota. Frequently however, the microbial population remain viable in the product but its proliferation is inhibited or retarded by the chemical preservatives. Most commonly used food chemical preservatives include, weak acids or their salts or esters such as lactic acid, citric acid, acetic acid, sodium benzoate, potassium sorbate, butylated hydroxyanisole (BHA), and butylatedhydroxytoluene (BHT). However, the presence of chemical residues in

foods and labeling of preservatives on food packages are major concerns to consumers. Therefore, there is need for naturally derived compounds and other natural products with antimicrobial properties (Gould, 1996).

2.6 Medicinal plants

Food safety is a major concern to both consumers and food industry as there are increasing number of reported cases of food associated infections. Most consumers prefer high quality, nutritious, and foods with prolonged shelf life with no chemical preservatives. Natural preservatives can then be used as additives instead of synthetic compounds while the microorganisms are eliminated and food is safe for consumption. The interest in plants with antimicrobial activities has increased due to the resistance of microorganisms to synthetic antibiotics, the potential health problem that could accompany the use of synthetic drugs, and the increasing costs of synthetic drugs for the maintenance of personal health.

Many spices which are herbal products and their essential oils extracts have been reported. The essential oils and terpenoid alcohols of spices contribute to their smell, taste and tactile sensation. Menthol, from mint, has a cooling effect as well as a characteristic fresh taste and smell (Shelef, 1983; Aktug and Karapinar, 1986; Arora and Kaur, 1999; Delgado *et al.,* 2004; Nassar- Abbas and Hakman, 2004). Also, cardamom, cloves, and some other spices and herbs contain eugenol and its smell is fragrant and aromatic. Ginger contains gingerols, zingiberene and other characteristic agents that have made it an important flavour in Asiatic and Arabic herbal traditions (Kovac *et al.,* 2004). Thai native herbs are becoming more widely used both in the food industry as flavouring agents (Siripongvutikorn *et al.,* 2005), as well as antimicrobial agents (Murakami *et al.,* 1994; Murakami *et al.,* 1995; Voravuthikunchai and Kitpipit, 2005; Voravuthikunchai and Limsuwan, 2006; Ifesan *et al*., 2009a; 2009b).

Indigenous plants have been used in herbal medicine for curing various diseases (Otshudi *et al.,* 1999; Essawi and Srour, 2000; Bonjar, 2003). Extracts of garlic, cinnamon, curry, mustard, basil, ginger and other spices and herbs has been observed to exhibit antimicrobial properties (Arora and Kaur, 1999; Marino *et al.,* 1999; Yeh and Liu, 2001; Siripongvutikorn *et al.,* 2005; Wang and Ng, 2005; Sujah,

2006; Kamatou *et al*., 2008). Antimicrobial activities of *Zataria multiflora* against foodborne bacteria was also reported (Fazeli *et al.,* 2007). The phenolic glycoside oleuropein from olives has been shown to possess antimicrobial properties against *S. aureus* and *Salmonella enteritidis* (Tranter *et al.,* 1993; Tassou and Nychas, 1994).

Many researchers have reported activities of medicinal plants on *S. aureus*. Khan *et al.,* (2001) and Somchit *et al.,* (2003) found that methanolic extract of *Cassia alata* could inhibit *S. aureus*. The combined effect of extracts from Chinese chive, cinnamon, corni fructus, and their purified-fractions against *S. aureus* in food system was shown by Mau *et al.* (2001). The antibacterial activities of essential oils of onions, garlic, and allicin from garlic against *S. aureus* were also reported (Kyung and Lee, 2001; Benkeblia, 2004). Phytochemicals such as olive extracts and extracts of *Helichrysum italicum* have been reported to prevent the growth, protein secretion, and inhibition of SEB and SEC production (Novick *et al.,* 2001; Nostro *et al.,* 2002). Methanolic extract of pomegranate was also effective at reducing or inhibiting SEA production (Braga *et al*., 2005).

2.7 Possible mechanism of action of medicinal plants

The antibacterial activity of chemical compounds present in plant extract or their essential oils cannot be attributed to one specific mechanism but there are several targets in the cell (Skandamis *et al*., 2001; Carson *et al*., 2002). Hydrophobicity of antibacterials has been thought to be an important characteristic which enables them to partition in the lipids of the bacterial cell membrane, disturbing the structures and rendering them more permeable (Knobloch *et al*., 1986; Sikkema *et al*., 1995). Cinnamon stick was found not only to possess high levels of cinnamaldehyde but also contained many proanthocyanidins which exhibited antibacterial properties. Observation of bacterial cells treated with these compounds under scanning electron microscope revealed physical damage and considerable morphological alteration to all tested bacteria (Shan *et al*., 2007). It is possible that the active components in cinnamon stick extract might bind to the cell surface and then penetrate to the target sites, such as the phospholipid bilayer of the cytoplasmic membrane and membrane-bound enzymes. The effects might include the inhibition of proton motive force, inhibition of the respiratory chain and electron transfer, and

inhibition of substrate oxidation (Farag *et al*., 1989; Denyer, 1990; Nychas, 1995). The treatment of *S. aureus* cells with tea tree oil and its components resulted in lysis and loss of membrane integrity which manifested by leakage of ions and inhibition of respiration, loss of 260-nm-absorbing material, increased susceptibility to salt (NaCl), and formation of mesosomes (Carson *et al.,* 2002; Carson *et al*., 2006). Ethanolic extract of galangal extract was reported to have inhibitory effect against *S. aureus* and the transmission electron microscopy revealed that the extract caused both outer and inner membrane damage and cytoplasm coagulation (Oonmetta-aree *et al.,* 2006).

2.8 *Eleutherine americana*

Eleutherine americana is an herbal plant whose red bulb is used in Asian cuisine. *Eleutherine americana* originates from tropical America where its elongated red tubers have been used by the local inhabitants to treat numerous diseases. The plant belongs to the Iridaceae family and it is widely cultivated in China for ornamentals and medicinal purposes. It has been cultivated in Javanese gardens and now can be found worldwide particularly in South Africa and rubber plantations in Java. In addition, it is used as a folk medicine for the treatment of cardiac diseases, especially coronary disorders (Ding and Huang, 1983), as diuretic, and emetic purgative (Johnson, 1999). It is being used as a traditional carminative and together with galangal to treat cold and nasal congestion in children (Saralamp *et al*., 1996).

Previously, our research group reported the antibiofilm and antiquorum sensing ability of a crude extract from *E. americana* against *Streptococcus pyogenes* (Limsuwan and Voravuthikunchai, 2008). The crude ethanolic extract from this extract produced good antibacterial activity against *S. aureus* isolated from foods (Ifesan *et al*., 2009a). In addition partially-purified fractions and pure compounds from *E. americana* demonstrated inhibition against methicillin-resistant *Staphylococcus aureus* obtained from foods (Voravuthikunchai *et al*., 2008; Ifesan *et al*., 2009b). Furthermore, we reported the application of the crude extract as a food additive in homemade salad dressing (Ifesan *et al*.,2009c), cooked pork (Ifesan *et al*., 2009d), and its inhibitory activities against lipase and protease enzymes *in vitro* and

anti-enterotoxin activity both *in vitro* and *in vivo* (Ifesan and Voravuthikunchai, 2009).

Several compounds including naphthoquinones, elecanicin, eleutherol, isoeleutherol-one as well as anthraquinones and their glycosides have already been isolated from the bulbs of this plant (Hara *et al*., 1997; Qui *et al*., 2005; Xu *et al.,* 2006), eleutherinoside A, and eleuthoside B (Paramapojn *et al.,* 2008). Active compounds from *E. americana* has been reported to display important biological activities. Eleutherol, eleutherin, and isoeleutherin showed both antifungal activity and enhancement of the blood current of the coronary artery (Zhengxiong *et al.,* 1984). The antifungal, antiviral, and anticancer activities of some of its partially purified fractions have been studied (Qui *et al*., 2005; Xu *et al*., 2006). Eleutherol, eleutherin, and isoeleutherin obtained from this plant extract were used for preparation of tablets for treatment of heart diseases such as angina pectoris (Ding and Huang, 1983). Furthermore, a new naphthoquinone, eleuthinone A, two new anthraquinones, eleuthraquinone A and B, and a naphthalene derivative, eleucanarol were isolated from the bulbs of *E. americana* (Hamtasin *et al*., 2008; Mahabusarakam *et al*., 2009).

The dichloromethane extract prepared from *Eleutherine bulbosa*, a bulb from the same family demonstrated fungitoxic activity in a direct bioautography assay against *Cladosporium sphaerospermum*. Similarly, naphthoquinones, such as, eleutherinone, eleutherin, isoeleutherin, and eleutherol were obtained from this species (Alves *et al*., 2003). All the isolated compounds were shown to exhibit strong antifungal activities except eleutherol. Dam and Mai (1990) reported the use of the rhizomes of *Eleutherine subaphylla* Gagnep. as an antibacterial and haemostatic agents.

Figure 1. *Eleutherine americana* bulb.

CHAPTER 3

MATERIALS AND METHODS

I. MATERIALS

1. Microorganisms

1.1 *Staphylococcus aureus*

1.1.1 *Staphylococcus aures* isolated from foods NPRC 401-506

1.2 Reference strains *Staphylococcus aureus*

1.2.1 ATCC 23235

1.2.2 ATCC 27664

1.2.3 ATCC 25923

2. Antibiotic

2.1 Antibiotics Discs (Oxoid)

2.1.1 Erythromycin 15 µg

2.1.2 Gentamicin 10 µg

2.1.3 Penicillin 1 µg

2.1.4 Oxacillin 10 µg

2.1.5 Tetracycline 30 µg

2.1.6 Trimethoprim-sulfamethoxazole 1.25/23.75 µg

2.1.7 Vancomycin 30 µg

2.2 Antibiotic

2.2.1 Vancomycin

2.2.2 Oxacillin

3. Media

1. Baird-Parker agar (BP) (Difco)
2. MacConkey agar (MCA) (Difco)
3. Mannitol Salt Agar (MSA) (Merck)
4. Mueller-Hinton Agar (MHA) (Difco)
5. Mueller-Hinton Broth (MHB) (Difco)

6. Nutrient Agar (NA) (Difco)
7. Plate Count Agar (PCA) (Difco)
8. Potato Dextrose Agar (PDA) (Difco)
9. Tryptic Soy Agar (TSA) (Difco)
10. Tryptic Soy Broth (TSB) (Difco)

4. Chemicals

1. Acetone (Fisher Scientific)
2. Ascorbic acid (Sigma)
3. Casein (Sigma)
4. Dichloromethane (Lab-Scan Analytical Science)
5. Dimethylsulfoxide (Sigma)
6. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma)
7. Ethylenediaminetetraacetic acid (EDTA)
8. Egg yolk emulsion (Difco)
9. 95% Ethanol (Lab-Scan Analytical Science)
10. Ferric chloride (Merck)
11. Folin-Ciocalteu's phenol reagent (Merck)
12. Gallic acid (Merck)
13. Glycerol (Vidhyasom)
14. Glucose (Merck)
15. Glutaraldehyde (Merck)
16. Hexane (Lab-Scan Analytical Science)
17. Hydrochloric acid (HCL) (BDH Laboratory)
18. Hydrogen peroxide (BDH)
19. Methanol (BDH)
20. Propylene oxide (Merck)
21. Rabbit Plasma (Difco)
22. SET-RPLA Kit (Oxoid)
23. Sodium carbonate (Na_2CO_3) (Merck)
24. Sodium chloride (NACI) (Merck)
25. Sodium hydroxide (NAOH) (Lab-Scan Analytical Science)
26. Tannin (Sigma-Aldrich, St Louis, Mo, USA)

27. Tween 80 (SDH Lab)
28. Thiobarbituric acid (TBA) (Merck)
29. Trichloroacetic acid (TCA) (Merck)
30. Vinegar (Pat Chun)
31. Voges-Proskauer medium (Difco)

5. Equipments

1. Autoclave (Tomy, ES 315)
2. Balance (Sartorius, BP 210S)
3. Beaker (Pyrex)
4. Centrifuge (Hitachi)
5. Duran bottle (Duran)
6. Filter paper disc 6 mm (Whatman)
7. Hot air oven (Binder, T410340)
8. Hot plate stirrer (Lab. Companion, HP 3000)
9. Hunter lab (Cielab)
10. Incubator (Heraeus, B 5100E)
11. Laminar air flow cabinet (Gelman, BH 143AS)
12. Light microscope (Olympus, CX31RBSFA)
13. Micropipette 1-10 µl, 2-20 µl, 20-200 µl, 100-1000 µl (Eppendorf)
14. Microscope
15. Microtiter plate 96 wells (Corning)
16. Microwave (Sharp)
17. Multichannel micropipette 20-200 µl (Finnpipette)
18. Petri dish (Anumbra)
19. pH Meter (Beckman)
20. Refrigerator (Sanyo)
21. Spectrophotometer (Shimadzu)
22. Transmission electron microscope
23. Test tube (Pyrex)
24. Ultramicrotome
25. Vernier caliper (Whale)
26. Vortex mixer (Vortex Genie 2, G 560E)

27. Water bath (Julabo, TW 20)

6. *Eleutherine americana*

Bulbs of *Eleutherine americana* were obtained from Songkla. Classified reference voucher specimens were deposited at the Herbarium of Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla.

7. Food Samples

Food samples were purchased over a period of three months from food centres in Prince of Songkla University and local market at Hat Yai, Songkla. All samples were taken to the laboratory immediately for analyses.

II. METHODS

1.0 Preparation of crude extract from *Eleutherine americana*

Bulbs of *E. americana* were washed with distilled water, dried in the oven (BINDER) at 60ºC, for 2 days, and then ground to obtain fine particles using a blender. They were soaked in different solvents including, 95% ethanol, hexane (Lab-Scan Analytical Sciences, Thailand), and acetone (Fisher Scientific UK Limited, England) in ratio 1:2 w/v (500g of extract : 1000 ml), mixed together, and left at room temperature for 7 days to allow proper dissolution of the active compounds in the plant extract. The extract was filtered by a Whatman filter paper 125 mm (Whatman Schleicher and Schuell) under vacuum at room temperature. The filterates were evaporated under reduced pressure in a rotary evaporator (BUCHI Rotavapor R-114, Switzerland) at 45ºC until the extracts became completely dry. After evaporation, a yellow-brown solid of hexane extract and brown solids of ethanol and acetone extracts were obtained and the extracts were stored at -20ºC until use. The percentage yield of the extract was obtained by dividing the final weight of the dried extract with the weight of dry plant material × 100. All extracts were dissolved in 10% dimethylsulfoxide (DMSO; Sigma-Aldrich, Germany) or 10% ethanol before use.

2.0 Free scavenging activities of crude extract from *Eleutherine americana*.

2.1 DPPH determination

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay was measured as described by Hutadilok-Towatana *et al*. (2006). About 0.5 ml extract solutions in 10% ethanol at different concentrations (0.003 - 0.031 mg/ml) were added to l ml of DPPH solution (0.2mM in 95% ethanol). Control was prepare by adding 10% ethanol to equal volume of DPPH solution. After mixing, the test tubes were incubated in the dark at room temperature for 30 min. The absorbance of the resulting solutions were read at 518 nm against a blank of 10% ethanol. The percent DPPH scavenged by the extract was calculated using the following equation:

% DPPH scavenging activity = (OD of control- OD of sample) /OD of control × 100.

IC_{50} value was calculated using vitamin E dissolved in ethanol as positive control.

IC_{50} value expresses the concentration of the extract needed to quench 50% radicals in the reaction mixture.

2.2 Hydroxyl free radical scavenging activity of the extract

This was determined according to Hutadilok-Towatana *et al.* (2006) with slight modifications. About 0.067 ml of the extract was mixed with 0.134 ml of 30 mM phosphate buffer, pH 7.4 containing 17 mM 2-deoxyribose. An aliquot of 0.007 ml of 0.3 mM $FeCl_3$ premixed with 1.2 mM EDTA was added, the mixture was vortexed, and the reaction was initiated by adding 0.067 ml of 0.6 mM ascorbic acid and 0.033 ml of 34 mM hydrogen peroxide. Samples were maintained at 37°C for 30 min, and then treated with 0.333 ml of 28% TCA and 0.333 ml of 1% TBA. Samples were heated at 90°C for 20 min, cooled, and the absorbance was read at 532 nm. Control sample was prepared by adding 10% ethanol instead of the extract. The result was expressed as IC_{50} using tannin as standard.

2.3 Determination of Total phenolics in the crude extract

Total phenolics were determined using Folin-Ciocalteu reagent (Singleton *et al*., 1999). First, the calibration curve of aqueous gallic acid solutions of

known concentrations was prepared. Folin-Ciocalteu's phenol reagent (5 ml) and 20% sodium carbonate solution (15 ml) were added to each 1 ml of gallic acid standard solution. The solutions were kept at room temperature (30ºC) for 90 min before measuring their absorbance at 760 nm by UV-VIS spectrophotometer. Clear solution of the crude extracts were prepared and mixed with Folin-Ciocalteu's phenol reagent and 20% sodium carbonate. Control with 10% ethanol was set up under the same condition. The mixtures were kept at room temperature for 90 min before measuring their absorbance at 760 nm. For blank, the distilled water was added to replace the standard solution. The total phenolic content in the extracts were calculated from the calibration curve and determined as percent phenolic contents (g phenolics/g crude extract).

3.0 Microbiological analyses

3.1 *Staphylococcus aureus* isolates

Twenty five grams of each of the food samples was suspended in 225 ml of buffered peptone water (Difco Labs, Division of Becton Dickinson and Co., Sparks, MD, USA) and aseptically homogenized in a stomacher. Serial decimal dilutions were prepared from this initial dilution. Each of the different dilutions (0.1 ml) was transferred onto the surface of Baird-Parker agar (Difco Labs) and incubated at 35ºC for 24 to 48 h. Typical colonies from the plates were selected on Tryptic Soy agar (TSA; Merck Germany) and incubated at 35ºC for 24 to 48 h (Atanassova *et al.,* 2001; Normanno *et al.,* 2007). Long term storage of isolates was kept at - 80ºC in brain heart infusion broth (BHIB; Difco Labs) containing 20% glycerol (Vidhyasom Co. Ltd., Thailand) as a cryoprotectant (Hammer *et al.,* 2005).

3. 2 Method of Identification of isolates

3. 2. 1 Biochemical tests

Staphylococcus aureus isolates were subjected to further tests for characterization. These include Gram staining, catalase reaction, production of acid

from mannitol salt agar (MSA; Merck Germany), anaerobic fermentation of mannitol, production of acetoin, and tube coagulase test (Schleifer, 1986).

3. 2. 2 Preliminary characterization of *Staphylococcus aureus* isolates

All *S. aureus* isolates from food and reference strains were characterized and screened for the production of virulence factors. Haemolysins, lecithinase, and lipase activity were investigated following the modified method of Udo and Jacob (2000) and Hammer *et al.* (2005). Inocula were prepared by subculturing isolates on TSA and incubated overnight at 35°C. Single colonies were then suspended in 0.85% NaCl (Merck Germany) and adjusted to a concentration of approximately 10^8 cfu/ml. Five microlitre of inoculum (10^4 cfu/ml) were spot-inoculated in radial patterns on the plate surfaces of each pre-dried plate. Haemolysins were detected by spot-inoculating isolates onto TSA agar containing 5% horse blood and incubated plates for 24 h at 35°C. After incubation, haemolytic isolates showed a zone of clearing in the blood agar adjacent to the bacterial growth. Egg yolk agar was used to investigate the lecithinase activity. Egg yolk agar plates were prepared by adding egg yolk emulsion at a final concentration of 10% (v/v) to nutrient agar (NA; Difco Labs) supplemented with 1% glucose (Merck Germany). Inoculated plates were incubated for 72 h at 35°C and isolates demonstrating opaque growth were considered lecithinase positive (Baron *et al*., 1994) . The nutrient agar plates containing 1% tween 80 (SDH Laboratories, England) (v/v) were used to study the lipase activity. The plates were incubated for 48 h at 35°C and colonies showing a clear zone adjacent to growth were recorded as lipase positive. A modified NA was prepared by adding 2% casein to the agar to study protease activity. The plates were incubated at 35°C for 24 h and isolates producing opalescent zones around the growth were protease positive.

4.0 Determination of antibacterial activity of crude extract on *Staphylococcus aureus* cells

4.1 Paper disc agar diffusion method (CLSI, 2006a)

Ten microlitre of crude extracts (250 mg/ml) were applied to 6 mm sterile filter paper discs (Macherey-Nagel GmbH) each disc contained 2.5 mg of the extract. Dry discs (dried at 35°C overnight) were applied to the surface of Muller Hinton agar (MHA; Difco Labs) plates that were seeded with 5 h broth culture of the tested bacteria (CLSI, 2006a). The plates were then incubated for 18-24 h at 35°C. The antibiotic discs (Oxoid Ltd, Basingstoke, UK) included, erythromycin (15 µg), gentamicin (10 µg), oxacillin (1 µg), penicillin (10 IU), sulfamethoxazole trimethoprim (25 µg), tetracycline (30 µg), vancomycin (30 µg) were used as control. Paper discs impregnated with 95% ethanol were used as control. The antibacterial activity was evaluated by measuring the diameter of inhibition zone. The experiment was performed in duplicate and the mean of the diameter of the inhibition zones were calculated.

4.2 Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the crude, partially-purified and pure compounds from *Eleutherine americana*

A modified microdilution method (CLSI, 2006b) was used to determine the MIC of *E. americana* extracts. Vancomycin (0.03-10 µg/ml) was used as reference standards (CLSI, 2006b). Twenty microlitre of medicinal plant extracts (2 fold dilution) at concentration ranging from 0.015 to 1.00 mg/ml and 80 µl of MHB were also added to the well. One hundred microlitre (1:200) of a 5 h culture of each bacterial isolates containing approximately 10^6 cfu were applied onto Muller Hinton broth (MHB; Difco Labs). The microtitre plates were then incubated at 35°C for 18-24 h. Minimal inhibitory concentrations were observed at least in duplicate as the lowest concentration of plant extracts that produce a complete suppression of colony visible growth. Minimal bactericidal concentrations were performed by streaking

contents from microtitre wells that gave MIC values on fresh MHA and incubated at 35°C for 24 h. Concentration at which there was no visible bacteria growth after 24 h incubation was regarded as MBC.

4.3 Kill curves in the presence of the crude extract and partially-purified fractions from *Eleutherine americana*

The modified method of Palombo and Semple (2001) was used. One thousand microlitre of 5 h culture grown in Tryptic Soy Broth (TSB; Merck Germany) was added to 1,250 µl TSB containing 250 µl of plant extract at MIC, 2MIC, and 4MIC, respectively in sterile test tubes. The tubes were incubated at 35°C, with shaking, and the growth of bacteria were measured by counting method after 0, 2, 4, 6, 8, and 24 h. Surviving bacteria were enumerated and control tubes without extract but with 10% ethanol were incubated under the same conditions. All assays were carried out in triplicate.

5.0 Production of staphylococcal enterotoxins

The production of enterotoxins A to D was determined using reversed passive latex agglutination method with the SET-RPLA kit (Oxoid Ltd, Basingstoke, UK). All *S. aureus* isolates were incubated into TSB (TSB; Merck Germany) with shaking aerobically at 35°C for 24-48 h. The cultures were centrifuged at 900 *g* for 20 min at 4°C. Microtitre plate was arranged so that each row consisted of 8 wells, and five rows of such 8 wells were used for each isolate. Twenty five microlitre of the diluent was dispensed into each well of the 5 rows, and 25 µl of the culture was added to the first well of each of the five rows. Doubling dilutions were performed along each of the five rows. The dilution was stopped at the 7th well so that the last well contained only the diluent. Latex sensitized with anti-enterotoxin (A-D) supplied with the kit was added into each well appropriately and the content was mixed. The plates were left undisturbed at room temperature for 20-24 h. Each well in each row was examined for agglutination according to the manual supplied with the kit.

6.0 The effects of extract on enzymes and enterotoxin of *Staphylococcus aureus*

6.1 The effects of crude extract on extracellular protease and lipase

The assay for measuring extracellular protease and lipase was based on the modified method described by Nostro *et al.* (2001). The subminimal inhibitory concentrations (subMICs): ½ and ¼ MIC of *E. americana* were estimated in nutrient agar with tween 80 for lipase activity and 2% casein for protease activity. The subMICs were obtained by incorporating the extract and appropriate melted agar medium (1:9 v/v) before pouring into petri dishes and allowed to gel. Five microlitre of inoculum adjusted to obtain 10^4 cfu/ml were spot-inoculated in radial patterns on the plate surfaces with and without extract and incubated for 24 h at 35°C for protease activity while 72 h for lipase activity determination. Total inhibition of activity was reported where no enzyme productions were observed compared with the control. The zone that surrounds the growth were measured using the vernier caliper.

Total inhibition of activity was reported where no enzyme productions were observed compared with the control. Partial inhibition of activity referred to isolates where inhibition observed is about 50% as compared with the control.

$$(\%\ \text{inhibition}) = \frac{\text{No of isolate that demonstrated inhibition either partial or total}}{\text{Total no of isolate tested}} \times 100$$

6.2 The effect of crude extract on enterotoxin production in broth system

Each dilution of crude extract from *E. americana* was added to TSB in order to obtain concentrations equal (1/4MIC, 1/2MIC, MIC, and 2MIC). Aliquots of overnight cultures adjusted with 0.5 McFarland to obtain 10^8 cfu/ml were inoculated into the tubes to obtain a final inoculum size of 10^6 cfu/ml. Tubes were incubated at 37ºC with shaking and samples were taken at 24 and 48 h respectively both for viable counts and enterotoxin determination. Production of enterotoxin A-D was determined using reversed passive latex agglutination method with the SET-RPLA kit (Oxoid). Five hundred microlitre of samples were removed from each tubes and were centrifuged at 900 *g* for 20 min at 4ºC. Two fold dilutions of the filterate (25 µl) were performed across two rows on a microtitre plate. This was mixed with an equal

volume (25 µl) of sensitized latex particles and allowed to react at room temperature. Positive and negative controls were also set up for each sample and the plates were incubated undisturbed at room temperature for 24-48 h.

6.3 Effect of crude extract on enterotoxin production in food system

Lean pork was purchased from the grocery and was transported to the laboratory immediately. The meat was cut manually into chunks and were rinsed under tap water. They were divided into four portions and were treated with extract by adding 1.00, 2.00, 4.00 mg/ml, respectively and the control which was treated with 10% ethanol. The treated chunks were then cooked in the microwave until the internal temperature of samples reached 80ºC. After cooling to room temperature, they were inoculated with *S. aureus* and mixed together using the stomacher, packaged into plastic bags and sealed. The samples were left on the table at an abused temperature (30°C) and were examined at every 2 h for both viable counts and enterotoxin determination. About 10 g of the meat was blended with 10 ml of normal saline (0.85% NaCl) in a blender and was centrifuged at 900 *g* for 20 min at 4ºC. The supernatant was filtered through a 0.45 µm membrane filter and the filterate was used for toxin assay.

7.0 Study of the effect of *Eleutherine americana* extract on *Staphylococcus aureus*

7.1 Loss of 260-nm-absorbing material

This experiment was done following Oonmetta-aree *et al*. (2002) with slight modifications. Bacteria cells were cultured on TSA plate and were incubated overnight. The cells were harvested and washed with normal saline by centrifugation at 10,000 *g* for 5 min. After centrifugation, the cells were collected and adjusted to achieve a concentration of approximately 10^9 cfu/ml. The inoculum was then mixed with the different extract concentrations (MIC, 2MIC, 4MIC) in the ratio 1:1 in the test tube while inoculum with 1% DMSO was set up as control. The suspensions were incubated at 35ºC for 0, 8 and 22 h. At each time intervals the suspensions were centrifuged as described above and absorbance was read at 260 nm using the spectrophotometer. Low molecular weight metabolites known to leak from cells

including nucleotides and their component structures such as purines, pyrimidines, amino acids were detemined by reading the optical density (OD) at 260 nm.

7.2 Test for salt tolerance

The ability of *S. aureus* cells treated with mixtures of plant extract and NaCl to grow on NA was investigated according to Carson *et al.* (2002) with slight modifications. Suspensions of bacteria were prepared (10^6 cfu/ml) and were treated with 1/2MIC, MIC, 2MIC of the extract and 1% DMSO. In other group, each extract concentration was combined with 7.5% NaCl and the control was set up without extract. The test tubes were incubated in shaker incubator at 35°C, and samples were removed at intervals, serially diluted, and streaked on NA. After incubation for 24 h, the numbers of cfu/ml on plates of NA were estimated and compared with the controls.

7. 3 Transmission electron microscopy (TEM)

Four millilitre of 5 h TSB culture ($1.5x10^6$ cfu/ ml) was added to 5 ml of TSB containing one ml of plant extract at MIC, 2MIC, and 4MIC concentrations, respectively in sterile test tubes. Controls without extract but with 1% DMSO was also set up. The tubes were incubated at 35°C with shaking for 12 h after which bacterial cells were collected by centrifugation at 10,000 *g* for 10 min. The pellets were washed two times with phosphate buffer solution (pH 7.2) and were fixed overnight in 2.5% glutaraldehyde at 4°C overnight. The pellets were processed in graded alcohols, propylene oxide, and araldite and were cured for 48 h at 60°C. Ultrathin sections were stained with uranyl acetate and lead citrate and were examined using transmission electron microscope (JEOL, JEM 2010, Japan).

7.4 Bacteriolysis

Bacteriolysis was carried out following the modified method from Carson *et al*. (2002). Suspensions of *S. aureus* were prepared as described above and the extract were added at concentrations equivalent to the MIC, 2MIC, 4MIC and the control which was treated with 1% DMSO. The suspensions were mixed with a vortex mixer and the OD_{620} was measured. Blanks were set up using dilutions of

extract and inoculum. Samples were taken immediately after the experiment was set up (0 h) and the test tubes were incubated in a shaker incubator at 35°C. Additional samples were taken for analysis at 8 h and 24 h respectively**.**

8.0 Determination of Cytotoxic Activity

8.1 Cytotoxicity against primate cell line

Crude extract was tested for its toxicity against vero cells following the modified method of Hunt *et al*. (1999). Green fluorescent protein (GFP) expressing vero cell line was generated in-house by stably transfecting the African green monkey kidney cell line (Vero, ATCC CCL-81), with pEGFP-N1 plasmid (Clontech). The cell line was maintained in minimal essential medium supplemented with10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g/l sodium bicarbonate and 0.8 mg/ml geneticin, at 37°C in a humidified incubator with 5% CO_2. The assay was carried out by adding 45 µl of cell suspension at 3.3×10^4 cells/ml to each well of 384-well plates containing 5 µl of *E. americana* extract previously diluted in 0.5% DMSO, and then incubated for 4 days at 37°C with 5% CO_2. Fluorescence signals are measured by using SpectraMax M5 microplate reader (Molecular Devices, USA) in the bottom-reading mode with excitation and emission wavelengths of 485 and 535 nm. Fluorescence signals at day 4 was subtracted from background fluorescence at day 0. The percentage of cytotoxicity was calculated by the following equation, where FU_T and FU_C represented the fluorescence units of cells treated with the extract and untreated cells, respectively.

$$\% \text{ cytotoxicity} = [1-(FU_T/ FU_C)] \times 100$$

IC_{50} values were derived from dose-response curves, using 6 concentrations of 2-fold serially diluted samples, by the SOFTMax Pro software (molecular device). Ellipticine and 0.5%DMSO were used as positive and negative controls, respectively.

9.0 Application of extract to model food systems.

9.1 Effect of heat treatment and change in pH on the extract stability

Five millilitre (1mg/ml) of the extract was adjusted to pH 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 with 1N HCI (BDH Laboratory, England) and 5N NaOH (Lab-Scan Analytical Sciences, Thailand) before they were used. In addition, at the various pH, the extract was either autoclaved, heated at 65ºC for 30 min or non heated before use. This was done to study the stability of the extract in the presence of heat and change in pH. The various treatments were allowed to cool down after which they were inoculated with approximately 10^6 cfu/ml of inoculum. The tubes were incubated at 4ºC, 10ºC and 35ºC, respectively and the growth of bacteria were measured by enumeration at 0 and 24 h. All assays were carried out in duplicate. Control tubes without extract but with 10% ethanol were incubated under the same conditions. Surviving bacteria were enumerated on NA and compared with the control.

9.2 Preparation of food samples

The effect of the crude extract on *S. aureus* cells inoculated into foods was carried out according to Bedie *et al.* (2001) and Kim *et al.* (2001) with slight modifications. Two food systems, home made salad dressing and pork meat were used.

9.2.1 Preparation of home made salad dressing

The salad dressing was prepared using egg yolk, sugar, and vinegar. The ingredients for salad dressing include, 2.5 kg hard-boiled egg yolk, 2.5 kg sugar (Mitr Phol pure refined sugar), 625 ml of rice vinegar (5% acidity) (Pat Chun brand), 312.5 ml of vegetable oil (refined palm olein), and 40 g of salt (iodized table salt). All the ingredients were blended together to obtain a smooth slurry and were divided into four equal fractions after which extracts were added at various concentrations of 0, 0.25, 0.50, and 1.0 mg/ml in the ratio 1:8. The salad dressings were then packaged separately for microbiological, sensory, and chemical tests. Samples for sensory and chemical tests were poured in bottles and stored at 4ºC while the portion for

antibacterial test was inoculated with *S. aureus* to obtain 10^6 cfu/ml and stored in sterile bottles at 4ºC and 10ºC respectively for 21 days.

9.2.2 Preparation of pork sample

Fresh lean pork was purchased from the departmental store and was transported to the laboratory immediately within 30 min. The meat was cut manually into chunks of about 2 ×2 ×2 cm. The pork contained 9.99% fat as determined by the Soxhlet extraction method (AOAC, 1995). Based on results of preliminary trials, varying concentrations of extract were used to treat the meat. The treatments were as follows; the control (10% ethanol), and three different extract concentrations, 2.7, 5.4, and 10.8 mg of extract were individually added to 100 g of meat. The various treatments were thoroughly mixed with sterile spoon to ensure uniform distribution of the added extract and meat was divided into four portions in sterile bowls. Each portion was steamed in the microwave (900 watts) until the internal temperature reached 80°C and was held for 2 min after which they were allowed to cool down at room temperature. They were then divided into separate groups for chemical, sensory, physical and microbiological analysis, packaged into plastic bags, sealed and stored at 4°C.

9.3 Microbiological analysis

Food samples were subjected to total plate, psychrophilic, coliform, and *S. aureus* counts according to the standard methods of the American Public Health Association (APHA, 1984). Twenty five g of each sample was aseptically weighed, and mixed with 225 ml of 0.1 % buffered peptone water. Samples were homogenized in a stomacher and serial dilutions were prepared. One hundred microlitre of each dilutions were poured on Baird-Parker for *S. aureus* count, plate count agar (PCA; Merck Germany) was used for total plate count and psychrophilic count while enumeration of coliforms was done using MacConkey agar (Merck Germany). All the plates were incubated at 35°C for 24 to 48 h except for psychrophilic counts which were incubated at 4°C for 10-14 days and counts were expressed as colony forming units per gram. The presence of yeast and mould was

enumerated by using potato dextrose agar with 10% tartaric acid (PDA, Difco) to adjust the pH. Plates were incubated at 25°C for 3-4 days.

9.3.1 Preparation of crude extract of *Curcuma longa* and *Allium sativum*

Crude extracts from *C. longa* and *A. sativum* were applied into pork to compare their effects with *E. americana* treatments. Crude ethanolic extract from *C. longa* was prepared following the method used to prepare crude *E. americana* extract, while water extract was prepared from garlic. Ten percent garlic extract was prepared by blending 10 g washed fresh garlic with 100 ml sterile distilled water. The suspension was filtered and stored at 4°C before use. The ratio of extract to meat is 1,500 ml:8,000 g. The treatments were as follows; the control (10% ethanol), 10% garlic and 2.7 mg of *E. americana* and *C. longa* extract were individually added to 100 g of meat, 750 ml of *E. americana* with either 750 ml of *C. longa* or garlic, and 500 ml of the 3 extracts added together for the final treatment. Each portion was steamed in the microwave (900 watts) until the internal temperature reached 80°C and was held for 2 min after which they were allowed to cool down at room temperature. They were then packaged into plastic bags, sealed and stored at 4°C for microbiological analysis.

9.4 Colour determination

Colour was measured with the use of colour meter (Hunter Lab Universal Software) using the L*, a*, and b* (lightness, redness, and yellowness) coordinates (CIELAB).

9.5 pH determination

The pH (Beckman pH Meter) of the salad dressing samples were done every 3 day through out the storage period directly using the samples. Ten gram of pork was homogenized with 50 ml of distilled water using Ultra homogenizer (Janke and Kenkel, IKA, Labor Tecnik, Germany) for 1 min after which the pH was read.

9.6 Thiobarbituric acid reactive substances (TBARs)

Thiobarbituric acid reactive substances in food samples were determined as described by Benjakul *et al*. (2005) with slight modifications. Determination of TBARs of salad dressing were carried out using samples with sugar and without sugar due to interference of sugar with colourings (Shlafer and Shepard, 1984). One gram of food sample was mixed with 4 ml of TBARs reagent containing 0.375% thiobarbituric acid (TBA, Merck), 15% trichloroacetic acid (TCA, Merck), and 0.25N HCL (BDH Lab.). The mixture was heated in boiling water for 10 min and then cooled with running water. The mixture was centrifuged (Hitachi, Tokyo, Japan) at 3600 *g* for 20 min. The supernatant was removed and absorbance was read at 532 nm using a UV-160 spectrophotometer (SHIMADZU). Thiobarbituric acid reactive substances was calculated from standard curve of malonaldehyde and expressed as mg malonaldehyde/kg sample.

9.7 Sensory evaluation

A total of 19 assessors (Staff and Graduate students) evaluated the food samples. Sensory tests were carried out on 0, 8, and 16 days of storage for salad dressing, while it was done for pork samples at 0, 3, 6, and 9 days. Samples were evaluated for the attributes of appearance, colour, viscousity, texture, taste, and overall acceptability using a 9-point hedonic scale, where 9 = extremely desirable, 5 = averagely desirable, and 1= extremely undesirable (Solokomas *et al*., 2008).

10.0 Further antimicrobial screening test

Eleutherine americana crude extract was further subjected to antimicrobial test using a number of food spoilage and foodborne pathogens. This include *Aspergillus niger*, *Bacillus cereus*, *Bacillus licheniformis, Bacillus subtilis, Bacillus spizizenii, Candida utilis, Candida albican, Citrobacter freundii*, *Erwinia* spp., *Escherichia coli*, *Klebsiella pneumoniae*, MRSA, *Microsporum gypseum*, *Penicillium* spp., *Pseudomonas aeruginosa*, *Rhizopus* spp., *Shigella bodyii*, *Staphylococcus aureus*, *Streptococcus* spp., *Trichoderma viride, Trichophyton rubrum*, and *Yersinia* spp. All the microorganisms were obtained from stock culture of Industrial Biotechnology Research Laboratory and Plant Phytochemistry

Laboratory, School of Biological Science, Universiti Sains Malaysia. The tests include susceptibility test using paper disc agar diffusion method (CLSI, 2006a), minimum inhibitory concentration determination using modified tube dilution method (CLSI, 2006b), and time-kill assay using the modified method of Lorian (1996) and Hammer *et al*. (2002).

11.0 Fractionation of crude extract

11.1 Column chromatography

Hexane extract was chromatographed on column chromatography over silica gel 100H using hexane–dichloromethane, dichloromethane–Methanol and Methanol as eluents. On the basis of their TLC characteristics, the fractions which contained the same major components were obtained to give fractions Ea1- Ea12 (Figure 2). All semi-purified fractions and pure compounds were obtained from Ass. Prof. Dr Wilawan Mahabusarakam and Miss Chulida Hamtasin.

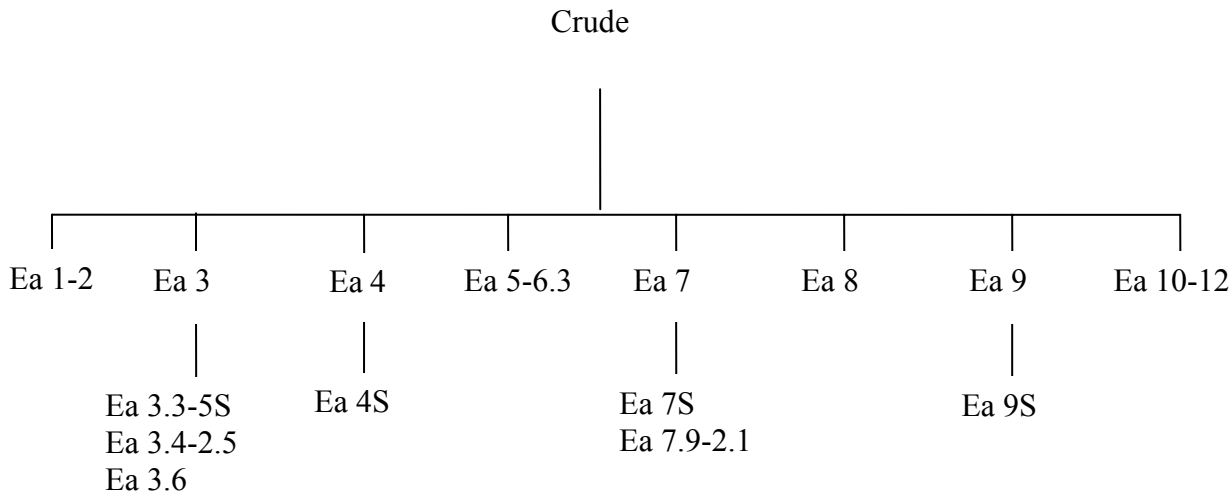


Figure 2. Schematic representations of the fractionation of the exudates from *Eleutherine americana*

12.0 Statistical analysis

Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Sciences package (SPSS 8.0 for windows, SPSS Inc, Chicago, IL). The experiments were determined in duplicates while means ± SD were calculated from triplicate determinations.

CHAPTER 4

4.0 RESULTS

4.1 Extract properties

The average percentage yield of the extract from the various solvents used are as follows, acetone, 3.86%, ethanol, 4%, and hexane, 3.92%. The scavenging effect of the crude ethanolic extract on 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydroxyl radicals were expressed as IC_{50} values (Table 5). The results indicated that the extract demonstrated good scavenging activities compared to the control. The extract produced IC_{50} values of 8.4 µg/ml and 0.78 mg/ml and on DPPH and hydroxyl free radicals, respectively. The total phenolic content of the ethanolic extract was determined using the Folin–Ciocalteu reagent and the crude extract yielded high phenolic content of 4.56 μmol gallic acid equivalent per milligram dried extract.

Table 5. Scavenging activities of crude ethanolic extract from *Eleutherine americana*

Test materials	[a]IC_{50} (mg/ml)	
	DPPH	Hydroxyl radical
E. americana	0.0084 ± 0.0007	0.78 ± 0.0212
Vitamin E[b]	0.023 ± 0.001	NA[c]
Tannin[b]	NA[c]	0.41 ± 0.01

[a]Sample concentration (mean triplicate determination ± SD) that produced 50% inhibition of radical.

[b]Standards dissolved in ethanol.

[c]Not applicable.

4.2 Food isolates

The types of ready-to-eat foods that were analyzed are presented in Table 6. All the food samples were bought from the food shops during lunch. *Staphylococcus aureus* growth on Baird-Parker plates were selected based on morphology and all the isolates were subjected to further biochemical tests for confirmation. Seventy-six out of 106 samples were contaminated with *S. aureus*. Seventeen of cake samples, (73.9%) which include éclairs, banana cake, and milk cake were contaminated with *S. aureus*. Twenty-eight out of 30 (93.3%) meat samples were contaminated. The meat samples include fried pork, fried chicken, and boiled chicken. Heavy contaminations were observed with both pork soup and papaya salad (100%) while the least contamination was recorded in chicken sausage (45.4%). Only 10 from 22 (45.4%) chicken sausages were contaminated while no microorganism was isolated from milk desserts.

Table 6. Sources of *Staphylococcus aureus* test isolates

Food types (%)	Number of samples	Numbers of samples with *S. aureus*
Cakes	23	17 (73.9%)
Chicken sausage	22	10 (45.4%)
Meat	30	28 (93.3%)
Milk dessert	5	0
Papaya salad	7	7 (100%)
Pork soup	9	9 (100%)
Steamed fish cake	10	5 (50.0%)
Total	106	76 (71.7%)

4.3 Phenotypic characteristics of food isolates

The characteristics of *S. aureus* isolated from the food samples were determined (Table 7). Three different colonies were frequently observed, some cells appeared small, black and surrounded with clear zones, big and black, while some are big, black and surrounded with clear zones. All the isolates were Gram-positive cocci when viewed under the microscope, and catalase positive. They all fermented mannitol salt agar, 94% were anaerobic mannitol fermenters and positive for acetoin production while 86% were positive for tube coagulase test.

Isolates were examined for extracellular enzyme production (Table 8). About 25% of the isolates secreted all the three enzymes protease, lipase, and haemolysin within the range of 1.14-2.00 cm. At least each of the isolates secreted one or two enzymes. Most isolates (81.1%) were capable of producing lipase enzyme. Contrastly, only a few isolates possessed lecithinase enzyme (10.3%).

Table 7. Biochemical characteristics of *Staphylococcus aureus* isolated from food

Biochemical tests	% Positive isolates
Catalase reaction	100
Growth on mannitol salt agar	100
Anaerobic fermentation of mannitol	94
Acetoin production from Voges-Proskauer medium	94
Tube coagulase test	86

Table 8. Enzyme activities *of Staphylococcus aureus* isolated from food

Enzyme production	% Positive isolates
Protease	36.1
Haemolysin	55.6
Lecithinase	10.3
Lipase	81.1

4.4 Antibiotic susceptibility patterns of food isolates

Figure 3 shows antibiotic susceptibility patterns of the 106 tested isolates. In the antimicrobial resistance trials, 85 (80.2%) isolates demonstrated antimicrobial resistance properties to at least one of the antibiotics, however, none was resistant to vancomycin. About 67 (63.2%) exhibited resistance to penicillin while 22 (20.75%) were resistant to oxacillin. Eighteen isolates (16.98%) were resistant to gentamicin, 36 (33.96%) were resistant to erythromycin, 43 (40.56%) of the isolates showed resistance to tetracycline, and 23 (21.69%) were resistant to sulfamethoxazole.

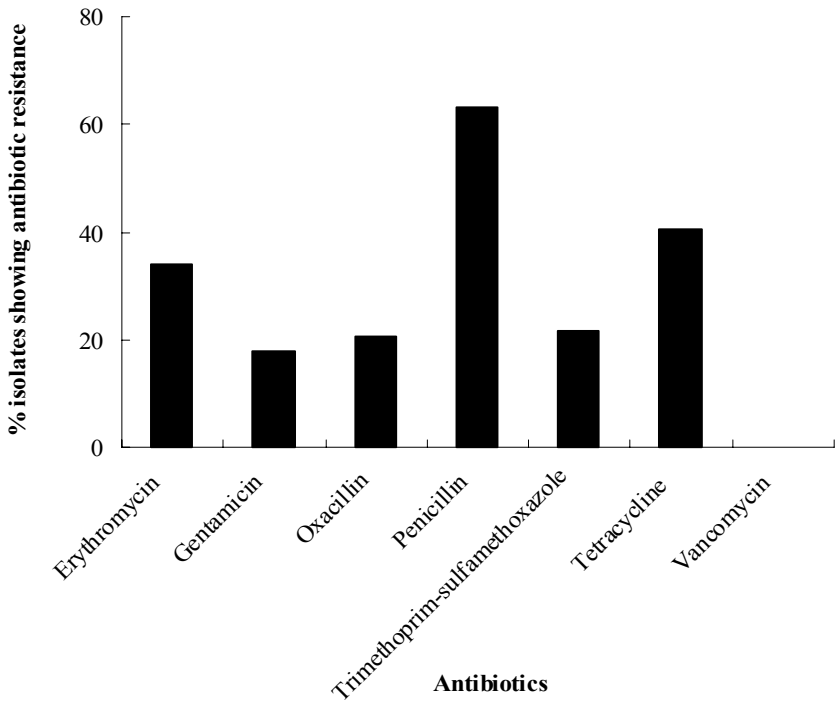


Figure 3. Antibiotic resistance patterns of *Staphylococcus aureus* isolated from food (n=106)

4.5 Paper disc agar susceptibility of *Eleutherine americana* on food isolates and reference strains

Table 9 shows the result of the agar disc susceptibility test of the crude extract from *E. americana*. Ethanol and hexane extracts were mixed together at same proportion to check if they could produce synergistic effect. The results of the susceptibility of the food isolates when subjected to statistical analysis revealed that the activities of the ethanol and acetone extract were significantly different from that of hexane and hexane+ethanol. Ethanolic extract was used for further studies because of the future plan to add the extract into food. Food isolates produced average inhibition zone between 14.5-15.7 mm, while the reference strains produced diameter range of 12.2-17.0 mm. All the isolates from food and reference strains were sensitive to the crude extract while no inhibition zones were obtained from the controls.

4.6 Antibacterial activity of the crude ethanolic extract from *Eleutherine americana* on *Staphylococcus aureus*

The MIC values of the food isolates ranged from 0.06-1.00 mg/ml (Table 10). Mean inhibitory concentration for 90% of isolates, MIC_{90} values was 0.5 mg/ml while the mean inhibitory concentration for 50% of isolates, MIC_{50} was at 0.25 mg/ml. Only two isolates (1.88%) had MIC value of 1.00 mg/ml. The MIC for all the reference strains was 0.25 mg/ml (Table 11), while the MBC was more than 1.00 mg/ml.

Table 9. Paper disc agar susceptibility test of extracts (2.5 mg/disc) of *Eleutherine americana* on *Staphylococcus aureus* isolates from food samples and reference strains

	Mean values of Inhibition zone (mm)			
S. aureus	Ethanol	Hexane	Acetone	Ethanol+Hexane
Food isolates	15.35 ± 0.15^{a}	14.51 ± 0.15^{b}	15.75 ± 0.14^{a}	14.59 ± 0.15^{b}
ATCC 25923	16.37 ± 0.08^{c}	16.37 ± 0.08^{c}	15.75 ± 0.02^{c}	15.87 ± 0.03^{c}
ATCC 23235	16.75 ± 0.14^{c}	12.25 ± 0.25^{c}	16.75 ± 0.25^{c}	17.00 ± 0.25^{c}
ATCC 27664	14.37 ± 0.37^{c}	12.50 ± 0.50^{c}	15.25 ± 0.25^{c}	14.50 ± 0.16^{c}

Values in the same row with different superscript (a-b) are significantly different ($p<0.05$).

[c]Mean values ± standard error of duplicate results.

No inhibition zones were observed from the controls.

Table 10. The minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) of the ethanolic crude extract from *Eleutherine americana* against *Staphylococcus aureus* isolated from foods

	Number of isolates (%)	
Concentration of *E. americana* (μg/ml)	MIC	MBC
62.5	4 (3.77%)	-
125	23 (21.69%)	-
250	42 (39.62%)	-
500	35 (33.01%)	-
1000	2 (1.88%)	20 (18.86%)
>1000	-	86 (81.13%)

- No isolate

4.7 Antibacterial activity of vancomycin antibiotic on food isolates and reference strains

The result of the MIC and MBC of the food isolates and reference strains against vancomycin antibiotic is shown on Table 12. The food isolates MIC values ranged from 0.03-0.12 µg/ml while the MBC was from 0.03->0.5 µg/ml. The MIC/MBC values for the reference strains were from 0.03-0.5/0.12-0.5 µg/ml.

Table 11. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the crude extract from *Eleutherine americana* against reference strains

S. aureus	MIC/MBC (µg/ml)
ATCC 25923	250/>1000
ATCC 23235[a]	250/>1000
ATCC 27664[a]	250/>1000

[a]Enterotoxin-producing reference strains

4.8 Time kill assay

Figure 4 shows time-kill curve of the three reference strains used in this study. Treatment with the crude extract at 4MIC (1 mg/ml) reduced the viability of *S. aureus* by at least 5 log cycle compared to the control and kept the inoculum at lag phase throughout the period of study. The extract at 2MIC inhibited the organisms by 3 log reduction while treatment with both MIC and 0.5MIC were different from control by either 1 or 2 log reductions.

Isolates from food were selected for the time-kill curve based on the MIC results (Figure 5). Isolate NPRC 411 had MIC value of 0.25 mg/ml and at 20 h the 4MIC concentration could reduce the bacteria cells by 5 log cycle (Figure 5A). Figure 5B showed NPRC 412 with MIC value of 0.5 mg/ml. NPRC 438 with MIC at 0.06 mg/ml at 4MIC produced 3 log reduction compared to the control at 24 h (Figure 5C). Figure 5D showed NPRC 500 with MIC of 1 mg/ml. At 2MIC and 4MIC, the extract yielded bacteriostatic effect on inoculum. The inoculum remained at lag phase of growth and there were 5-6 log reduction when compared with the control. The results indicated that each isolate behaved differently at above MIC concentrations.

Table 12. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of vancomycin against *Staphylococcus aureus* isolated from foods and reference strains

S. aureus	[a]MIC_{50}	[b]MIC_{90}	MIC range	MBC range	MIC breakpoint	
(Vancomycin)	µg/ml	µg/ml	µg/ml	µg/ml	Susceptible	Resistant
Food isolates (n=106)	0.06	0.06	0.03-0.12	0.03->0.5	≤4	≥32
ATCC 25923	NA	NA	0.03	0.25		
ATCC 23235	NA	NA	0.03	0.12		
ATCC 27664	NA	NA	0.5	0.5		

[a] antibiotics concentration at which 50% of the isolates were inhibited

[b] antibiotics concentration at which 90% of the isolates were inhibited

NA- Not applicable

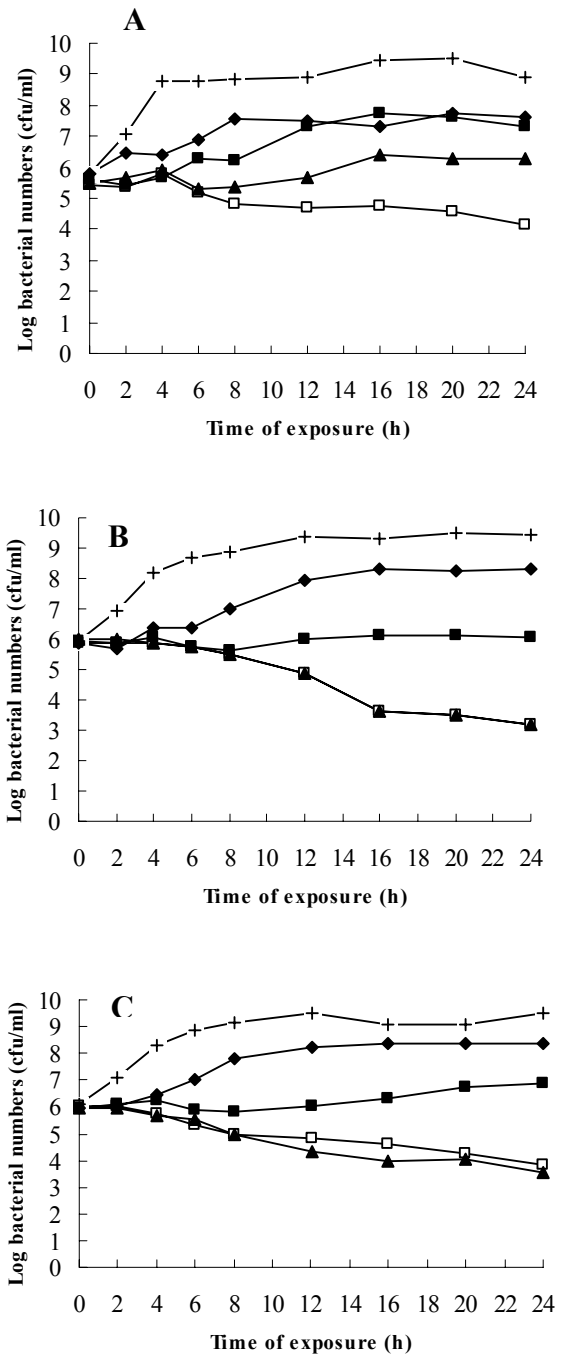


Figure 4. Time-kill curve of *Staphylococcus aureus* ATCC 25923 (A), ATCC 23235 (B), and ATCC 27664 (C) after treatment with the ethanolic extract of *Eleutherine americana*. Control suspensions (+), 4MIC (□), 2MIC (▲), MIC (■) and 0.5MIC (♦). The lower detection threshold was 10^2 cfu/ml

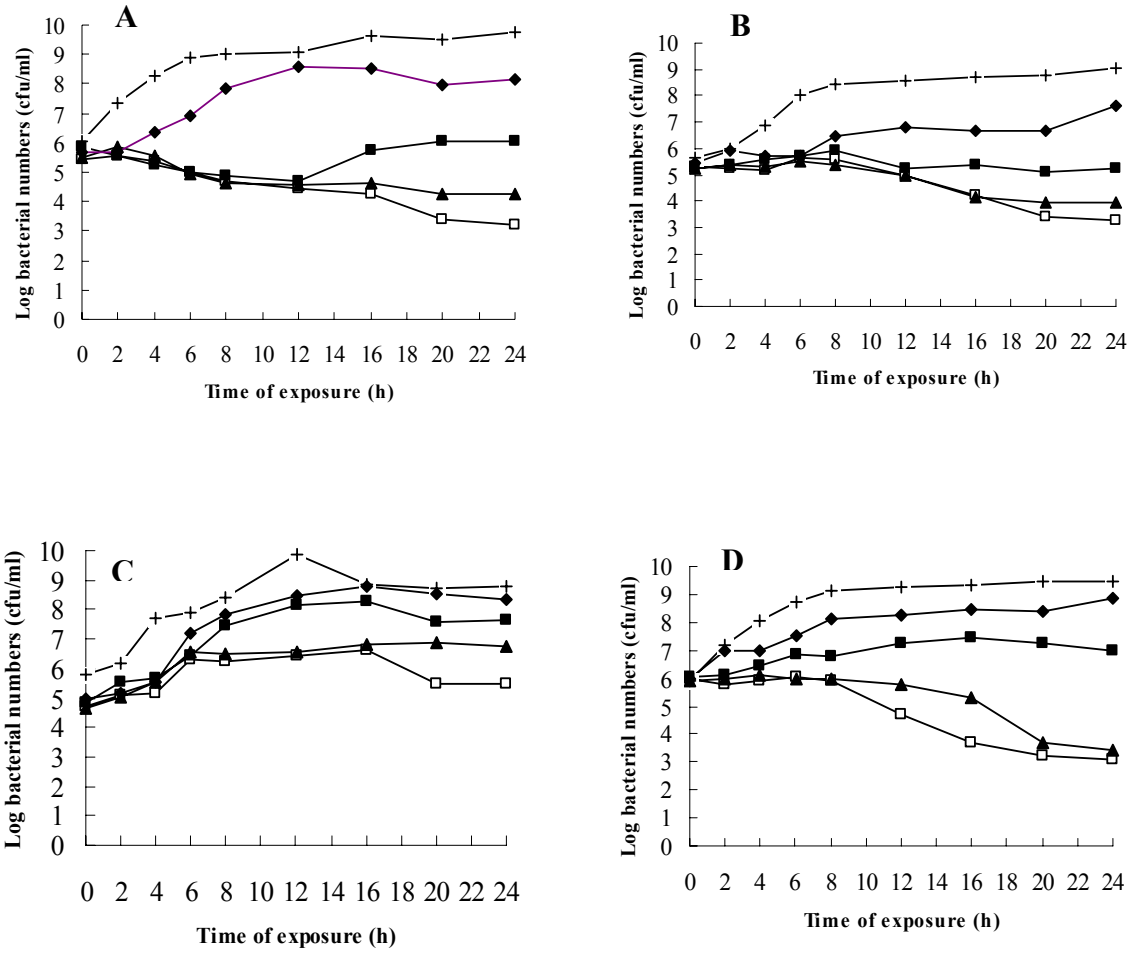
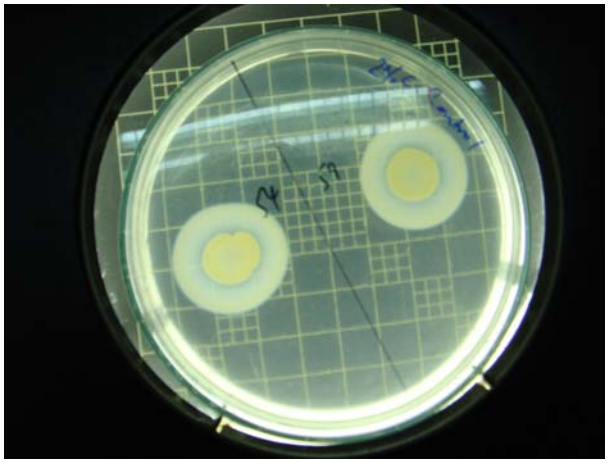


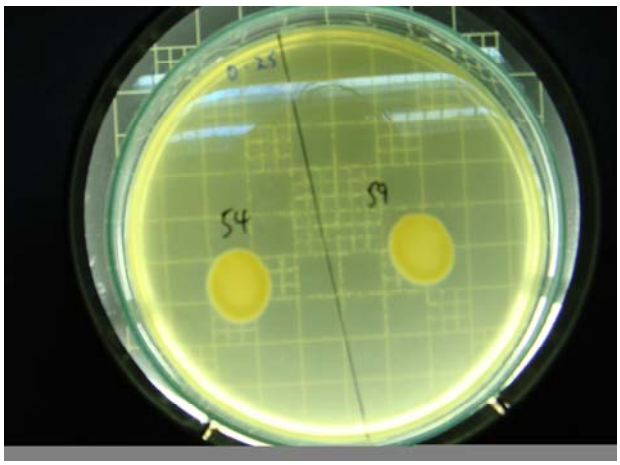
Figure 5. Time-kill curve of *Staphylococcus aureus* NPRC 411 (A), NPRC 412 (B), NPRC 438 (C), and NPRC 500 (D) after treatment with the ethanolic extract of *Eleutherine americana* respectively. Control suspensions (+), 4MIC (□), 2MIC (▲), MIC (■) and 0.5MIC (♦). The lower detection threshold was 10^2 cfu/ml

4.9 Anti-enzymatic activity of crude ethanolic extract

The inhibitory effect of *E. americana* crude extract on staphylococcal extracellular enzyme was studied. Eleven food isolates were selected with the three reference strains used in this study. The results revealed that at subMIC levels (62.5-125 µg/ml), the plant extract demonstrated inhibitory activities against both lipase and protease enzymes (Table 13). The inhibitory activities of the extract on the two enzymes tested are either total or partial. Extract activity on lipase enzyme produced a total inhibition of 36.3% at ½MIC and 18.1% at ¼MIC, while for protease a total inhibition of 27.2% was obtained at ½MIC and 9.0% at ¼MIC. In addition, the extract could totally inhibit protease enzyme production by ATCC 27664 at both ½MIC and ¼MIC.



A. Control



B. 1/2MIC extract treatment

Figure 6. Inhibitory effect of *Eleutherine americana* at subminimal inhibitory concentration on protease enzyme production by *Staphylococcus aureus* NPRC 454 and NPRC 459. The control (A), and treated (B)

Table 13. Inhibition (%) of enzyme activity of *Staphylococcus aureus* by *Eleutherine americana* extract at subminimal inhibitory concentrations

	1/2MIC (125 μg/ml)		1/4MIC (62.5 μg/ml)	
S. aureus	Lipase	Protease	Lipase	Protease
Food isolates (n=11)	36.3[a] (54.5)[b]	27.2[a] (72.7)[b]	18.1[a] (63.6)[b]	9.09[a] (81.8)[b]
ATCC 23235	P[b]	P[b]	P[b]	P[b]
ATCC 27664	P[b]	T[a]	P[b]	T[a]
ATCC 29523	P[b]	P[b]	P[b]	P[b]

[a]Total inhibition

[b]Partial inhibition

4.10 Anti-enterotoxin activity of crude ethanolic extract from *Eleutherine americana*

Evaluation of toxin production (A-D) by *S. aureus* in the presence of *E. americana* extract revealed that the ability to produce toxin decreases as extract concentration increases. At subinhibitory concentrations, the extract could inhibit enterotoxin production compared to the control titre value despite the slight reduction observed in the bacterial growth (Tables 14-16). The inhibitory effect of the extract on enterotoxins A and D produced by NPRC 468, a food isolate and ATCC 23235 followed the same pattern. Enterotoxin productions were observed at MIC but at very low titre value compared with the controls. However, Table 14 revealed that 250 µg/ml of the extract (MIC) could inhibit enterotoxin C production and at 2MIC there were no enterotoxin production from all the *S. aureus* tested at 24 h in the presence of viable cells.

Table 14. Effect of different concentrations of *Eleutherine americana* on enterotoxin A and C produced by *Staphylococcus aureus* NPRC 468

Extract (µg/ml)	Time (h)	Viable count log (cfu/ml)	Enterotoxin A titre[a]	Enterotoxin C titre[a]
0	24	9.87	>128	128
	48	9.50	>128	128
62.5	24	8.95	32	4
	48	9.10	32	4
125	24	8.61	16	4
	48	8.93	16	4
250	24	7.72	4	ND[b]
	48	8.00	4	ND[b]
500	24	3.55	ND[b]	ND[b]
	48	ND[b]	ND[b]	ND[b]

[a]Reciprocal of last dilution that produced an agglutination.

[b]Not detected.

Limit of detection of the RPLA test is 0.5 ng/ml.

Table 15. Effect of different concentrations of *Eleutherine americana* on enterotoxin B produced by *Staphylococcus aureus* NPRC 502

Extract (µg/ml)	Time (h)	Viable count log (cfu/ml)	Enterotoxin B titre[a]
0	24	9.33	>128
	48	9.24	>128
62.5	24	8.47	32
	48	8.84	32
125	24	7.84	8
	48	8.02	8
250	24	7.09	2
	48	7.25	2
500	24	3.96	ND[b]
	48	ND[b]	ND[b]

[a]Reciprocal of last dilution that produced an agglutination.

[b]Not detected

Limit of detection of the RPLA test is 0.5 ng/ml

Table 16. Effect of different concentrations of *Eleutherine americana* on enterotoxin D produced by *Staphylococcus aureus* ATCC 23235

Extract (µg/ml)	Time (h)	Viable count log (cfu/ml)	Enterotoxin D titre[a]
0	24	9.43	>128
	48	9.26	>128
62.5	24	8.86	32
	48	9.10	32
125	24	8.61	16
	48	8.86	16
250	24	6.03	4
	48	6.76	4
500	24	3.55	ND[b]
	48	ND[b]	ND[b]

[a]Reciprocal of last dilution that produced an agglutination.

[b]Not detected.

Limit of detection of the RPLA test is 0.5 ng/ml.

4. 11 Inhibitory activity of the crude extract in food system

The food system study revealed that different time is required for the synthesis of enterotoxin A-D (Table 17). Enterotoxin A was produced at 6 h, followed by D at 8 h while both B and C were not detected until 24 and 20 h, respectively. Enterotoxin B was detected in the control after 24 h and was not found in the samples treated with extract until 30 h in the pork treated with 1.0 mg/ml. The extract could delay toxin C production for 4 h at 4MIC and 8MIC repectively, while enterotoxin was not detected from sample treated with 16MIC at 48 h.

Table 17. Inhibitory effect of the various concentration of crude extract from *Eleutherine americana* against enterotoxins produced by *Staphylococcus aureus* inoculated into cooked pork and stored at room temperature 30°C

S. aureus	Toxin	Control	4MIC	8MIC	16MIC
NPRC 468	SEA	6[a]/8.22[b]	8/8.03	14/8.00	14/7.91
NPRC 502	SEB	24[a]/9.73[b]	30/8.57	ND[c]/7.71	ND[c]/6.98
NPRC 468	SEC	20[a]/10.11[b]	24/8.40	24/8.09	ND[c]/7.01
ATCC 23235	SED	8[a]/9.30[b]	8/9.28	8/9.05	8/7.84

[a]Time enterotoxin was detected (h)/[b]Number of cells (cfu/g) at the time of detection

[c]Enterotoxin not detected at 48 h

SE, Staphylococcal enterotoxin

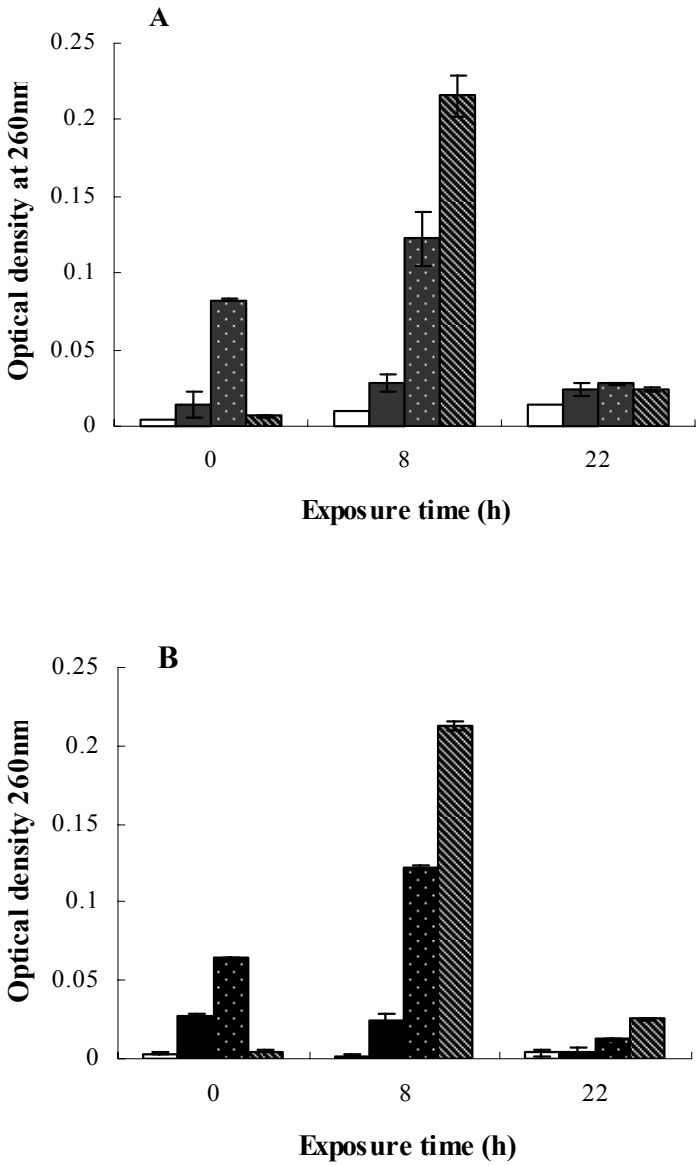


Figure 7. Absorbance of the cell materials at 260 nm released from *Staphylococcus aureus* ATCC 27664 (A) and NPRC 500 (B) after treatment with MIC (black bars), 2MIC (black bars with white dots), 4MIC (black bars with diagonal stripes) or with 1% DMSO , control (white bars). The data are expressed as means ± standard errors

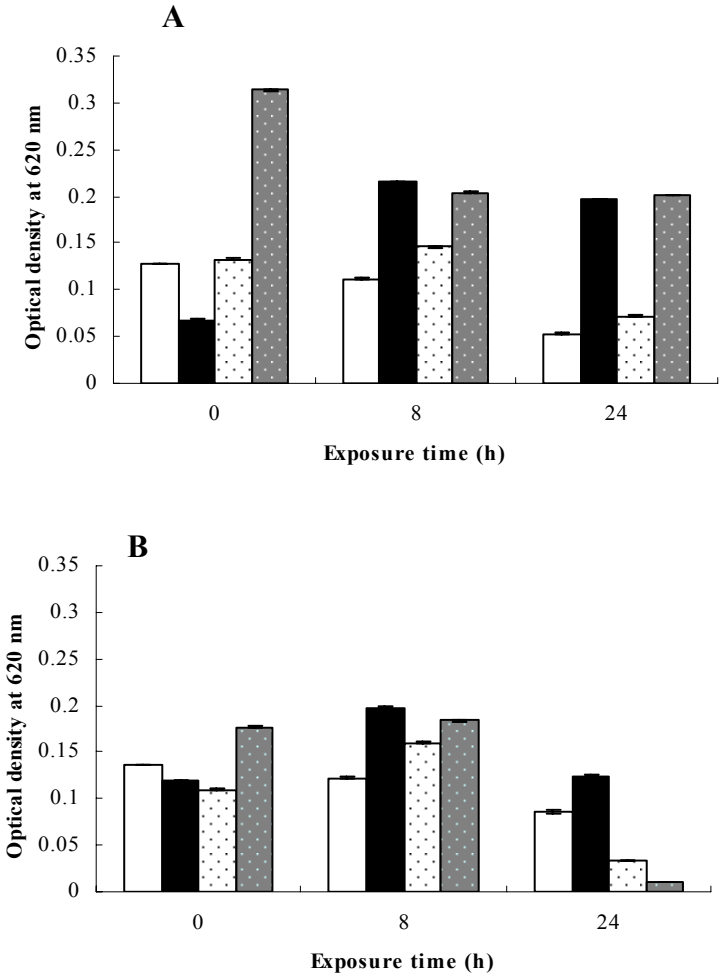


Figure 8. Optical density (OD_{620}) of suspensions of *Staphylococcus aureus* ATCC 27664 (A) and NPRC 500 (B) treated with and without various concentrations of crude extract from *Eleutherine americana.* Control treated with 1% DMSO (white bars), and after treatment with MIC (black bars), 2MIC (white bars with black dots), 4MIC (grey bars with white dots). The data are expressed as means ± standard errors

4.14 Effect of crude extract from *Eleutherine americana* on *Staphylococcus aureus* cell membrane

The results of the effect of crude extract from *E. americana* cell membrane of *S. aureus* is shown on Figure 7. The effect of the highest concentration of the extract (1 mg/ml) at 8 h resulted in higher cell materials which produced higher OD_{260} compared with the control and at 22 h lower OD values were recorded for all treatments.

4.15 Effect of crude extract on *Staphylococcus aureus* cell wall

The results obtained from measurement of optical density at 620 nm indicated that treated cells produced higher OD values compared with the control (Figure 8A). However, the treatment of the food isolate NPRC 500 with the extract produced slight reduction of the OD values at 2MIC and 4MIC at 24 h (Figure 8B). This experiment may indicate that primary mechanism of action of *E. americana* crude extract is not cell wall lysis.

4.16 Effect of crude extract from *Eleutherine americana* on *Staphylococcus aureus* ultrastructure

Exposure of *S. aureus* to crude extract from *E. americana* and observed under transmission electron microscope revealed that the cells undergone some ultrastructural changes. Figure 9A revealed the control which was treated with 1% DMSO, while Figure 9B and C represented the cells treated with 2MIC and 4MIC of the extract. It was observed that the treatment with the crude extract resulted in enlargement of the cell, and increased thickness of the cell membrane when compared to the control. In addition, it could be noticed that there was a type of splitting of cell materials to the outer surface of the disruptured membrane (Figure 9C and D).

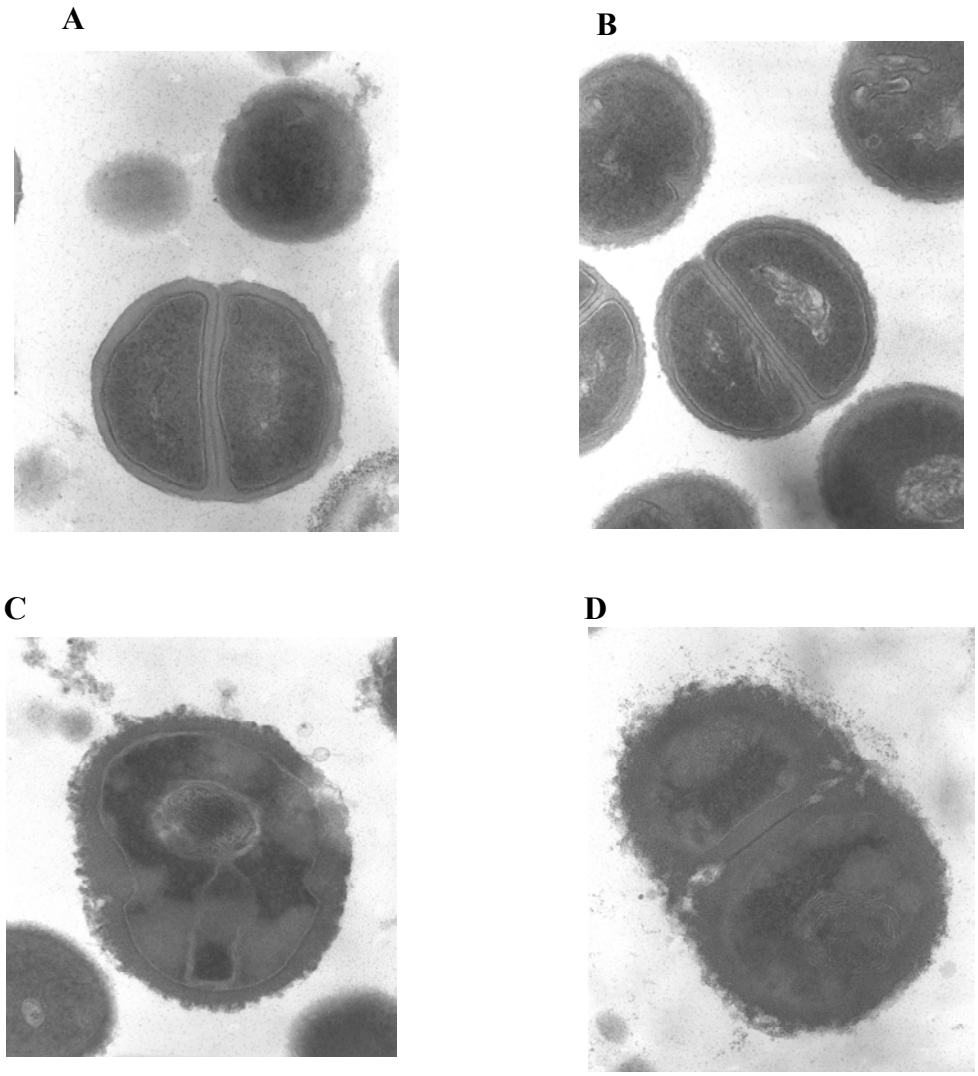


Figure 9. Observation of *Staphylococcus aureus* ATCC 27664 under Transmission Electron Micrograph at 12 h after treated with 1% DMSO (A) control, and treatment with different crude ethanolic extract from *Eleutherine americana*, 0.25 mg/ml (B), 0.5 mg/ml (C), and 1.0 mg/ml (D). Magnification: ×50,000

4. 17 Cytotoxicity activity

Cytotoxicity test revealed that *E. americana* crude extract is cytotoxic to vero cells (African green monkey kidney) with IC_{50} of 4.82 µg/ml. However, the result of the susceptibility test of the extract against *Lactobacillus* spp. showed that the extract possessed no antibacterial activity against *Lactobacillus* spp.

4.18 Additive effect of extract and salt on *Staphylococcus aureus*

Ability of *S. aureus* strains to grow on nutrient agar after treated with crude ethanolic extract of *E. americana* and 7.5% NaCl was examined. The extract concentrations used were 1/2MIC, MIC, and 2MIC. Figure 10 showed enterotoxin-producing ATCC 27664 treated with crude extract, (Figure 10A), and after treated with mixture of extract and NaCl (Figure 10B). Addition of extract and salt resulted to total inhibition at 2MIC (Figure 10B). Food isolate, NPRC 425, behaved differently in that addition of NaCl to extract was able to reduce the inoculum size by 1 or 2 log at all extract concentration (Figure 10D). ATCC 25923 and NPRC 500 showed similar behaviour to the treatment (Figure 11).

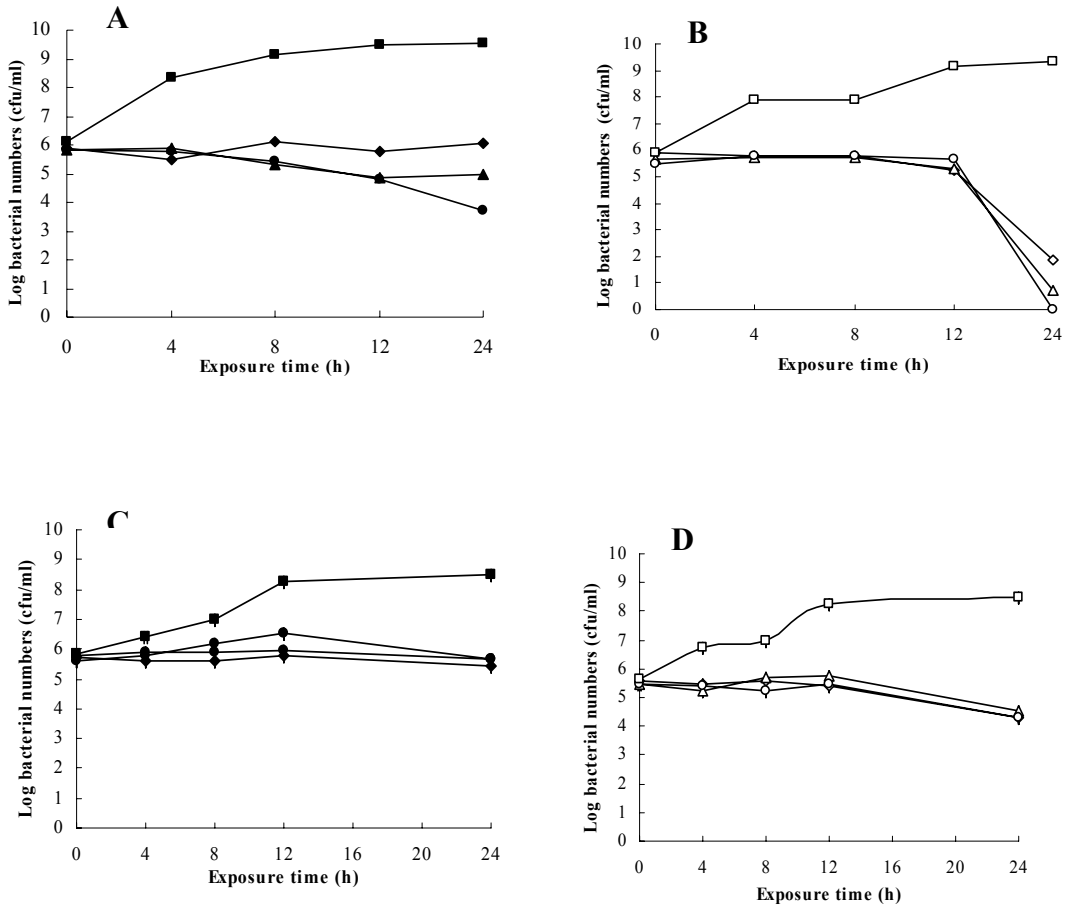


Figure 10. Number of *Staphylococcus aureus* cells, ATCC 27664 (A) and NPRC 425 (C) able to form colonies on nutrient agar after treated with crude extract of *Eleutherine americana* (closed symbols), and ATCC 27664 (B) and NPRC 425 (D), after treated with crude extract + 7.5% NaCl (open symbols). The rhombus (1/2MIC), triangle (MIC), circle (2MIC), and the square (control)

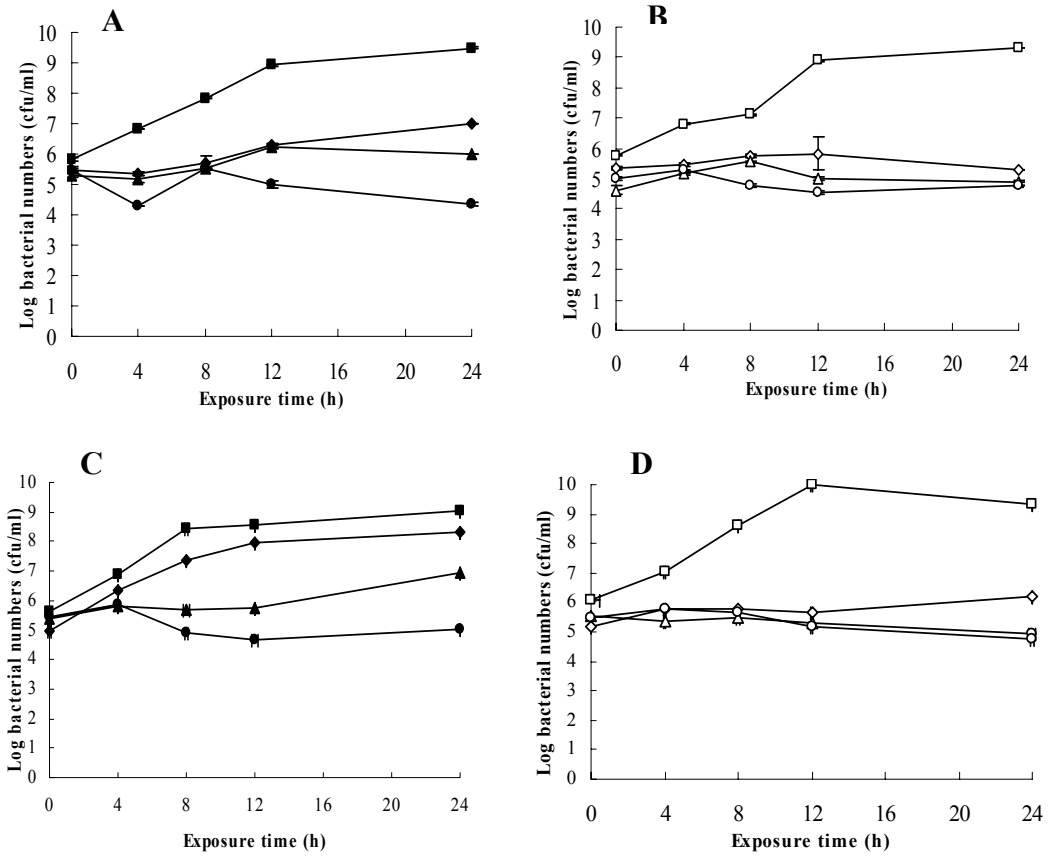


Figure 11. Number of *Staphylococcus aureus* cells ATCC 25923 (A) and NPRC 500 (C) able to form colonies on nutrient agar after treated with crude extract of *Eleutherine americana* (closed symbols) and ATCC 25923 (B) and NPRC 500 (D), after treated with crude extract + 7.5% NaCl (open symbols) The rhombus (1/2MIC), triangle (MIC), circle (2MIC), and the square (control)

4.19 Stability study of the crude extract

The effect of change in pH and incubation temperature on the extract activity in the broth system is shown in Figure 12. The result revealed that when the extract was incubated at 35ºC (Figure 12A) the extract could reduce the inoculum size of *S. aureus* by at least 3 to 6 log at 24 h contrary to what obtained at temperatures 10 and 4 ºC, respectively (Figure 12B and 12C).

4. 20 Application of the extract into salad dressing

4.20.1 Antibacterial activity of the extract in the salad dressing

Figure 13 revealed the effect of *E. americana* extract on the salad dressing inoculated with *S. aureus* and stored at 4 and 10 ºC, respectively. It was observed that there were reductions in the bacterial load of both the control and the treatments through out the storage period (Figure 13A). All the extract concentrations had inhibitory effect on the bacteria producing about 3 log reductions from day 0 to day 21. The antibacterial activity of the extract in the treatments stored at 10ºC is also shown in Figure 13B. At this temperature the extract activity was better than at 4ºC because no *S. aureus* were detected as from day 15 at both 0.5 mg/ml and 1.0 mg/ml extract concentrations. The total viable counts (on plate count agar) obtained from the salad dressing during the 21 day storage is shown on Figure 14A and 14B. At 4ºC storage temperature (Figure 16A), no microbial counts were detected again in all the treatments at 21 day. Similarly, at 10ºC (Figure 14B), no microorganisms were found from both salad dressing with 0.5 mg/ml and 1.0 mg/ml extract concentration as from 15 day and at 21 day for sample with 0.25 mg/ml extract.

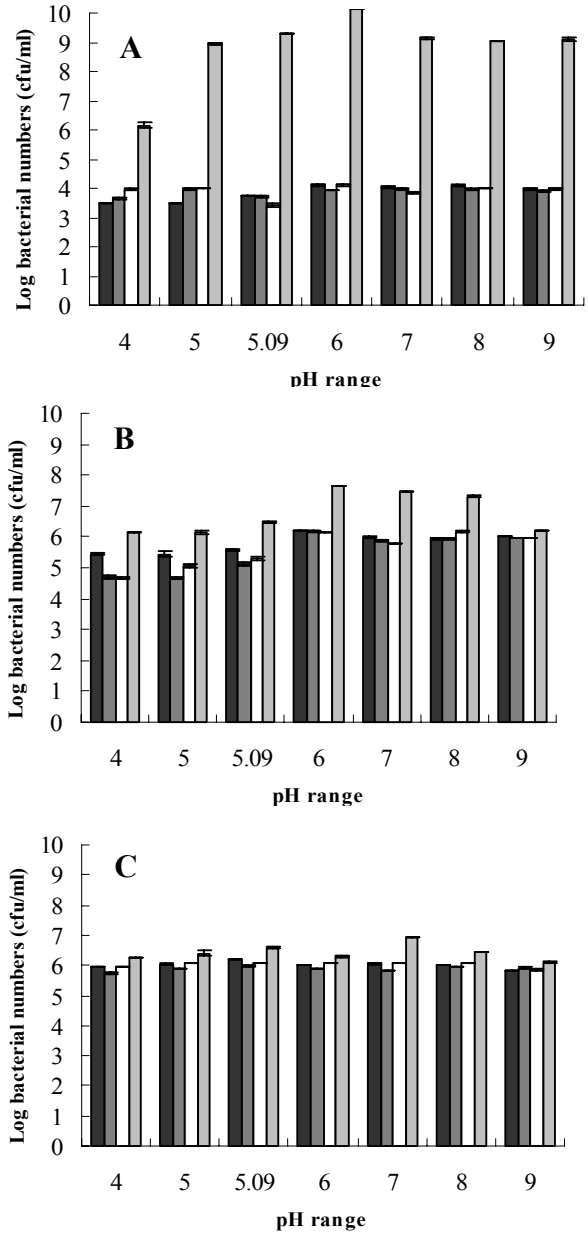


Figure 12. Antibacterial activity of crude extract from *Eleutherine americana* at 1 mg/ml against ATCC 27664 at various pH without heat treatment (black bars), pasteurized (dark grey bars), autoclaved (white bars), after incubation at 35ºC (A), 10ºC (B), and 4ºC (C) for 24 h. The grey bars represent control. Each symbol indicates the means ± SEs for three replicates

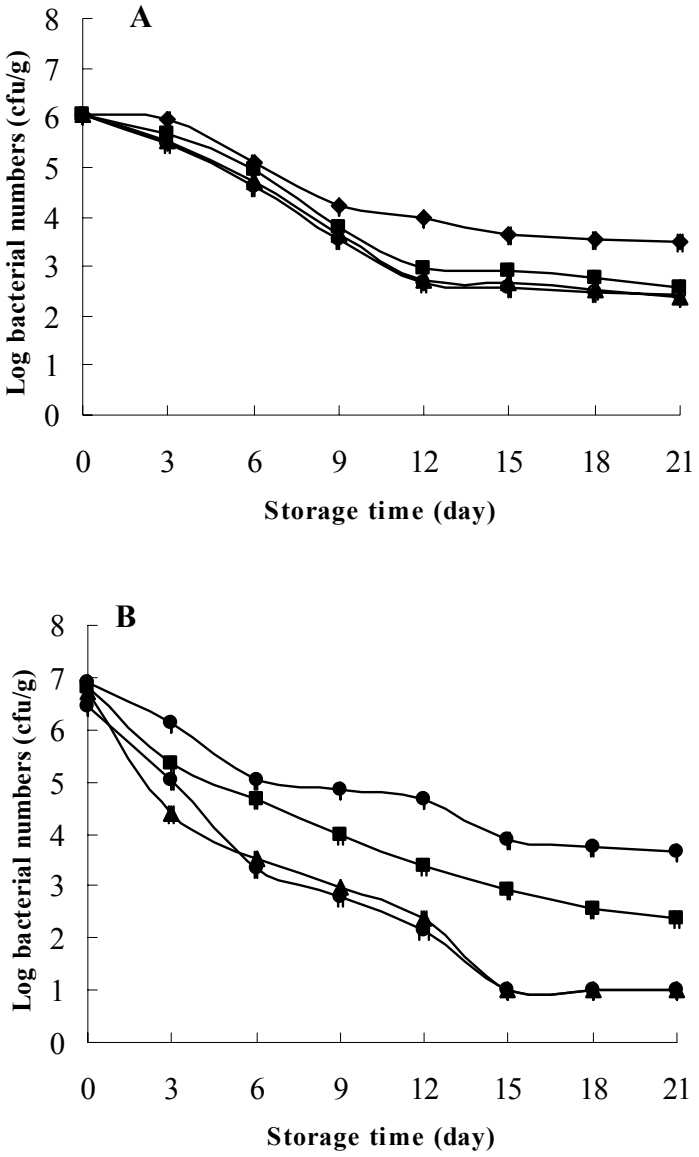


Figure 13. Viable counts on Baird-Parker showing inhibitory effect of crude extract from *Eleutherine americana* on *Staphylococcus aureus* ATCC 27664 inoculated into home made salad dressing and stored at 4ºC (A), and 10ºC (B) for 21 days. Samples without extract (control; closed rhombus) and those with 0.25 mg/ml (closed square), 0.5 mg/ml (closed triangle) and 1.0 mg/ml (open circle) extract. The limit of detection was 10^1 cfu/g

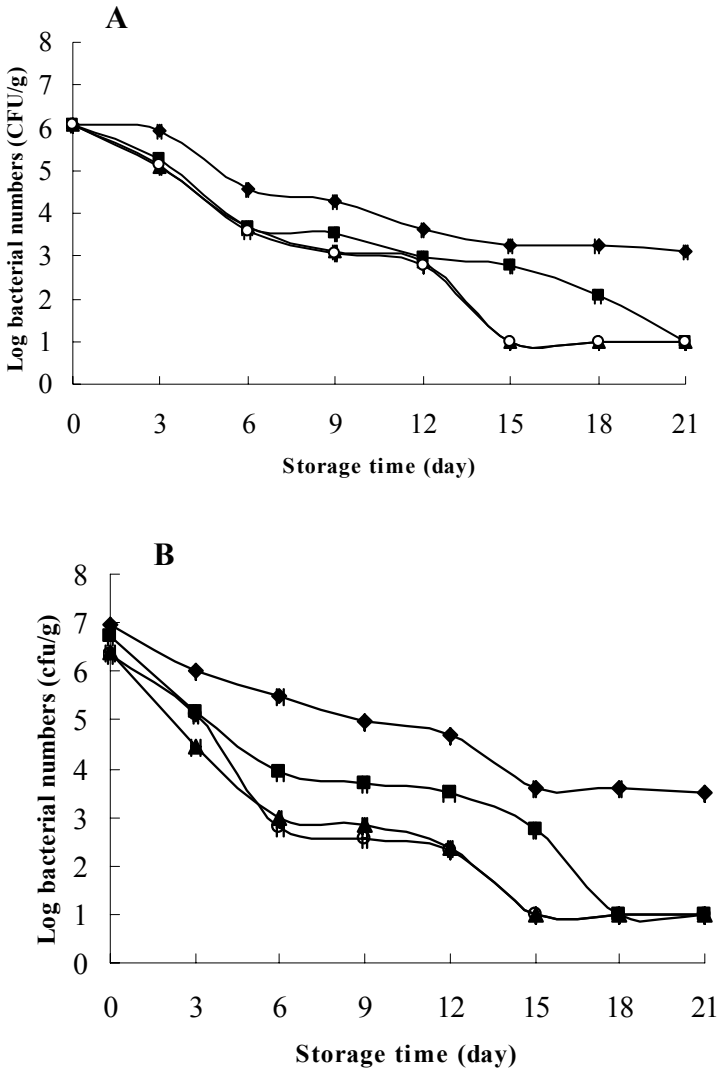


Figure 14. Viable counts on plate count agar obtained from home made salad dressing inoculated with *Staphylococcus aureus* treated with extract from *Eleutherine americana* and stored at 4ºC (A) and 10ºC (B) for 21 days. Samples without extract (control; closed rhombus) and those with 0.25 mg/ml (closed square), 0.5 mg/ml (closed triangle), and 1.0 mg/ml (open circle) extract. The limit of detection was 10^1 cfu/g

4.20.2 Effect of crude extract on the pH and water activity of salad dressing

The pH value of the salad dressings with or without extract is presented on Table 18. On the 0 day the pH values of the control sample and treatments were not significantly different, however, as storage days increased the values changed. The pH values ranged from 5.89 to 4.14 from day 0 to day 21 with the control significantly lower than the treatments. The water activity values of the control sample was 0.88, while the treatments ranged from 0.88-0.91.

Table 18. pH values of home made salad dressing treated with natural extract from *Eleutherine americana*

	Treatments (Salad dressing + extract)			
Storage time (day)	Control	0.25 mg/ml	0.5 mg/ml	1.0 mg/ml
0	5.56 ± 0.01^{aEF}	5.49 ± 0.38^{aB}	5.84 ± 0.03^{aG}	5.71 ± 0.07^{aG}
3	5.49 ± 0.03^{bDE}	5.67 ± 0.02^{cBCD}	5.50 ± 0.02^{bE}	5.43 ± 0.03^{aE}
6	5.61 ± 0.06^{aG}	5.76 ± 0.05^{bCD}	5.57 ± 0.04^{aF}	5.65 ± 0.00^{aF}
9	5.54 ± 0.01^{cF}	5.89 ± 0.03^{dD}	5.47 ± 0.02^{bE}	5.36 ± 0.02^{aD}
12	5.47 ± 0.01^{cD}	5.77 ± 0.01^{dCD}	5.37 ± 0.01^{bD}	5.20 ± 0.01^{aC}
15	5.35 ± 0.01^{cC}	5.61 ± 0.01^{dBC}	5.30 ± 0.01^{bC}	$5.18 \pm 0.01^{a\,C}$
18	$4.14 \pm 0.01^{a\,B}$	$4.31 \pm 0.01^{c\,A}$	$4.30 \pm 0.01^{c\,A}$	4.27 ± 0.01^{bA}
21	4.21 ± 0.01^{aA}	4.36 ± 0.01^{bA}	4.37 ± 0.01^{bB}	4.37 ± 0.01^{bB}

Values (average of three readings ± standard deviation) in the same row with different superscript (a-d) are significantly different ($p<0.05$).

Values in the same column with different superscript (A-G) are significantly different ($p<0.05$).

4.20.3 Effect of crude extract on TBARs of salad dressing

Lipid oxidation of the salad dressings without sugar were monitored every 3 day during the period of study (Table 19). Considering the samples without sugar, the TBARs of the control and treatments were not significantly different at day 0, however, as from the 3rd day of storage the values were increasing with storage days, while the extract demonstrated antioxidant activity. TBARs of samples with sugar were higher ranging from 14.36-9.50 compared to the samples without sugar.

Table 19. TBARS (mg malonaldehyde/kg) values of home made salad dressing without sugar treated with natural extract from *Eleutherine americana* and stored at 4ºC for 21 days

	Treatments (Salad dressing + extract)			
Storage time (day)	Control	0.25 mg/ml	0.5 mg/ml	1.0 mg/ml 0
0	3.47 ± 0.68^{a}	2.92 ± 0.73^{a}	2.64 ± 0.19^{a}	2.58 ± 0.03^{a}
3	3.57 ± 0.04^{c}	3.37 ± 0.18^{c}	2.81 ± 0.24^{b}	2.22 ± 0.12^{a}
6	3.93 ± 0.47^{b}	3.19 ± 0.13^{a}	3.19 ± 0.18^{a}	2.64 ± 0.26^{a}
9	5.81 ± 0.11^{c}	4.51 ± 0.38^{b}	2.99 ± 0.26^{a}	2.67 ± 0.06^{a}
12	4.58 ± 0.29^{b}	3.46 ± 0.42^{a}	3.07 ± 0.27^{a}	3.01 ± 0.21^{a}
15	5.81 ± 0.47^{c}	4.96 ± 0.49^{c}	3.44 ± 0.47^{b}	2.46 ± 0.35^{a}
18	6.25 ± 0.22^{c}	5.08 ± 0.35^{c}	3.81 ± 0.57^{b}	3.06 ± 0.28^{a}
21	6.56 ± 0.34^{d}	5.27 ± 0.15^{c}	3.73 ± 0.29^{b}	3.02 ± 0.24^{a}

Values (average of three readings ± standard deviation) in the same row with different superscript are significantly different ($p<0.05$)

4.20.4 Effect of extract on the colour of salad dressing

The effect of extract on the colour of the salad dressing is shown on Table 20. Addition of extract at all concentrations significantly increased the lightness (L*) values of the salad dressing. The L* value of the control was maintained until the 21st day whereas those of the treated samples varied throughout storage. The redness (a*) values of the samples decreased with the addition of the extract compared to the control and were significantly different at each extract concentration (Table 21). In addition, significant differences were observed in the yellowness (b*) values of the salad dressing compared to control and this was stable throughout the storage period.

Table 20. Effect of *Eleutherine americana* extract on the colour of home made salad dressing stored at 4ºC for 21 days

	Treatments (Salad dressing + extract)			
Storage time (day)	Control	0.25 mg/ml	0.5 mg/ml	1.0 mg/ml
[1]L *-value				
0	60.00 ± 0.05^{a}	62.34 ± 0.02^{d}	61.13 ± 0.07^{c}	60.35 ± 0.08^{b}
3	52.38 ± 0.04^{a}	54.57 ± 0.07^{d}	54.14 ± 0.05^{c}	53.42 ± 0.05^{b}
6	60.90 ± 0.10^{a}	62.07 ± 0.03^{c}	61.43 ± 0.07^{b}	60.85 ± 0.11^{a}
9	60.13 ± 0.13^{a}	62.05 ± 0.04^{d}	61.31 ± 0.02^{c}	60.79 ± 0.01^{b}
12	60.75 ± 0.04^{a}	62.43 ± 0.09^{b}	60.98 ± 0.27^{a}	60.69 ± 0.07^{a}
15	61.54 ± 0.01^{a}	63.24 ± 0.04^{d}	62.93 ± 0.06^{c}	61.91 ± 0.04^{b}
18	61.38 ± 0.07^{a}	64.44 ± 0.19^{c}	62.44 ± 0.05^{b}	62.71 ± 0.02^{b}
21	62.31 ± 0.02^{b}	63.35 ± 0.03^{c}	62.38 ± 0.43^{b}	61.72 ± 0.05^{a}
[2]a*-value				
0	18.04 ± 0.12^{d}	16.03 ± 001^{b}	16.00 ± 0.15^{a}	16.19 ± 0.05^{c}
3	18.36 ± 0.16^{c}	16.40 ± 0.03^{a}	16.83 ± 0.02^{a}	17.58 ± 0.50^{b}
6	18.47 ± 0.11^{d}	16.31 ± 0.02^{a}	16.66 ± 0.10^{b}	17.18 ± 0.12^{c}
9	16.94 ± 0.27^{c}	15.92 ± 0.11^{a}	16.44 ± 0.07^{b}	17.35 ± 0.02^{d}
12	17.78 ± 0.16^{b}	16.33 ± 0.23^{a}	16.44 ± 0.49^{a}	17.39 ± 0.21^{b}
15	17.71 ± 0.03^{b}	16.54 ± 0.10^{a}	18.04 ± 0.07^{c}	17.70 ± 0.14^{b}
18	18.00 ± 0.30^{b}	16.81 ± 0.06^{a}	17.53 ± 0.83^{ab}	17.75 ± 0.12^{b}
21	18.47 ± 0.11^{c}	17.24 ± 0.13^{a}	18.40 ± 0.25^{c}	17.80 ± 0.21^{b}
[3]b*-value				
0	69.30 ± 0.07^{d}	60.86 ± 0.04^{c}	55.97 ± 0.14^{b}	54.51 ± 0.04^{a}
3	69.22 ± 0.05^{d}	59.97 ± 0.95^{c}	57.14 ± 0.03^{b}	55.18 ± 0.08^{a}
6	69.77 ± 0.09^{d}	59.61 ± 0.13^{c}	56.34 ± 0.22^{b}	54.44 ± 0.14^{a}
9	68.66 ± 0.37^{d}	60.71 ± 0.09^{c}	56.77 ± 0.02^{b}	55.58 ± 0.10^{a}
12	70.57 ± 0.05^{d}	60.83 ± 0.10^{c}	56.95 ± 0.45^{b}	56.21 ± 0.07^{a}
15	69.90 ± 0.11^{d}	61.65 ± 0.06^{c}	58.68 ± 0.05^{b}	56.28 ± 0.05^{a}
18	67.12 ± 0.62^{d}	60.24 ± 0.26^{c}	58.31 ± 0.95^{b}	55.80 ± 1.39^{a}
21	69.94 ± 0.09^{d}	61.04 ± 0.11^{c}	57.74 ± 0.15^{b}	55.49 ± 0.01^{a}

Values (average of three readings ± standard deviation) in the same row with different superscript are significantly different ($p<0.05$).

[1]Degree of lightness of the samples.

[2]Redness /greenness (+) = red, (-) = green.

[3]Yellowness/ blueness (+) = yellow; (-) = blue.

4.20.5 Sensory evaluation of the salad dressing

The salad dressing samples, with or without extract is shown on Figure 15. Sensory attributes of the salad dressing with or without extract at 0 day are presented on Table 21. The results showed that there were no significant differences for texture, taste, and overall acceptability in the treatments.

Figure 15. Samples of salad dressing served to panelists for sensory test

Table 21. Mean ranks for quality attributes of home made salad dressing treated with crude extract from *Eleutherine americana* and stored at 4ºC

Treatments		Attributes					
(salad dressing + extract)		Appearance	Colour	Viscousity	Texture	Taste	Overall acceptability
0 day	0.25 mg/ml	6.36 ± 0.95^{ab}	6.26 ± 1.28^{b}	6.52 ± 1.26^{ab}	6.47 ± 1.42^{a}	6.52 ±1.21^{a}	6.52 ± 1.12^{a}
	0.50	6.21 ± 1.03^{b}	6.26 ± 1.09^{b}	6.84 ± 0.76^{a}	6.63 ± 1.11^{a}	6.57 ± 1.01^{a}	6.63 ± 0.95^{a}
	1.00	6.15 ± 0.89^{b}	5.57 ± 1.38^{b}	6.68 ± 0.82^{a}	6.26 ± 1.24^{a}	5.78 ± 1.31^{a}	5.89 ± 1.14^{a}
	Control	7.10 ± 0.93^{a}	7.31 ± 0.67^{a}	7.05 ± 1.22^{a}	6.68 ± 1.37^{a}	6.47 ± 1.38^{a}	6.57 ± 1.34^{a}
8th day	0.25 mg/ml	7.41 ± 0.79^{a}	7.41 ± 0.62^{a}	7.17 ± 0.72^{a}	7.17 ± 0.80^{a}	7.17 ± 0.80^{a}	7.23 ± 0.66^{a}
	0.50	7.41 ± 0.71^{a}	7.47 ± 0.71^{a}	7.35 ± 0.93^{a}	7.35 ± 0.93^{a}	7.23 ± 0.75^{a}	7.35 ± 0.86^{a}
	1.00	7.17 ± 0.88^{a}	7.05 ± 0.74^{a}	7.11 ± 1.05^{a}	7.12 ± 0.92^{a}	6.82 ± 1.33^{a}	7.17 ± 0.86^{a}
	Control	7.17 ± 1.07^{a}	7.41 ± 1.12^{a}	7.17 ± 1.42^{a}	6.94 ± 1.74^{a}	6.82 ± 1.23^{a}	7.17 ± 0.95^{a}
16th day	0.25 mg/ml	7.00 ± 1.22^{a}	6.76 ± 1.39^{a}	6.64 ± 0.72^{a}	6.64 ± 1.32^{a}	6.64 ± 1.41^{a}	6.97 ± 1.17^{a}
	0.50	7.17 ± 0.72^{a}	6.88 ± 0.99^{a}	6.88 ± 1.16^{a}	7.00 ± 0.86^{a}	7.05 ± 1.71^{a}	7.23 ±1.09^{a}
	1.00	7.23 ± 0.90^{a}	7.05 ± 1.34^{a}	7.17 ± 1.01^{a}	6.88 ± 1.16^{a}	6.70 ± 1.68^{a}	7.05 ± 1.59^{a}
	Control	6.88 ± 1.61^{a}	7.00 ± 1.36^{a}	6.64 ± 1.83^{a}	6.64 ± 1.64^{a}	6.76 ± 2.06^{a}	6.76 ±1.52^{a}

Values in the same column (each storage day) with different superscript are significantly different ($p<0.05$).

4.21 *Eleutherine americana* extract as an additive in cooked pork

4.21.1 Effect of extract on the pH and TBARs of cooked pork

The probable use of crude extract from *E. americana* in cooked pork was also examined. Effect of the extract on the pH and TBARs of the cooked pork stored at 4°C is presented in Table 22. At 0 day, the pH of the treatments were significantly higher than the control sample. During the storage for 3 and 6 days, there was no significant difference in the samples, while some differences were noticed on day 9. The result of the TBARs revealed that the extract could delay the lipid oxidation process.

4.21.2 Effect of crude extract on colour and shear force of cooked pork

The result obtained from the effect of the extract on colour changes of the pork revealed that the treatments significantly different from the control while the extract did not have any effect on the shear force values (Table 23).

Table 22. pH and TBARs values of pork cooked with natural extract from *Eleutherine americana* during storage at 4°C

	Treatments (cooked meat + extract mg/100 g)			
Storage time (day)	Control	2.7	5.4	10.8
pH values				
0	5.96 ± 0.05^{b}	6.02 ± 0.03^{a}	6.06 ± 0.02^{a}	6.06 ± 0.02^{a}
3	6.05 ± 0.02^{a}	6.07 ± 0.01^{a}	6.04 ± 0.01^{a}	6.06 ± 0.01^{a}
6	5.94 ± 0.02^{a}	5.89 ± 0.01^{b}	5.95 ± 0.01^{a}	5.97 ± 0.01^{a}
9	5.93 ± 0.04^{b}	5.92 ± 0.01^{b}	5.95 ± 0.01^{ab}	5.99 ± 0.01^{a}
TBARs values (mg malonaldehyde/kg)				
0	6.67 ± 0.83^{a}	2.53 ± 0.49^{b}	1.32 ± 0.76^{c}	1.03 ± 0.27^{c}
3	8.60 ± 0.10^{a}	6.24 ± 0.72^{b}	3.92 ± 0.54^{c}	3.04 ± 0.37^{c}
6	9.33 ± 0.16^{a}	7.67 ± 0.56^{b}	3.90 ± 0.41^{c}	3.67 ± 0.06^{c}
9	11.34 ± 0.37^{a}	8.15 ± 0.30^{b}	4.64 ± 0.65^{c}	2.72 ± 0.69^{c}

Values in the same row are means ± SD from triplicate determinations with different superscript are significantly different ($p<0.05$).

TABLE 23. Colour changes and shear force values of the pork meat cooked with *Eleutherine americana* extracts during storage at 4ºC

	Treatments (cooked pork + extract mg/100 g)			
Storage time (day)	Control	2.7	5.4	10.8
[1]L *-value				
0	63.50 ± 0.05[a]	59.03 ± 0.04[c]	62.05 ± 0.07[b]	55.11 ± 0.05[d]
3	60.24 ± 0.05[b]	60.79 ± 0.02[a]	58.64 ± 0.11[c]	54.94 ± 0.10[d]
6	61.62 ± 0.09[c]	63.77 ± 0.06[a]	63.00 ± 0.01[b]	58.18 ± 0.02[d]
9	63.58 ± 0.02[a]	58.26 ± 0.03[c]	61.58 ± 0.02[b]	57.87 ± 0.04[d]
[2]a*-value				
0	0.71 ± 0.04[d]	2.01± 0.06[c]	3.00 ± 0.09[b]	3.73 ± 0.10[a]
3	0.79 ± 0.06[d]	1.71 ± 0.03[c]	2.73± 0.15[b]	3.45 ± 0.07[a]
6	1.22 ± 0.12[d]	2.01 ± 0.10[c]	2.52 ± 0.05[b]	3.47 ± 0.06[a]
9	1.26 ± 0.07[d]	2.03 ± 0.07[c]	2.39 ± 0.05[b]	3.27 ± 0.05[a]
[3]b*-value				
0	17.18 ± 0.03[d]	18.13 ± 0.03[c]	21.29 ± 0.02[a]	20.27 ± 0.03[b]
3	16.63 ± 0.02[c]	18.93 ± 0.03[b]	19.00 ± 0.10[b]	19.48 ± 0.03[a]
6	16.69 ± 0.02[d]	18.83 ± 0.03[c]	20.85 ± 0.03[a]	19.49 ± 0.05[b]
9	17.50 ± 0.01[d]	18.05 ± 0.03[c]	19.89 ± 0.02[a]	19.21 ± 0.04[b]
Shear force values (kg)				
0	3.62 ± 0.77[a]	3.64 ± 0.58[a]	3.49 ± 0.59[a]	3.74 ± 0.82[a]
3	4.04 ± 0.65[a]	3.93 ± 0.68[a]	3.88 ± 0.93[a]	3.77 ± 0.93[a]
6	3.89 ± 0.76[a]	4.32 ± 0.62[a]	3.87 ± 0.92[a]	4.29 ± 0.81[a]
9	3.91 ± 0.79[a]	3.75 ± 0.48[a]	4.10 ± 0.91[a]	4.05 ± 0.87[a]

Values in the same row are means ± SD from triplicate determinations with different superscript are significantly different ($p<0.05$).

[1]Degree of lightness of the samples.

[2]Redness /greenness (+) = red, (-) = green.

[3]Yellowness/ blueness (+) = yellow; (-) = blue.

4.21.3 Antibacterial activity of the crude extract against *Staphylococcus aureus* inoculated into cooked pork

The inhibitory effect of crude extract from *E. americana* against *S. aureus* inoculated into boiled pork and stored in the refrigerator for 9 days is shown on Figure 16. There were no yeast and mould, psychrophilic, and coliform growth in the pork samples during the course of study. The addition of the extract at 10.8, 5.4, and 2.7 mg produced 0.57, 0.47, 0.41 log differences, respectively when compared with the control at 9 days. Furthermore, the antibacterial effect of *E. americana* extract in combination with crude ethanolic extract of *Curcuma longa* and water extract of garlic was carried out. The result obtained from the combined treatments was not different to the observation made when *E. americana* alone was used (Figure 17).

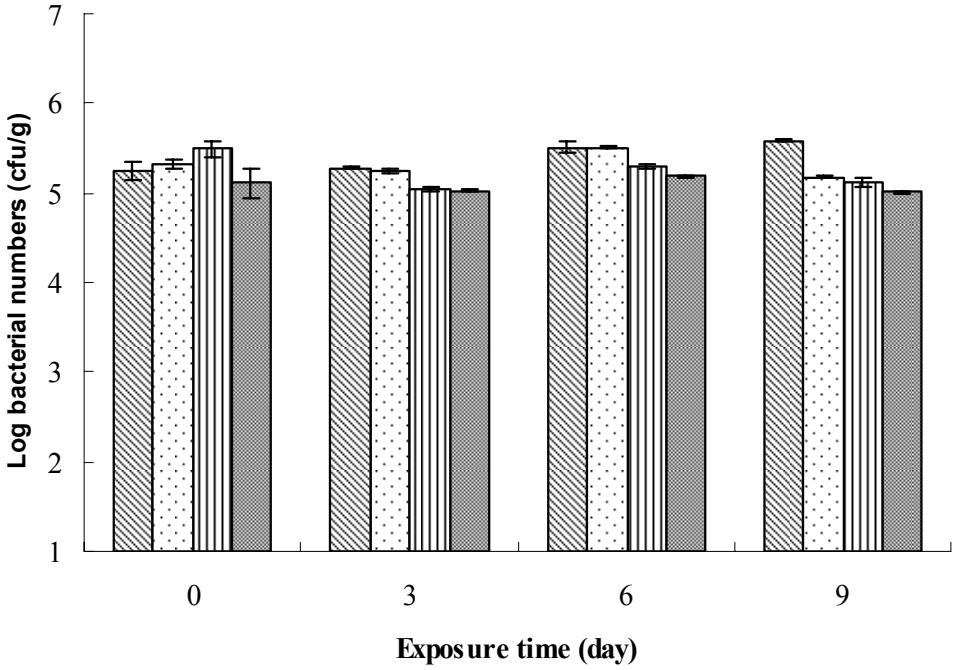


Figure 16. Antibacterial effect of crude extract from *Eleutherine americana* on *Staphylococcus aureus* inoculated into cooked pork and stored at 4°C. Control (bar with diagonal stripes), 2.7 mg/100 g (white bar with black dots), 5.4 mg/100 g (white bar with lines) and 10.8 mg/100 g (dark grey bars). The limit of detection was 10^1 cfu/g

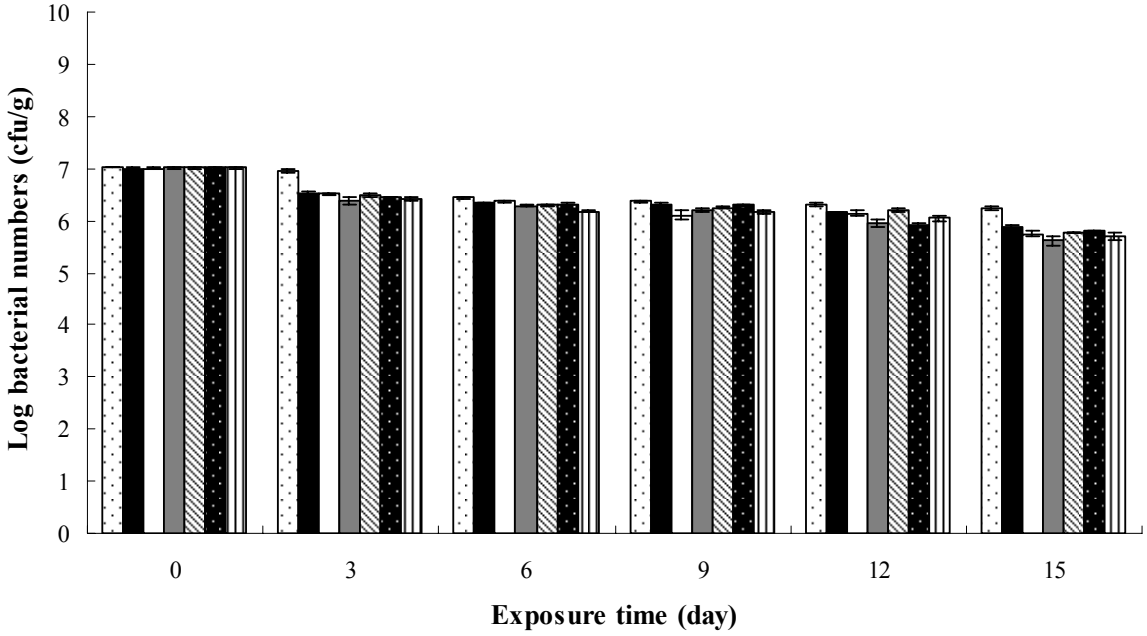


Figure 17. Antibacterial effect of crude extract from *Eleutherine americana* (Ea), *Curcuma longa* (C), and *Allium sativum* (G) on *Staphylococcus aureus* inoculated into cooked pork and stored at 4°C. Control (white bar with black dots), Ea (black bars), Ea + C (white bars), Ea +G (grey bars), C (white bars with black stripes), G (black bars with white dots), and Ea +G + C (white bars with lines). The limit of detection was 10^1 cfu/g

4.21.4 Sensory evaluation of the pork cooked with crude extract from *Eleutherine americana*

Figure 18 shows the representative sample of the pork served to the panelists on the days for sensory tests. The effect of the extract treatment on the sensory scores of the samples from 0 to 9 day is shown on Table 24. The effect of the extract treatment on the sensory scores of the samples from 0 to 9 day revealed that there was no significant difference in the treatments and control until day 9.

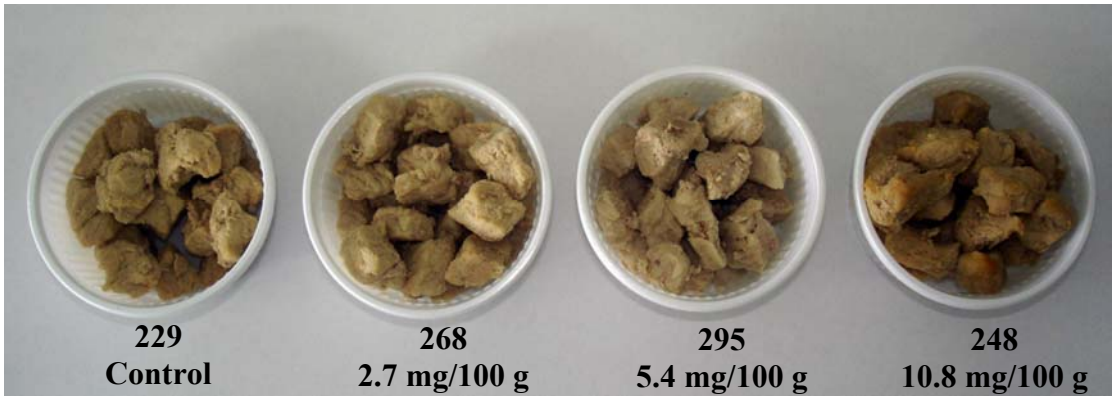


Figure 18. Samples of cooked pork served to panelist for sensory test

Table 24. Effect of *Eleutherine americana* extract on sensory score of cooked pork during storage at 4ºC

	Treatments	Attributes				
	(Pork + extract mg/100 g)	Appearance	Colour	Texture	Taste	Overall acceptability
0 day	2.7	6.90 ± 1.25[a]	6.75 ± 1.48[a]	6.25 ± 1.55[a]	6.30 ±1.36[a]	6.80 ± 1.36[a]
	5.4	7.00 ± 1.29[a]	6.75 ± 1.61[a]	6.45 ± 1.39[a]	6.00 ± 1.80[a]	6.55 ± 1.46[a]
	10.8	6.25 ± 1.94[a]	6.40 ± 1.72[a]	5.65 ± 1.49[a]	5.65 ± 1.46[a]	6.10 ± 1.25[a]
	Control	6.80 ± 1.36[a]	6.75 ± 1.44[a]	6.10 ± 1.33[a]	6.05 ± 1.53[a]	6.40 ± 1.35[a]
3rd day	2.7	6.67 ± 1.46[a]	6.33 ± 1.60[a]	7.00 ± 1.67[a]	6.83 ± 1.47[a]	7.16 ± 1.83[a]
	5.4	6.83 ± 1.63[a]	6.67 ± 1.61[a]	6.83 ± 1.60[a]	6.16 ± 1.60[a]	6.66 ± 1.50[a]
	10.8	6.67 ± 1.03[a]	6.67 ± 1.03[a]	6.83 ± 1.47[a]	6.00 ± 0.89[a]	6.33 ± 1.21[a]
	Control	6.67 ± 1.63[a]	6.67 ± 1.63[a]	6.00 ± 1.89[a]	6.33 ± 1.86[a]	6.50 ± 1.37[a]
6th day	2.7	6.87 ± 1.55[a]	6.75 ± 1.03[a]	6.50 ± 1.19[a]	6.25 ± 1.28[a]	6.62 ± 0.91[a]
	5.4	6.62 ± 0.74[a]	6.37 ± 0.92[a]	6.00 ± 1.30[a]	6.12 ± 0.99[a]	6.25 ± 0.70[a]
	10.8	6.87 ± 0.83[a]	6.62 ± 0.74[a]	6.50 ± 0.92[a]	6.50 ± 1.41[a]	6.80 ± 0.64[a]
	Control	6.12 ± 2.15[a]	6.12 ± 1.24[a]	6.00 ± 1.06[a]	5.50 ± 1.06[a]	5.80 ± 1.12[b]
9th day	2.7	6.80 ± 1.32[a]	6.47 ± 1.60[a]	6.47 ± 1.50[a]	6.28 ± 1.30[a]	6.31 ± 0.95[a]
	5.4	6.71 ± 1.30[a]	6.09 ± 1.81[a]	6.33 ± 1.90[a]	6.19 ± 1.43[a]	6.54 ± 1.18[a]
	10.8	6.52 ± 1.36[a]	6.66 ± 1.06[a]	6.47 ± 1.12[a]	6.14 ± 1.45[a]	6.57 ± 1.24[a]
	Control	5.33 ± 2.15[b]	5.33 ± 1.71[b]	5.19 ± 1.99[b]	5.08 ± 1.45[b]	5.19 ± 1.53[b]

Values in the same column (each storage day) with different superscript are significantly different ($p<0.05$)

4.22 Antimicrobial screening test for *Eleutherine americana*

The results of the antimicrobial screening test on food spoilage and foodborne pathogens for the crude ethanolic extract from *E. americana* are shown on Tables 25 and 26. Figures 19 and 20 demonstrated the time-kill assay results. The results from this screening test revealed that *E. americana* possessed antifungal activities.

Table 25. Paper disc agar susceptibility test of *Eleutherine americana* extracts (2.5 mg/disc) on food spoilage and foodborne pathogens

Test microorganisms	Zone of inhibition[a] (mm)
Bacteria	
Bacillus cereus 10876	13.0
Bacillus licheniformis 12759	19.5
Bacillus spizizenii 6633	18.5
Bacillus subtilis	15.5
Citrobacter freundii	-
Erwinia spp.	14.5
Escherichia coli	-
Klebsiella pneumoniae 13883	-
Methicillin resistant *Staphylococcus aureus* (MRSA)	19.0
Pseudomonas aeruginosa 27853	-
Shigella bodyii 9207	-
Staphylococcus aureus 12600	15.0
Streptococcus spp.	20.0
Yersinia spp.	-
Fungi	
Aspergillus niger	15.0
Microsporum gypseum	-
Penicillium spp.	16.0
Rhizopus spp.	17.0
Trichoderma viride	-
Trichophyton rubrum	-
Yeasts	
Candida utilis	-
Candida albican	-

[a] Mean values from duplicate results

- no inhibition

Table 26. Minimal inhibitory concentration (MIC) of ethanol extracts of *Eleutherine americana* on food spoilage and foodborne pathogens

Test microorganisms	MIC (mg/ml)
Bacteria	
Bacillus cereus 10876	0.125
Bacillus licheniformis 12759	0.125
Bacillus spizizenii 6633	0.25
Bacillus subtilis	0.125
Erwinia spp.	0.25
MRSA	0.125
Staphylococcus aureus 12600	0.25
Streptococcus spp.	0.25
Fungi	
Aspergillus niger	1.0
Penicillium spp.	0.25
Rhizopus spp.	0.25

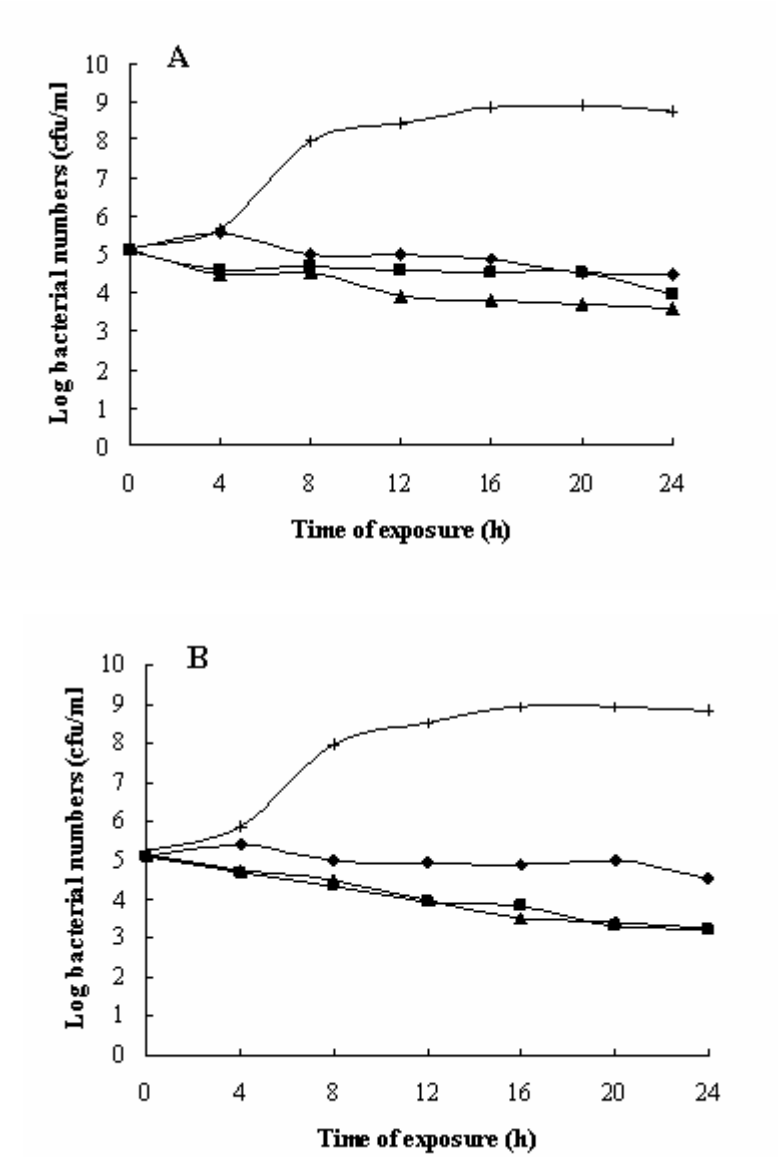


Figure 19. Time-kill curve of *Bacillus cereus* 10876 (A), and *Erwinia* spp. (B) after treatment with crude extract from *Eleutherine americana*. The control suspensions (+), 2MIC (▲), MIC (■), and 1/2MIC (◆). The lower detection threshold was 10^2 cfu/ml

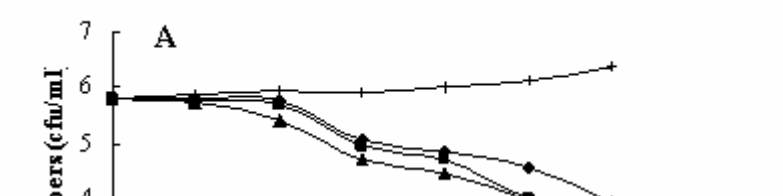


Figure 20. Time-kill curve of *Rhizopus* spp. (A), and *Aspergillus niger* (B) after treatment with crude extract from *Eleutherine americana*. The control suspensions (+), 2MIC (▲), MIC (■), and 1/2MIC (◆). The lower detection threshold was 10^2 cfu/ml.

4.12 Antibacterial activity of the partially-purified fractions obtained from crude extract of *Eleutherine americana*

Partially-purified fractions (Figure 2) of the crude extract were examined for their antibacterial activities against *Staphylococcus aureus* reference strain ATCC 25923 (Table 27). Fractions Ea6.3 produced MIC value of 0.125 mg/ml with MBC value of 1.0 mg/ml, fraction Ea9 yielded an MIC value of 0.25 mg/ml, and MBC value of 0.5 mg/ml, while fraction Ea6.3 produced the best antibacterial activity having MIC value of 0.125 mg/ml, and MBC value of 0.25 mg/ml. In addition, methicillin (oxacillin) resistance *S. aureus* isolated from food samples (Table 28) were subjected to antibacterial test using partially-purified fractions, Ea6.3 and Ea9.0 (Table 29). About 33% of the isolates from meat samples were methicillin-resistant, followed by cakes (26%), papaya salad and steamed fish cake (22.2%), and the least were found in pork soup and chicken sausage (11.1%).

Fraction Ea6.3 exhibited improved antibacterial activity producing MIC/MBC values of 0.25/0.5 mg/ml against the enterotoxin-producing reference strains, ATCC 23235 and ATCC 27664 (Table 29). The MIC value for the MRSA ranged from 0.125-0.5 mg/ml while the MBC was 0.25->1.00 mg/ml. Fraction Ea9.0 yielded MIC values 0.125-0.25 mg/ml and the MBC values are 0.5->1.00 mg/ml, while for ATCC 27664, MIC/MBC value was 0.125/1.00 mg/ml.

Table 27. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the partially-purified fractions from *Eleutherine americana* on *Staphylococcus aureus* (ATCC 25923)

Fractions	MIC/ MBC (mg/ml)
Ea3.3	0.5/ >1.0
Ea3.4	0.25/ >1.0
Ea3.6	0.125/ >1.0
Ea4S	>1/>1.0
Ea5.3	0.125/ 1.0
Ea6.3	0.125/ 0.25
Ea7S	0.5/ >1.0
Ea7.9	0.0625/ >1.0
Ea9	0.25/ 0.5
Ea9S	0.5/ >1.0
Ea10.5	0.0625/ >1.0
Ea11	0.5/ >1.0
Ea12	1.0/ >1.0

Table 28. Sources of methicillin-resistance *Staphylococcus aureus* (MRSA) isolates

Food type	Number of samples	Numbers of samples with MRSA (%)
Cakes	23	6 (26.0%)
Meat	30	10 (33%)
Pork soup	9	1(11.1%)
Papaya salad	7	2 (22.2%)
Steamed fish cake	10	2 (22.2%)
Chicken sausage	22	1 (11.1%)
Milk dessert	5	0
Total	106	22 (20.75%)

Table 29. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the partially-purified fractions from *Eleutherine americana* on Methicillin-resistance *Staphylococcus aureus* (MRSA) from foods

S. aureus	Partially-purified fractions MIC/MBC) mg/ml	
	Ea6.3	Ea9.0
MRSA (n = 22)	0.125-0.5/ 0.25->1.0	0.125-0.25/0.5->1.0
ATCC 23235[a]	0.25/ 0.5	0.125/ >1.0
ATCC 27664[a]	0.25/ 0.5	0.125/ 1.0

[a]Enterotoxin- producing strains

4.13 Kill curve in the presence of partially-purified fractions

The enterotoxin-producing reference strain treated with fraction Ea6.3 of the extract at 4MIC (1 mg/ml) and 2MIC (0.5 mg/ml), resulted in bactericidal activity at 20 and 24 h, respectively (Figure 21A). The MRSA isolates, NPRC 421 (Figure 21B) and NPRC 461 (Figure 21C) were totally eliminated both at 2MIC and 4MIC at 24 h. The antibacterial activity of fraction Ea9.0 is equally shown on Figure 22. The fraction produced lethal effect on the enterotoxin-producing reference strain at 4MIC at 24 h (Figure 22A), while for MRSA isolates (Figure 22B and 22C) the fraction reduced the inoculum by 7 log at 4 MIC concentration at 24 h relative to the control.

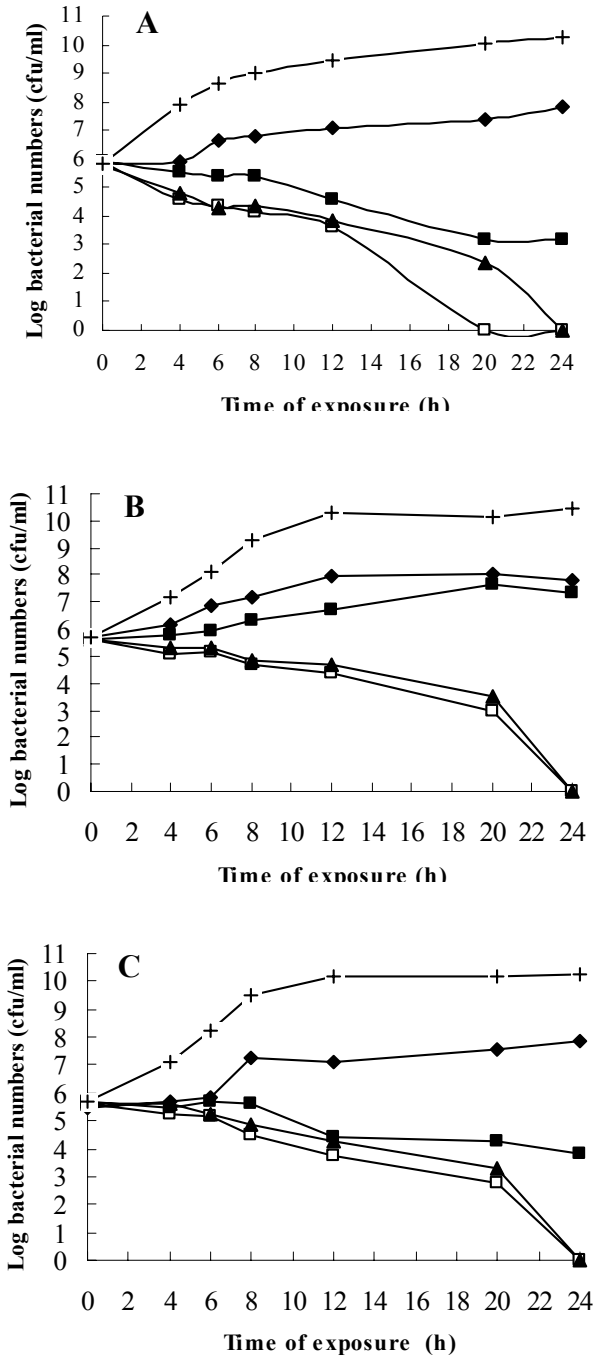


Figure 21. Time-kill curve of *Staphylococcus aureus* ATCC 27664 (A), NPRC 421 (B), and NPRC 461 (C) after treatment with the partially-purified fraction, Ea6.3, from *Eleutherine americana*. Control suspensions (+), 4MIC (□), 2MIC (▲), MIC (■) and 0.5MIC (♦). The lower detection threshold was 10^2 cfu/ml

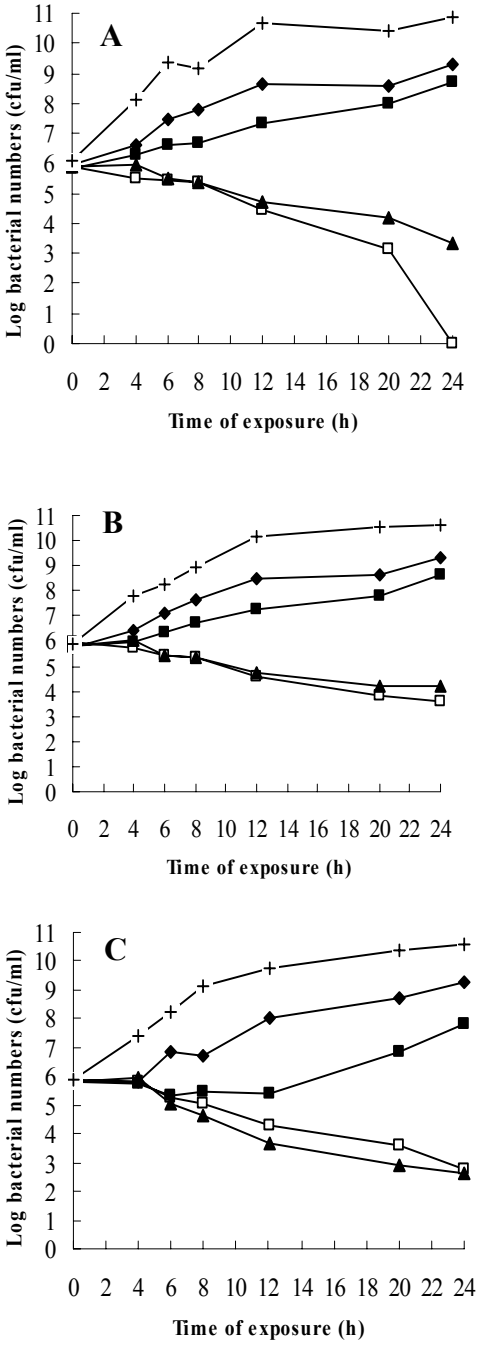


Figure 22. Time-kill curve of *Staphylococcus aureus* ATCC 27664 (A), NPRC 421 (B), and ATCC NPRC 461 (C) after treatment with the partially-purified fraction, Ea9.0, from *Eleutherine americana*. Control suspensions (+), 4MIC (□), 2MIC(▲), MIC (■) and 0.5MIC (♦). The lower detection threshold was 10^2 cfu/ml

Table 30. Minimal inhibitory concentration (MIC) of the pure compounds from *Eleutherine americana* on *Staphylococcus aureus*

	MIC (µg/ml)	
Compounds	ATCC 27664	ATCC 25923
Ea3.3	31.25	125
Ea3.4	125	250
Ea3.6	125	125
Ea4S	>1000	>1000
Ea7S	500	500
Ea9S	500	500
Ea7662	>1000	>1000

The MIC values of the pure compounds are presented in Table 30, while the names and structures of the compounds are shown on Table 31. The pure compounds were tested against two reference strains and the MIC values ranged from 0.125->1.0 mg/ml.

Table 31. Pure compounds identified from *Eleutherine americana*

Name of compound	Structure
Hongconin	(1*R*,3*R*)5,10-Dihydroxy-9-methoxy-1,3-dimethyl-1*H*-naphtho[2,3-*c*]pyran-4(3*H*)-one.
Anthraquinone	4,8-dihydroxy-3-methoxy-1-methylanthraquinone-2-carboxylic acid methyl ester.
Anthraquinone	8-hydroxy-3,4-dimethoxy-1-methylanthraquinone-2-carboxylic acid methyl ester
Eleutherol	4-hydroxy-5-methoxy-3-methylnaphtho[2,3-*c*]furan-1(3*H*)-one
Eleutherin	(1*R*,3*S*)3,4-dihydro-9-methoxy-1,3-dimethyl-1*H*-naphtho[2,3-*c*]pyran-5,10-dione.
Elecanacin	1,2,3a,4a-Tetrahydro-6-methoxy-4-methyl-10*H*naphtho[2,3 :2,3] cyclobuta [1,2-*b*]furan-5,10(3a*H*)-dione.
Isoeleutherin	(1*R*,3*R*)3,4-Dihydro-9-methoxy-1,3-dimethyl-1*H*naphtho[2,3 *c*]pyran-5,10-dione.
Erythrolaccin	1,3,6-trihydroxy-8-methylanthraquinone.
Eleuthinone A*	[8-methoxy-1,4-dioxo-3-(2-oxopropyl)-1,4-dihydronaphthalen-2-yl]acetic acid methyl ester.
Eleuthraquinone A*	2-acetyl-3-hydroxy-8-methoxy-1-methylanthraquinone.
Eleuthraquinone B*	3,6,8-trihydroxy-4-methoxy-1-methylanthraquinone-2-carboxylic acid methyl ester.
Eleucanarol*	3,4-dihydroxy-5-methoxy-3-methylnaphtho[2,3-c]furan-1(3H)-one.

Source: Hamtasin et al., 2007; Mahabusarakam *et al*., 2009.

*New compounds isolated by Hamtasin et al., 2007; Mahabusarakam *et al*., 2009.

CHAPTER 5

DISCUSSION

The scavenging activities of crude extract on DPPH and hydroxyl radicals were expressed as IC_{50} values (Table 5). DPPH is a stable free radical and has been used to evaluate free radical scavenging activity of natural antioxidant (Bondet *et al.,* 1997). It is a compound that possesses a proton free radical with a characteristic absorption, which decreases significantly on the exposure to proton radical scavengers (Yamaguchi *et al.,* 1998). It was observed that *E. americana* extract was able to reduce the DPPH radical from purple colour to yellow-coloured diphenylpicrylhydrazine. The reduction of alcoholic DPPH solution in the presence of hydrogen-donating antioxidant is due to the formation of the non-radical form, DPPH-H (Shon *et al.,* 2003). Hydroxyl radical is known to be capable of abstracting hydrogen atoms from memberane lipids and thus bring about peroxide reactions of lipids (Yen and Hsieh, 1997). The ability of *E. americana* crude extract to quench the hydroxyl radical seems to be related directly to prevention of propagation in the process of lipid oxidation. The scavenging activities demonstrated by the extract could be as a result of several naphthoquinones that have been reported to be present in the bulb (Zhengxiong *et al*., 1984; Hara *et al.,* 1997; Qui *et al*., 2005; Xu *et al*., 2006; Paramapojn *et al*., 2008; Hamtasin *et al*., 2007; Ifesan *et al*., 2009b; Mahabusarakam *et al*., 2009). The high free radical scavenging activity indicates that *E. americana* extract is rich in antioxidant phytochemicals such as phenolic as evident from the result of total phenolic content. Generally, natural antibacterials possessing the strongest antibacterial properties against pathogens have been reported to contain a high percentage of phenolic compounds such as carvacrol, eugenol and thymol (Dorman and Deans, 2000; Juliano *et al*., 2000; Lambert *et al*., 2001).

Staphylococcus aureus is present on the skin and mucosa of humans, animals and in the environment (Jay, 2000; Lee, 2003). As a consequence, food products may originally be contaminated during or after processing. *Staphylococcus aureus* has been isolated from several foods, such as, meat and meat products,

vegetables, fish products and fermented food products (Wieneke *et al*., 1993; Tamarapu *et al*., 2001; Aycicek *et al*., 2005; Normanno *et al*., 2005; Normanno *et al*., 2007). The result from this finding conform to other previous reports where high percentages of *S. aureus* were obtained from meat (Lee, 2003; Normanno *et al*., 2007). Dewaal and Bhuiya (2007) reported that the most common hazard bacteria in pork was *S. aureus*. In a study of food poisoning in England, the most prevalent contaminated foods (75%) were meat , ham, poultry, or their products (Wieneke *et al*., 1993). The milk dessert purchased during the period of study had no *S. aureus* contamination. This may be as a result of the pasteurization treatment given to the dessert before they were packaged in plastic containers.

Growth of *S. aureus* is encouraged in foods kept at slightly elevated temperatures. All the food samples bought except the sausage and milk samples were kept at room temperature (30ºC) which is likely to support the growth of any *S. aureus* that survived the cooking processes or recontamination from the body of the handler after processing (Jay, 2000; Bremer *et al*., 2004). The main reservoir for staphylococci is the human being, the hand and the mucous membranes of the nasopharynx are predominant sites of colonization (Soriano *et al*., 2002). Lack of personal hygiene among food handlers, poor hand and surface hygiene has also been reported as factors that contribute to foodborne illness (Cogan *et al*., 2002).

A microbiological examination of the nose, throat, hands and nail samples of food handlers from cafeterias of a Chilean University revealed that enterotoxigenic staphylococci was found in 41% of the male food handers, while 57% was obtained from the female food handlers (Soto *et al*., 1996). In addition, Lues and Tonder (2007) obtained 88 and 48% *S. aureus* from the hands and aprons of food handlers, respectively. Contrastly, analysis of specimens from food-producing animals, including milk and meat samples revealed that about 15 strains harboured *mecA* gene and concluded that contaminated foods of animal origin may be a source of MRSA infection for human (Lee, 2003). Animals and pests are recognized carriers of enterotoxigenic staphylococci, and the presence of animals, whether domestic (Fukuda *et al*., 1984), or wild (Valle *et al*., 1990), should not be permitted in foodservice establishments. It is possible that the sanitation or temperature control or both were inadequate and under this suitable condition *S. aureus* present in foods can

produce enterotoxin which when consumed would result to staphylococcal food poisoning. It is thus important that stringent hygiene and sanitary standards be maintained throughout the food chain at all times. This could suggest that natural preservatives such as spices and plant extracts should be used as additives in ready-to-eat foods to suppress or control the growth of foodborne pathogens in cases of temperature abuse.

The isolation medium used in this study was the Baird-Parker after which isolates were subjected to series of traditional biochemical tests for the identification of *S. aureus* (Schleifer, 1986). Baird-Parker agar (BPA) is the recommended medium for recovery of *S. aureus* from contaminated food sources (Vanderzant and Splittstoesser, 1992). The medium was developed from a tellurite-glycine formulation and subsequently improved reliability for isolation of coagulase-positive *S. aureus* from foods (Baird-Parker, 1962). Staphylococci that contain lecithinase break down the egg yolk and clear zones appear around the colonies (Difco, 1998) while presence of lipase is shown as an iridescent film in and immediately surrounding colonies, visible by reflected light (Matos *et al*., 1995). *Staphylococcus aureus* which failed to produce halo on Baird-Parker were subcultured on mannitol salt agar, tested for mannitol fermentation and finally tube coagulase test (Adesiyun *et al*., 1999; Sandel and McKillip, 2004). The use of anaerobic utilization of mannitol to distinguish *S. aureus* from other staphylococci has been recommended (STSM, 1965). Strains of *S. intermedius* do not utilize mannitol anaerobically (Hajek, 1976). The National Mastitis Council recommends that the acetoin test (Voges-Proskauer test) could be used as an additional means to differentiate *S. aureus* from coagulase-positive *S. hyicus* and *S. intermedius* that do not produce acetoin (Schleifer, 1986). Result from this research is in close agreement with results from other studies that demonstrated that greater than 90% of *S. aureus* produced acetoin from glucose (Harmon *et al*., 1991; Roberson *et al*., 1992). In this study, 94% of the isolates were positive for anaerobic fermentation of mannitol and this agrees with Roberson *et al*. (1992), and Hajek (1976). The importance of this study is that in laboratories where the technology for sophisticated techniques like zymotying, ribotying, plasmid typing are not available, combination of these phenotypical characteristics could be used (Adesiyun *et al*., 1999).

One of the key factors enabling *S. aureus* to survive, colonize, proliferate and cause human infections is the expression of virulence factors. All isolates produced at least two or more of the virulence factors tested (Table 8). The result showed that lipase enzyme was produced more than any other enzyme. This corresponds with Hammer *et al*. (2006) where 32 out of 44 strains tested were positive for lipase enzyme. Staphylococci possess lipolytic enzymes which render them resistant to the bactericidal lipids of human skin (Kusumaningrum *et al*., 2003). Furthermore, bacterial lipases have been reported and can survive pasteurization temperatures (McKellar and Cholette, 1986; Andrews *et al*., 1987), thus they are heat stable, which is the reason for them being a main source of spoilage in heated fat-containing food products (Braun *et al*., 2001).

S. aureus produces several extracellular proteases, including metallo-, serine and cysteine proteases (Dubin, 2002). The function of all proteases is to cleave proteins and thus may inactivate key proteins and antimicrobial peptides involved in host defenses. About 36.1% of the food isolate from this study synthesized protease (Table 8), and this may be an indication that the isolates could be virulent. The result showed that 55% demonstrated haemolysis on blood agar. This is lower than the result obtained from hospital isolates where 70% haemolysin was recorded (Udo and Jacob, 2000). The least synthesized enzyme was lecithinase (10.3%) and this also corresponds with report of Hammer *et al*. (2006), where 15% of the isolates produced lecithinase enzyme. The reason for lower lecithinase production in this study may be due to the fact that the *S. aureus* isolates are from foods. Human strains and strains biotyped as human biotypes had higher percentage of lecithinase enzyme than bovine isolates or biotypes (Matos *et al*., 1995).

A relatively high number of isolates tested in this study were resistant to one or two antibiotics commonly used in the therapeutic protocols of many human infections (Figure 3). Normanno *et al*. (2007) also reported that about 68.8% strains isolated from meat and dairy products showed antimicrobial resistance properties to all the antibiotics tested except vancomycin. This study revealed that MRSA could be isolated from food. Thus, multiresistance was demonstrated by the food isolates (Normanno *et al*., 2007; Pesavento *et al*., 2007). Extended use and misuse of antibiotics in animal husbandry may contribute to the increasing incidence of MRSA

strains (Valsangiacomo *et al*., 2000; Lee, 2003). This can foster antibiotic resistance in bacteria which are common in animals and that cause disease in humans (Copert, 1988; Feinman, 1998). Contaminated foods of animal origin may serve as a source of MRSA infection to humans (Lee, 2003; Kitai, 2005). The sources of MRSA in the food samples may be through the food handlers (Jones *et al*., 2002), which may require a better sanitary education focusing on their potential role as reservoirs of foodborne pathogen.

Plants contain a number of organic components including alkaloids, flavones, phenols, quinones, terpenoids, and tannins, all of which are known to possess antibacterial activity (Cowan, 1999). The results of the agar susceptibility test and MIC of the crude extract is within the range that has been reported by other researchers where medicinal plants have been shown to possess inhibitory activities against *S. aureus*. Crude extract from *Cinnamomum burmanii* produced MIC and MBC above 250 mg/ml against *S. aureus* (Shan *et al*., 2007). Khan *et al.,* (2001) found that methanolic extract of *Cassia alata* could inhibit the bacterium at a concentration of 4 mg/ml while both water and ethanolic extract of same plant inhibited *S. aureus* at about 10 mg/ml (Somchit *et al.,* 2003). The crude extract of *E. americana* used in this study can compete successfully with the previous plant extracts reported with MIC values of 0.06-1.00 mg/ml.

The effectiveness of an antimicrobial agent is measured by its ability to inhibit or kill bacteria. Time-kill curve is an example of bactericidal activity expressed as the rate of killing by a fixed concentration of an antimicrobial agent and its one of the most reliable method for determining tolerance (Nostro *et al*., 2001). Large numbers of isolates tested demonstrated that the extract kept the inoculum at lag phase and brought about at least 5-6 log cycle reduction at 4MIC after 24 h exposure. The data obtained from this study could be described as the antibacterial effect of the extract which depended on the exposure time and the bacterial cell concentration.

Subminimal inhibitory concentration (SubMIC) of *E. americana* demonstrated inhibition against protease and lipase enzyme secretion (Table 13). The ability of the plant extract to inhibit lipase and protease enzyme production could be as a result of interaction between the extract and the *S. aureus* cells. Anti-enzymatic

activity of the extract could be attributed to its bioactive compounds. Naphthoquinones are polyphenols known to form soluble complexes of high molecular weight with proteins, thereby, reacting with the cell enzymes in the cytoplasm and the cell wall. Suppression of exoprotein synthesis was observed with antibiotics that inhibit protein synthesis (Herbert *et al*., 2001). The effect of certain antibiotics on the expression of staphylococcal and streptococcal virulence factors in relation to the severity of infection has been studied (Gemmell *et al*., 1995; Herbert *et al*., 2001). Antimicrobials at subinhibitory concentrations have been shown to affect the host-bacterium relationship, especially with regard to inflammatory cell function (Ohlsen *et al*., 1997; Gemmell, 1984). The ability of subMIC of *Helichrysum italicum* extract on enzymatic activity of *S. aureus* was reported (Nostro *et al.*, 2001). Lipophilic character of some flavonoids present in plant extract has been suggested to be responsible since these compounds act on the cytoplasmic membrane. Lipophilic compounds in the cytoplasmic membrane of bacteria has effect on the structural and functional properties of the membrane which become increasingly permeable to protons and ions and lose their integrity (Sikkema *et al*., 1995).

Evaluation of enterotoxins (A-D) produced by *S. aureus* in the presence of *E. americana* extract revealed that the ability to produce toxin decreases as extract concentration increases (Tables 14-16). The ability of *E. americana* crude extract to inhibit staphylococcal enterotoxins could also be explained following its anti-enzymatic activity and the bioactive compounds present in the extract. Anti-enterotoxin activity of plant extracts or their oils are documented (Nostrol *et al*., 2002; Braga *et al*., 2005; Hammer *et al*., 2006). In addition, phytochemicals have been demonstrated to prevent the growth and protein secretion of *S. aureus* (Novick *et al*., 2001). Observations common to all the plant extracts reported were that the extract treatment caused decrease in number of viable cells and this was followed by reduced enterotoxin or inhibition of enterotoxin production.

The study of the enterotoxin production in the food system revealed that different time is required for the synthesis of enterotoxins A-D (Table 17). Enterotoxin A was produced at 6 h, followed by D at 8 h while both B and C were not noticed until 24 and 20 h respectively. Pereira *et al*. (1991) reported that some strains of *S. aureus* produce detectable amounts of enterotoxins after 24 h. Contrastly, in

cultures grown in enriched medium as well as in mushroom contaminated with *S. aureus* and incubated at 37°C, SEA was detected after 2 h (Rasooly and Rasooly, 1998). In this study we used cooked pork and incubated at 30°C, SEA was produced at 6 h. These findings may suggest that the type of medium or substrate used and the incubation temperature are determining factors of the time for staphylococcal enterotoxin synthesis.

The result obtained revealed that the extract could delay the enterotoxin production in the food compared to the control. Enterotoxin A production was observed after 6 h of incubation in the control sample, while there were no toxins in all the samples treated with the plant extract. However, at 8 h toxin A was found in the sample treated with 1.0 mg/ml (4MIC), while toxin productions were delayed at higher extract concentrations 2.0 and 4.0 mg/ml (8MIC and 16MIC) until 14 h. Enterotoxin B was detected in the control after 24 h and was not found in the samples treated with 1.0 mg/ml extract until 30 h. However, there was no SEB detected in samples treated with 8MIC and 16MIC at 48 h. The extract delayed toxin C production for 4 h at 4MIC and 8MIC repectively, while enterotoxin was not detected from sample treated with 16MIC at 48 h. The extract produced no effect against enterotoxin D production in the pork.

At high levels of fat or protein bacteria are protected from the action of natural antimicrobials (Aureli *et al.*, 1992). This may explain why higher concentrations of *E. americana* were needed in the pork sample which contained about 10% fat to delay toxin production compared to what obtained in the broth. Nevertheless, the extract could delay SEA-SEC production compared with the control when the pork was stored at an abused temperature. The ability of *E. americana* extract to delay toxin production in the pork could as well be related to its bioactive compounds.

Cell materials including nucleic acid, metabolites, purines, and pyrimidines which were released into the suspension as the result of leakage of the cytoplasmic membrane was determined by measuring the OD at 260 nm. The presence of these materials in a suspension indicates damage to the cell at the membrane level (Woo *et al.,* 2000). Figure 7 revealed that *E. americana* could have damaging effect on the staphylococcal cytoplasmic membrane while higher OD

values were obtained after 8 h compared to the control. After 8 hr most cell content can be found in the media at 4MIC both in Figure 7A and B, however, after 22 hr there was no cell content available any more in the medium. The reason for this may be that the content released from the cells had undergone reactions with the extract and they were not sensitive again to OD measurement. The main bioactive compounds in the extract is quinones and they are known to complex irreversibly with nucleophilic amino acids in proteins often leading to inactivation of the protein and loss of function (Stern *et al*., 1996). Our result is also in agreement with Oonmetta-aree *et al.* (2006), where the cell content observed at 260 nm decreased as the time increased. Antimicrobials that acted on the bacterial cytoplasmic membrane and induced loss of 260 nm absorbing material, including, lemon grass (Onawunmi and Ogunlana, 1987) tea tree oil (Carson *et al*., 2002), and galangal (Oonmetta-aree *et al.,* 2005) have been reported.

The ability of the *E. americana* extract to cause the release of cytoplasmic content and damages to the cell membrane may be attributed to its bioactive compounds. Considering the large number of different groups of chemical compounds present in plant extract or their 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 (Skandamis *et al*., 2001; Carson *et al*., 2002). We may infer that the mechanism of action of this extract would therefore be similar to other phenolics which are generally considered to be the disturbance of the cytoplasmic membrane, disruption of proton motive force, electron flow, active transport and coagulation of cell contents (Denyer and Hugo, 1991b; Sikkema *et al*., 1995; Davidson, 1997).

Denyer and Hugo (1991a) reported that although a certain amount of leakage from bacterial cells may be tolerated without loss of viability, extensive loss of cell contents or the exit of critical molecules and ions would lead to cell death. In addition, there are some evidences from studies with tea tree oil and *E. coli* that cell death may occur before lysis (Gustafson *et al*., 1998). It is possible that the treatment of cells with the extract might induce the release of membrane-bound cell wall autolytic enzymes, which may eventually result in lysis (Gilbert, 1984; Carson et al., 2002). In addition, the reduction of the OD value observed (Figure 8B) could have

been because of lysis due to weakening of the cell wall. We may explain our finding from this work following same pattern as the result from loss of cytoplasmic substances measured at 260 nm indicated that *E. americana* caused leakage of the cytoplasm without causing gross cell wall damage.

When bacteria are exposed to antibiotics at low concentrations, changes in bacterial morphology, ultrastructure, biochemistry, and multiplication rate have been observed (Charache, 1968; Klainer and Perkins, 1971; Lorian, 1978). The effect of antibiotics on the morphology and ultrastructure of bacteria can be classified according to the target where the alteration occurs. Changes in the structure of the cell wall of *S. aureus* was observed when treated with ß-Lactam antibiotics (Lorian, 1996). The cells were found to increase in size with thickened and multiple cross walls compared to the control. Observation of the cells of *S. aureus* under transmission electron microscope after treated with *E. americana* at 2MIC and 4MIC revealed an alteration in the outer membrane's integrity. The bacterial cells increased in size with thickened cell wall when compared to the control (Figure 9). Similarly, alteration of the outer membrane's integrity with damaged cell membranes and cell wall have been reported with oregano essential oil (Lambert *et al*., 2001), tea tree oil (Carson *et al*., 2002), galangal ethanolic extract (Oonmetta-aree *et al*., 2006).

The inhibitory effect of salt on microorganisms has been discussed in the literatures (Hajmeer, 2001; Hajmeer and Marsden, 2002; Jay, 2000), as it basically exerts a drying effect on both food and microorganisms. However, *S. aureus* has the ability to grow in the presence of high concentration of salt (Jay, 2000; Aycicek *et al*., 2005). Halotolerance in *S. aureus* is determined by an ability to accumulate osmoprotective molecules such as choline, glycine betaine, and L-proline (Graham and Wilkinson, 1992; Amin *et al*., 1995). This makes it possible for *S. aureus* to contaminate salt-rich foods and produce enterotoxin which may result to food poisoning. It was observed that growth of *S. aureus* in TSB+1% DMSO and TSB+7.5% w/v NaCl without extract (control) (Figures 10 and 11) were characterized by distinct absence of lag phase, and reached highest inoculum size of about 9 log. However, presence of extract at both MIC and 2MIC concentration produced an extended lag phase and kept the inoculum size at or below log6. It can be concluded that combined effect of the extract at all extract concentrations used and salt (Figure

12B), could be synergistic, while observation from (Figures 10D, 11B and 11D) are additives.

Similar result was reported by Carson *et al*. (2002) where treatment of *S. aureus* cells with tea tree oil and its components significantly reduced the ability of the survivors to form colony on media containing NaCl. In addition, Stapleton *et al*. (2006) demonstrated how epicatechin gallate was able to extend staphylococcal lag phase in the presence of NaCI. The minimal concentration of *E. americana* required to produce a reduced growth rate in the presence of 7.5% NaCI was 0.125 mg/ml which is at 1/2MIC, while concentration above MIC 0.5 mg/ml resulted in total elimination of the isolate within 24 h (Figure 10B). Combination of antibacterials having biochemically or physicochemically complementary mechanisms of action may lead to synergistic activity (Denyer and King, 1988; Lehmann, 1988; Pons *et al.,* 1992). This attribute may suggest that the extract could be used as a preservative in salt-containing foodstuffs where *S. aureus* is a major problem.

Cytotoxicity activity of *E. americana* crude extract could be due to the anthraquinones present in the extract. Xu *et al*. (2006) reported that a yellow needle structure was obtained as a pure compound and it is closely related to anthraquinones. This compound was found to inhibit the proliferation of human erythroleukemia cancer cell line. However, several active compounds from *E. americana* have been reported to display important biological activities. Eleutherol, eleutherin, and isoeleutherin showed both antifungal activity and enhancement of the blood flow of the coronary artery (Zhengxiong *et al.,* 1984). Eleutherin, another compound, was found to form a type of noncleavable complex with human topoisomerase II, and isoeleutherine showed inhibitory activity against human immunodeficiency virus (Hara *et al.,* 1997).

The effect of heat treatment, changes in pH, and incubation temperature on the extract activity in the broth system is shown in Figure 12. The original pH of the extract is 5.09, the antibacterial activity of extract appeared to be stable despite the change in pH. Generally, after heating, the antibacterial activity of the extract remained stable or was even slightly enhanced. In addition, the result shows that the extract demonstrated stability to heat treatments. The aromatic stability of naphthoquinones which are abundant in *E. americana* has been reported

(Brimble *et al*., 1999). The pH independence and thermal stability of the extract is due to the stability of some of its bioactive compounds. The thermal stability of *E. americana* would allow it to be a potential food additive in food processing where heat treatment is used.

The inhibitory activity of the extract is dependent on the relationship between temperature and growth rate constant of microorganisms. At high temperatures, growth rate increases with increasing temperatures, and as growth occurred the extract could diffuse into the cell component, interfere with the various enzymes and produce inhibitory activity. However, at low temperatures, two factors govern the point at which growth occurs. Reaction rates for the individual enzymes in the organism become much slower, and low temperatures reduce the fluidity of the cytoplasmic membrane, thus interfering with transport mechanisms (Mossel *et al*., 1995). Microbial cells respond to a decrease in temperature by inducing a set of cold shock proteins, and these proteins are thought to play a role in the protection of cell damage caused by temperature reductions (Lee, 2003). *Staphylococcus aureus* are known to produce these shock proteins (Cordwell *et al*., 2002), and as the temperature is lowered the plasma membrane undergoes a phase transition in which solute transport is limited. The minimum growth temperature of *S. aureus* was 5-10ºC (Hintlian and Hotchkiss, 1987) and if there was no growth the extract could not penetrate into the cells to cause inhibition. Conclusively, at the refrigeration temperature, the extract was able to maintain the bacterial at the lag phase and the inhibitory activity at 10ºC was slightly better than at 4ºC.

Raw and minimally processed salad vegetables are usually sold to the consumer in a ready-to-eat form and depend on refrigeration as the main means of preservation (European Commission, 2004). Prepared salads are considered safe to eat by consumers (Food Standards Agency, 2007), and its development has also been towards obtaining salad meals by adding a variety of ingredients such as cheese, salmon, crayfish and pasta to produce a balanced meal. However, between 1992-2006 general outbreaks of infectious intestinal diseases reported to Health Protection Agency revealed that 82 (4%) of these outbreaks were linked to prepared salads (Little and Gillespie, 2008). Cross-contamination, infected food handlers and inappropriate storage temperatures have been reported as contributory faults to

foodborne outbreaks. *Staphylococcus aureus* is one of the bacteria that have been implicated in salad contamination and the likely source is the food handler. Soriano *et al*. (2002) reported detection of staphylococci in 17.3% of Russian salad, and 18.9% of vegetable salad. Staphylococcal enterotoxins are heat resistant (ICMSF, 1996) and foods containing the enterotoxin usually appear and taste normal. Such high levels of *S. aureus* contamination raise concerns and needed an intervention which can possibly be offered by the salad dressing.

Salad dressings are characterized with low pH, reduced water activity, storage under refrigeration (Smittle, 1977; Radford and Board, 1993), and this has contributed to the microbial safety of these commodities. Many herbs and spices are known to possess antimicrobial activity (Gill *et al.,* 2002; Chun *et al*., 2004; Fisher and Phillips, 2006) and natural products are used increasingly as alternatives or as supplements to diet. The addition of essential oils from oregano to salad dressing (Davidson, 1997; Koutsoumanis *et al*., 1998), garlic, ginger mustard, and clove to mayonnaise (Leuschner and Zamparini, 2002) for safety has been reported. Such natural supplements meet the current demands of consumers thus minimize the use of chemical preservatives and improve the taste of the food.

Figure 13A showed the effect of *E. americana* extract on the salad dressing inoculated with *S. aureus* and stored at 4ºC. It was observed that there were reductions in the bacterial load of both the control and the treatments through out the storage period. All the extract concentrations had inhibitory effect on the bacteria producing about 3 log reductions from day 0 to day 21. The antibacterial activity of oregano in mayonnaise was described by Skandamis and Nychas (2000), where reductions of the inoculum size was also observed in the control sample, and this was attributed to the inhibitory action of lemon juice used to reduce the pH of the mayonnaise. The survival of many pathogens in acidic products such as salad dressings and mayonnaise depends on a variety of extrinsic factors such as temperature and intrinsic factors, for example, the acidulant used and the oil (Radford and Board, 1993; Erickson *et al*., 1995). Therefore, the use of natural additive such as *E. americana* can be considered as an additional hurdle for the safety, acting in combination with low pH, water activity and storage temperatures.

The antibacterial activity of the extract in the treatments stored at 10ºC is also shown in Figure 13B. At this temperature the extract activity was better than at 4ºC because no *S. aureus* were detected as from day 15 at both 0.5 and 1.0 mg/ml extract concentrations. A marked decrease in the bacterial population was observed in home made taramasalad (Koutsoumanis *et al*., 1999) and mayonnaise (Skandamis and Nychas, 2000) when stored at low pH and high temperatures of 10-22ºC, while lower temperatures, 4-5ºC, protected *Salmonella* spp. The result obtained from this study can follow the same explanation where survival of *S. aureus* was greater as the temperature decreased, probably due to the lower metabolic activity at decreased temperatures. In addition, the protective effect of low temperatures on survival of acidification may arise from alteration of the kinetics of protein denaturation (Brown and Booth, 1991). Inability of *S. aureus* to grow on PCA as from day 15 (Figure 14) may be an indication that the cells were injured but were able to recover on Baird-Parker which is an enriched media. There was no detection of coliforms, psychrophiles, yeasts and molds during the period of storage. The absence of coliforms signifies that all ingredients used for the salad dressing were free from feacal contamination.

The pH value of the salad dressings with or without extract is presented in Table 18. The pH values ranged from 5.89-4.14 from day 0 to day 21 with the control significantly lower than the treatments. Microbial inhibition in salad dressings and mayonnaise has been attributed to the synergistic effect of low pH and the antimicrobial properties of undissociated acetic acid (Delaquis *et al*., 1999). Weak acids are lipid soluble and equilibrate across the membrane by simple diffusion and at low concentration, certain acids, for example, acetic acid may also be actively transported by inducible permeases (Casal *et al*., 1996). The pH can interact with factors such as water activity, salt, temperature, redox potential and preservatives to inhibit growth of pathogens (Mossel *et al*., 1995). The pH values are within the range previously reported in home made taramasalad supplemented with oregano essential oil (Koutsoumanis *et al*., 1999) but higher than the value obtained when synthesized sucrose and methylglucose fatty acid monoesters were used as additives in salad dressing (Yang *et al*., 2003).

Lipid oxidation of the salad dressings were monitored every 3 day during the period of study. For salad dressing without sugar (Table 19), thiobarbituric acid reactive substances of the control and treatments were not significantly different at day 0, but as from the 3rd day of storage the values were increasing with storage days, while the extract demonstrated antioxidant activity. Samples with sugar produced higher TBARs ranging from 14.36-9.50 compared to the samples without sugar. This is due to the interference of sugar with the colourings (Shlafer and Shepard, 1984) and thus resulted in high TBARs values. In lipid oxidation, free radicals abstract hydrogen from a fatty acid double bond to produce fatty acid free radicals, which react with oxygen to produce fatty acid hydroperoxide. This is unstable and decomposes rapidly to shorter chain hydrocarbons such as malonaldehyde and the final products can be determined as TBARs (Benjakul *et al*., 2005). Polyphenolic compounds are primarily responsible for the antioxidant activity of natural extract (Cuppett, 2001), thus in the presence of *E. americana* extract used in this study, the propagation step of lipid peroxidation might be inhibited, resulting in lower oxidation in the samples with extract.

The addition of extract at all concentrations significantly increased the lightness (L*) values of the salad dressing. The L* value of the control was maintained until the 21st day whereas those of the treated samples varied throughout storage. The redness (a*) values of the samples decreased with the addition of the extract compared to the control and were significantly different at each extract concentration (Table 20). In addition, significant differences were observed in the yellowness (b*) values of the salad dressing compared to control and this was stable throughout the storage period. The differences observed in the colour of the treatments and the control is due to the colour of the extract, and the colour was well accepted by the consumer as observed from the sensory tests.

The results of sensory test revealed that there were no significant differences for texture, taste, and overall acceptability in the treatments. The appearance and colour of the control sample were scored higher than the treatments with extract, though the samples with extract were also scored above average. It is possible that the panelists are more familiar with the salad dressing without the extract since the addition of extract reduced the yellowness normally expected from the egg

yolk. The mean scores for the quality attributes of the salad dressing on the 8th storage day was an improvement over the 0 day (Table 21). There were no significant differences in the scores of all the attributes both for the control and the samples with extract. Furthermore, the scores were higher than the scores on 0 day for all the treatments which showed that the samples were more preferred at day 8. This may be explained that as the storage days increased, there were chemical reactions taking place in the salad dressing due to the presence of the vinegar and the extract. Some of the panelists also commented that there were reductions in the egg flavour in the treated samples compared to the control sample. This may be an additional advantage of using the extract as an additive as it was able to reduce the egg flavour in the product. The sensory scores on day 16 revealed that addition of the extract did not have any negative effect on the consumer acceptability as there was no significant difference between the control and samples with extract. The result of the sensory test indicated that the use of *E. americana* extract as additive in the home made salad dressing is acceptable during all the occasions of the sensory analysis.

The growing interest in convenience foods is making ready to eat meat products to become more desirable and well known. Lipid oxidation is a process by which quality loss of muscle foods occur causing chemical spoilage in food systems. Precooked meat especially pork is susceptible to lipid oxidation compared to those of beef and sheep (Kanner, 1994; Channon and Trout, 2002; Jayathilakan *et al*., 2007) because of its relatively high content of unsaturated fatty acids (Enser *et al.*, 1996). In addition, the warmed over flavour develops in few hours when the cooked meat is stored at 4°C (Tims and Watts, 1958), together with microbial contamination (Delaquis *et al*., 1999).

The use of chemicals to enhance food safety is of great interest to the food industry, however, consumers have grown concerns about the health risks associated with the use of these chemicals. It has been demonstrated that butylated hdroxytoluene may cause internal and external haemorrhaging at high dose which could cause death in some strains of mice and guinea pig (Shahidi and Wanasundara, 1992). The use of natural preservatives to improve the shelf life of meat products is a promising technology as many herbs, spices and their essential oils have demonstrated antioxidant (Rey *et al*., 2005; Banon *et al*., 2007; Carpenter *et al*., 2007; Jayathilakan

et al., 2007; Juntachote *et al*., 2007) and antimicrobial properties (Ahn *et al.,* 2007; Solomakos *et al*., 2008; Gutierrez *et al.*, 2008).

The effect of the extract on the pH and TBARs of cooked pork stored at 4°C is presented in Table 22. Generally, there was decrease in the pH values of all the samples from 0 to 9 day. During the storage for 3 and 6 days, there was no significant difference in the pH of the treated samples and control, until day 9 when the pH of the samples treated with 10.8 mg and 5.4 mg of the extract were significantly higher than the others. The pH of cooked pork samples in this study ranging from 5.92-6.07 is within the range reported in the literatures where plant extract were used to treat cooked pork (McCarthy *et al*., 2001; Ahn *et al.*, 2007; Carpenter *et al*., 2007). In addition, a decline in pH of pork patties from 5.7 to 5.5 over a 12 day storage period after treatment with grape seed extract and bearberry whose pH values were 3.84 and 4.71 and it was concluded that the pH of the pork was unaffected by the addition of the extracts (Carpenter *et al*., 2007).

At day 0, the control was significantly higher than the treatments and reached the standard limit by day 3 (Wong *et al*., 2001). As the storage days increased the lipid oxidation in the samples increased. Phospholipids are the primary substrates of lipid oxidation and the membrane phospholipids which are high in polyunsaturated fatty acids are responsible for the initial development of oxidation in cooked meat products during storage (Gray and Pearson, 1987). The ability of the extract to act as an antioxidant is attributed to several naphthoquinones which possess OH groups that are likely responsible for its ability to retard lipid oxidation by scavenging free radicals as evidenced by the DPPH and hydroxyl radical activities. A direct relationship has been found between the phenolic content and antioxidant capacity of plants (Al-Mamary *et al*., 2002). Natural polyphenolics demonstrated their beneficial health effects by their antioxidant activity. These compounds are able to remove free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce α-tocopherol radicals, and inhibit oxidases (Amic *et al*., 2003).

Treatment of the pork samples with the crude extract from *E. americana* resulted in colour changes that are significantly different from the control (Table 23). The addition of the extract increased the redness (a^*) value of the meat significantly as the extract concentration increases. Colour is an important visual cue

involved in consumer perception of acceptable meat quality (Faustman and Cassens, 1990) and attractive food colour could result to increased consumption. Relationship between colour stability with lipid oxidation and natural pigment is established (Jakobson and Bertelsen, 2000; Maccini and Hunt, 2005). The retention of the red colour of cooked pork treated with *E. americana* may result from its antioxidative effect and its contribution to pigment. Similar observations were made by Rey *et al*. (2005), and Carpenter *et al*. (2007) who reported the ability of beetroot extract and grape seed extract to reduce lipid oxidation and increase meat colour in cooked pork patties. In addition to antioxidant activity, *E. americana* extract could act as a potential natural colour enhancer of red meat and meat products.

The result obtained from the shear force values revealed that the treatments were not significantly different from the control sample. It may be concluded that the extract produced no tenderizing effect on the meat sample.

It was observed that the inhibitory activity of the extract in the pork was lower compared to what was obtained in the broth system (Figure 16). However, the extract in combination with the refrigeration temperature was able to prevent the growth of bacteria during the storage period. Antimicrobial activities in the food system are influenced by many factors, such as, the food components, pH, and storage temperature. Aureli *et al.* (1992) reported that high levels of fat and or protein in the food could serve as shield for bacteria against the action of natural antibacterials. More nutrients are available in foods compared to laboratory media and this may enable bacteria to repair damaged cells faster (Gill *et al.,* 2002). In addition, lower water content of food compared to laboratory media may hamper the progress of antibacterial agents to the target site in the bacterial cell (Smith-Palmer *et al*., 2001).

There were no significant differences in the sensory scores until day 9 when all the attributes in the control sample, including appearance, colour, texture, taste, and overall acceptability were significantly lower than the treatments. The possible reason for this may be due to high lipid oxidation value obtained on the last day of the experiment. Precooked meat products are susceptible to lipid oxidation, which resulted in rapid formation of rancid flavour known as warmed-over flavour during refrigerated storage (Tims and Watts, 1958; Love, 1988). It was observed that the increase in the red colour of the meat due to the effect of the extract was well

accepted (Table 24). From the sensory scores we can conclude that addition of *E. americana* at all concentration used did not adversely affect the sensorial properties of cooked pork in this study.

We may infer from this screening result that *E. americana* extract demonstrated a broad spectrum activity. The highest zone of inhibition of 20 mm was observed with *Streptococcus* spp, followed by *Bacillus licheniformis*, and MRSA with 19 mm, and the least was *Bacillus cereus* producing 13 mm diameter (Table 25). The MIC values ranged from 0.125 -1.0 mg/ml, with the highest value from *A. niger* (Table 26). Time–kill studies carried out against *B. cereus, Erwinia* spp, *A. niger*, and *Rhizopus* spp revealed that the crude extract exhibited both bacteriostatic and fungistatic activities (Figures 19 and 20). Antibacterial activity demonstrated by the extract showed that it was able to prolong the lag phase at all extract treatments and caused 5 log reduction in growth at 4MIC when compared to the control. The fungistatic activity of the extract showed that the extract at 4MIC brought about reductions of 3 log at 4 mg/ml and 1 mg/ml against *A. niger*, and *Rhizopus* spp, respectively. Daferera *et al*. (2000) reported that fungitoxic activity of thyme, oregano and dictamus oils may have been due to formation of hydrogen bonds between the hydroxyl group of oil phenolics and active sites of target enzymes. We reported the scavenging property of this extract against DPPH and hydroxyl free radicals and its high percentage of total phenol (Ifesan *et al*., 2009d). Antifungal activity of *E. americana* could be attributed to several bioactive compounds present in the crude extract. These compounds belonging to either naphthoquinones, naphthols or anthraquinones demonstrated antifungal and anticancer activity (Xu *et al*., 2006). Similarly, eleutherinone obtained from *E. bulbosa* was reported to possess fungitoxic activity (Alves *et al*., 2003).

Partially-purified fractions obtained from crude extract of *E. americana* were examined for their antibacterial activities against *S. aureus* reference strain ATCC 25923 (Table 27). Only the reference strain was used first for the screening of the activities of the fractions due to the quantities available. The MIC values obtained from all the fractions were lower than or same as that of the crude with the exception of fractions Ea4S and Ea12. Methicillin-resistance isolates from the food samples were subjected to antibacterial test with the partially-purified

fractions which produced better inhibitory activities (Ea6.3 and Ea9.0). The increasing occurrence of MRSA has made therapy more difficult (CDC, 1997), and with the rise in bacterial resistance to antibiotics there is a considerable interest in the development of other classes of antimicrobials for the control of infection. The presence of MRSA in foods may demonstrate its spread into the community. This may result to increasing impact of health care-associated organisms in settings and among populations previously considered to be unaffected by this antimicrobial resistant pathogen. The ability of crude extract and partially-purified fractions from *E. americana* to inhibit MRSA is an indication that it can serve as an alternate therapy.

Staphylococcus aureus becomes methicillin resistant by the acquisition of the *mecA* gene, which encodes a penicillin binding protein (PBP2a) with a low affinity for β-lactams (Chambers, 1997). PBPs are membrane bound *DD*-peptidases that evolved from serine proteases, and their biochemical activity is similar to that of the serine proteases (Waxman and Strominger, 1983; Ghuysen, 1994). It is possible that the bioactive compounds present in the extract inhibited the synthesis of PBP2a. In our study *E. americana* extract demonstrated anti-protease enzyme activity (Ifesan and Voravuthikunchai, 2009). In addition, quinones can bind to bacterial adhesins and complex with cell wall, thus inactivating enzymes (Cowan, 1999). Therefore, the inhibitory activity demonstrated by crude extract from *E. americana* could be explained by the bioactive compounds that have been isolated from the extract.

It is expected that the semi-purified fractions of *E. americana* should contain bioactive compounds than the crude extract as observed from the results of the time-kill curve in the presence of fractions (Figures 21 and 22). The improved antibacterial activity of the partially-purified fractions of *E. americana* may be attributed to the naphthoquinones, anthraquinones and naphthalene derivatives (Hamtasin *et al*., 2007; Mahabusarakam *et al*., 2009). In addition, bioactive compounds from bulbs of *E. americana* belonged to either naphthoquinones, such as elecanacin, eleutherine, and isoeleutherine (Hara *et al*., 1997), naphthols or anthraquinones (Zhengxiong *et al*., 1984; Xu *et al*., 2006), eleutherinoside A, and eleuthoside B (Paramapojn *et al.,* 2008). Medicinal plants rich in naphthoquinones have been reported to possess antibacterial activity (Ambrogi *et al*., 1970; Machado *et*

al., 2003). Naphthoquinone derivatives can act as antibacterial agents by participating competitively in electron transport with the cell components (Holmes *et al.,* 1964). Quinones, especially benzoquinone and naphthoquinone derivatives have been reported to possess biologically important compounds, such as vitamin K group, and the Q coenzyme. Other derivatives of quinones were reported to exhibit antimicrobial activities against bacteria and fungi (Akiya, 1956; Holmes *et al.*, 1964). In addition to providing a source of stable free radicals, quinones are known to complex irreversibly with nucleophilic amino acids in proteins often leading to inactivation of the protein and loss of function (Stern *et al*., 1996). Phytochemicals possess active compounds that could compete with synthetic chemical substances and they also have an advantage over antibiotics in being able to inhibit antibiotic resistant strains.

CONCLUSIONS

This study was carried out with relatively high numbers of isolates (106) and from different food sources demonstrated that the ethanolic extract of *E. americana* produced inhibitory effect on *S. aureus* isolated from food. At the beginning of this study we were not expecting such a high rate of contamination of the foods. This could be an indication that the food handlers needed to be given better education in sanitary practices focusing on their potential roles as reservoir of foodborne pathogen. The ability of *E. americana* crude extract to inhibit MRSA, inhibit or delay some virulence factors by *S. aureus* could present it as a novel antibacterial agent. The increase in demand for natural products in foods led to efforts in finding alternatives or minimize the use of synthetic chemical preservatives.

Eleutherine americana extract possessed high phenolic content and demonstrated good scavenging properties *in vitro* and in food systems. The extract was found to possess radical scavenging attributes which enabled it to delay lipid oxidation in salad dressing and pork samples. Results obtained in this study indicated that crude extract from *E. americana* could be used as an additive in salad dressing to complement vinegar which is used as a preservative in the product. Addition of this natural product in lower concentration reduced lipid oxidation, as well as improved the taste, colour, and acceptability of the product. In addition, the crude extract could be a promising novel antioxidant to prevent lipid oxidation and a potential natural colour enhancer of red meat and meat products. The response of the panellist as revealed by the sensory tests indicated that the extract was well accepted as an additive both in salad dressing and pork. However, the antibacterial effect observed in pork was mild and combination with refrigeration storage would be needed to obtain a safe product. Furthermore, future studies could include study on the application of the partially-purified fractions to food considering the toxicity of the crude extract to vero cells.

It may be concluded that phenolics not only attack cell wall and cell membrane thereby affecting their permeability and release of intracellular constituents but they also interfere with membrane functions including electron transport, nutrient uptake, protein, nucleic acid synthesis, and enzyme activity. Thus phenolic compounds could have several invasive targets that can lead to the inhibition of bacteria.

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Appendix 1. Sources and biochemical tests of food isolates

Isolates	Catalase reaction	Mannitol salt agar	Mannitol fermentation	Production of acetoin	Coagulase reaction	Food
NPRU 401	+	+	+	-	+	eclairs
NPRU 402	+	+	+	+	+	eclairs
NPRU 403	+	+	+	+	+	milk cake
NPRU 404	+	+	+	+	+	banana cake
NPRU 405	+	+	+	+	-	fried pork
NPRU 406	+	+	+	+	-	fried chicken
NPRU 407	+	+	+	+	+	fried chicken
NPRU 408	+	+	+	+	+	pork soup
NPRU 409	+	+	+	+	+	papaya salad
NPRU 410	+	+	+	+	+	fried pork
NPRU 411	+	+	+	+	+	dessert
NPRU 412	+	+	+	+	+	fried pork
NPRU 413	+	+	+	+	+	fried pork
NPRU 414	+	+	+	+	+	fried pork
NPRU 415	+	+	+	+	-	eclairs
NPRU 416	+	+	-	+	+	eclairs
NPRU 417	+	+	+	+	+	milk cake
NPRU 418	+	+	+	+	+	milk cake
NPRU 419	+	+	+	+	+	fried pork
NPRU 420	+	+	+	+	+	fried pork
NPRU 421	+	+	+	+	+	fried pork
NPRU 422	+	+	+	+	+	pork soup
NPRU 423	+	+	+	+	+	pork soup
NPRU 424	+	+	+	+	+	papaya salad
NPRU 425	+	+	+	+	+	papaya salad
NPRU 426	+	+	+	+	+	boiled chicken

Isolates	Catalase reaction	Mannitol salt agar	Mannitol fermentation	Production of acetoin	Coagulase reaction	Food
NPRU 427	+	+	+	+	+	pork soup
NPRU 428	+	+	+	+	-	boiled chicken
NPRU 429	+	+	+	+	+	boiled chicken
NPRU 430	+	+	+	+	+	boiled chicken
NPRU 431	+	+	+	+	+	boiled chicken
NPRU 432	+	+	+	+	+	boiled chicken
NPRU 433	+	+	+	+	+	boiled chicken
NPRU 434	+	+	+	+	-	fried pork
NPRU 435	+	+	+	+	+	fried pork
NPRU 436	+	+	+	+	+	chicken sausage
NPRU 437	+	+	+	-	+	milk cake
NPRU 438	+	+	+	+	+	milk cake
NPRU 439	+	+	+	+	-	boiled chicken
NPRU 440	+	+	+	+	-	boiled chicken
NPRU 441	+	+	+	+	+	eclairs
NPRU 442	+	+	+	+	+	papaya salad
NPRU 443	+	+	+	+	+	eclairs
NPRU 444	+	+	-	+	+	pork soup
NPRU 445	+	+	+	+	+	pork soup
NPRU 446	+	+	+	+	+	pork soup

Isolates	Catalase reaction	Mannitol salt agar	Mannitol fermentation	Production of acetoin	Coagulase reaction	Food
NPRU 447	+	+	+	+	+	banana cake
NPRU 448	+	+	-	+	+	papaya salad
NPRU 449	+	+	+	+	+	chicken sausage
NPRU 450	+	+	+	+	+	eclairs
NPRU 451	+	+	+	+	+	eclairs
NPRU 452	+	+	+	+	+	eclairs
NPRU 453	+	+	+	+	+	eclairs
NPRU 454	+	+	+	+	+	papaya salad
NPRU 455	+	+	+	+	+	boiled chicken
NPRU 456	+	+	+	+	+	boiled chicken
NPRU 457	+	+	+	+	+	pork soup
NPRU 458	+	+	+	+	+	fried chicken
NPRU 459	+	+	+	+	+	fried chicken
NPRU 460	+	+	+	+	+	boiled chicken
NPRU 461	+	+	+	+	+	boiled chicken
NPRU 462	+	+	+	+	-	papaya salad
NPRU 463	+	+	+	+	+	papaya salad
NPRU 464	+	+	+	+	-	papaya salad
NPRU 465	+	+	+	+	+	boiled chicken
NPRU 466	+	+	+	+	+	boiled chicken
NPRU 467	+	+	+	+	+	fried chicken
NPRU 468	+	+	+	-	+	fried chicken

Isolates	Catalase reaction	Mannitol salt agar	Mannitol fermentation	Production of acetoin	Coagulase reaction	Food
NPRU 469	+	+	+	+	+	papaya salad
NPRU 470	+	+	+	+	+	fish cake
NPRU 471	+	+	+	+	-	eclairs
NPRU 472	+	+	+	+	+	eclairs
NPRU 473	+	+	+	+	+	eclairs
NPRU 474	+	+	+	+	+	eclairs
NPRU 475	+	+	+	+	-	fried chicken
NPRU 476	+	+	+	+	+	boiled chicken
NPRU 477	+	+	+	+	+	boiled chicken
NPRU 478	+	+	+	+	+	chicken sausage
NPRU 479	+	+	+	+	+	banana cake
NPRU 480	+	+	+	+	+	banana cake
NPRU 481	+	+	+	+	+	banana cake
NPRU 482	+	+	+	+	+	banana cake
NPRU 483	+	+	+	+	-	boiled chicken
NPRU 484	+	+	+	+	+	chicken sausage
NPRU 485	+	+	+	+	+	chicken sausage
NPRU 486	+	+	+	+	+	papaya salad
NPRU 487	+	+	+	+	+	boiled chicken
NPRU 488	+	+	+	+	+	boiled chicken
NPRU 489	+	+	+	+	+	fish cake

Isolates	Catalase reaction	Mannitol salt agar	Mannitol fermentation	Production of acetoin	Coagulase reaction	Food
NPRU 490	+	+	+	+	+	boiled chicken
NPRU 491	+	+	+	+	+	pork soup
NPRU 492	+	+	+	+	+	pork soup
NPRU 493	+	+	+	+	-	chicken sausage
NPRU 494	+	+	+	+	+	chicken sausage
NPRU 495	+	+	+	+	+	milk cake
NPRU 496	+	+	+	+	+	milk cake
NPRU 497	+	+	+	+	+	fried pork
NPRU 498	+	+	+	+	+	chicken sausage
NPRU 499	+	+	+	+	+	chicken sausage
NPRU 500	+	+	+	+	+	fish cake

Appendix 2. Mean inhibition zone (mm) of extracts (2.5 mg/disc) of *Eleutherine americana* on *S. aureus* isolates from food samples

Isolates	Ethanol+Hexane	Hexane	Acetone	Ethanol
NPRC 401	15.87	16.37	15.75	16.37
NPRC 402	15.12	17.25	18.00	16.37
NPRC 403	12.87	13.12	14.50	16.00
NPRC 404	13.75	12.12	15.62	16.12
NPRC 405	16.00	16.12	16.50	17.37
NPRC 406	15.75	18.50	20.00	20.12
NPRC 407	12.50	13.25	14.87	15.62
NPRC 408	15.37	14.12	15.75	16.62
NPRC 409	14.87	15.87	16.75	18.62
NPRC 410	13.12	13.12	14.62	13.87
NPRC 411	12.37	13.00	14.00	12.87
NPRC 412	15.12	14.50	17.37	16.25
NPRC 413	12.00	12.75	14.50	13.12
NPRC 414	13.50	14.37	14.37	14.12
NPRC 415	12.87	13.37	14.37	13.62
NPRC 416	12.87	13.25	14.12	13.62
NPRC 417	13.62	13.87	14.87	15.00
NPRC 418	12.87	13.75	15.00	13.62
NPRC 419	13.25	13.37	14.50	13.12
NPRC 420	12.50	13.37	13.37	13.37
NPRC 421	12.50	15.12	14.87	14.12
NPRC 422	18.12	17.50	19.75	18.87
NPRC 423	13.87	14.37	14.37	13.87
NPRC 424	12.37	13.37	13.75	13.00
NPRC 425	14.50	14.37	16.37	15.50
NPRC 426	13.13	14.12	14.62	14.00

Isolates	Ethanol+Hexane	Hexane	Acetone	Ethanol
NPRC 427	14.12	12.50	16.62	13.62
NPRC 428	12.62	13.25	14.37	13.87
NPRC 429	13.50	14.50	15.62	14.25
NPRC 430	12.25	13.25	13.87	12.75
NPRC 431	11.75	12.12	13.00	12.87
NPRC 432	13.62	13.75	14.50	15.75
NPRC 433	19.00	18.87	19.50	19.50
NPRC 434	17.25	17.75	19.00	17.50
NPRC 435	17.87	17.62	18.75	18.87
NPRC 436	17.12	16.62	17.75	16.37
NPRC 437	16.87	17.25	17.37	17.00
NPRC 438	12.62	12.00	13.50	13.50
NPRC 439	12.75	12.50	13.25	12.87
NPRC 440	12.25	13.00	14.25	12.87
NPRC 441	14.37	14.12	15.50	15.75
NPRC 442	13.62	13.75	14.25	13.75
NPRC 443	12.62	12.87	12.50	12.00
NPRC 444	12.00	12.37	12.62	12.25
NPRC 445	12.75	13.12	15.25	14.37
NPRC 446	15.87	13.87	16.75	15.00
NPRC 447	12.87	12.37	14.50	14.25
NPRC 448	13.25	12.37	14.25	14.12
NPRC 449	15.12	13.50	17.00	16.00
NPRC 450	13.87	12.50	15.50	13.87
NPRC 451	15.62	16.50	17.75	18.25
NPRC 452	17.12	15.12	16.25	17.25
NPRC 453	17.50	16.37	17.50	17.25
NPRC 454	16.50	14.75	17.50	15.87
NPRC 455	16.50	15.25	16.75	16.50
NPRC 456	12.50	12.12	13.87	12.12

Isolates	Ethanol+Hexane	Hexane	Acetone	Ethanol
NPRC 457	14.87	14.00	15.25	18.50
NPRC 458	15.50	13.62	15.25	15.37
NPRC 459	16.50	14.50	16.50	15.25
NPRC 460	16.37	16.37	16.62	15.62
NPRC 461	15.87	16.00	17.25	18.00
NPRC 462	17.12	14.75	16.87	18.00
NPRC 463	12.75	12.37	12.75	12.00
NPRC 464	16.25	15.37	18.00	17.50
NPRC 465	12.25	12.37	13.75	12.87
NPRC 466	15.00	15.62	16.50	16.12
NPRC 467	15.25	15.75	18.25	17.25
NPRC 468	15.75	16.50	16.87	15.50
NPRC 469	15.62	15.00	16.37	15.25
NPRC 470	12.50	12.00	13.00	12.75
NPRC 471	15.25	16.00	16.25	16.87
NPRC 472	17.00	15.75	17.25	17.25
NPRC 473	16.25	15.75	15.75	15.75
NPRC 474	15.75	15.62	17.12	15.87
NPRC 475	15.50	14.75	15.37	14.37
NPRC 476	19.62	19.75	19.87	19.75
NPRC 477	14.75	16.87	16.00	15.87
NPRC 478	14.12	13.87	15.00	14.62
NPRC 479	15.25	15.87	15.50	15.00
NPRC 480	13.75	14.25	15.62	14.50
NPRC 481	16.50	15.50	16.62	16.25
NPRC 482	15.25	16.25	18.12	17.12
NPRC 483	14.37	15.00	16.62	15.75
NPRC 484	14.25	13.37	16.25	15.25
NPRC 485	15.62	15.87	17.50	15.87
NPRC 486	11.00	12.37	12.25	12.25

Isolates	Ethanol+Hexane	Hexane	Acetone	Ethanol
NPRC 487	13.00	14.00	14.50	15.12
NPRC 488	13.62	14.12	14.12	15.62
NPRC 489	14.37	14.87	16.25	15.75
NPRC 490	15.75	16.25	17.12	16.62
NPRC 491	15.12	16.12	17.50	16.42
NPRC 492	16.25	16.12	18.62	17.50
NPRC 493	15.50	15.25	16.50	15.62
NPRC 494	11.50	11.125	12.37	12.25
NPRC 495	13.12	12.37	15.00	13.75
NPRC 496	15.25	15.12	17.25	16.62
NPRC 497	18.00	16.75	18.50	17.50
NPRC 498	19.87	18.12	18.62	19.25
NPRC 499	12.37	12.87	13.87	13.62
NPRC 500	13.12	13.00	14.25	14.87
NPRC 501	13.12	13.12	14.87	14.12
NPRC 502	16.25	16.00	17.00	16.50
NPRC 503	15.00	12.62	14.37	15.50
NPRC 504	15.50	13.75	14.62	14.50
NPRC 505	14.50	13.12	14.12	14.12
NPRC 506	14.50	13.00	14.37	15.50

Appendix 3. Enzyme activities of *Staphylococcus aureus* isolates (cm)

Isolates	Haemolysin	Lecithinase	Lipase	Protease
NPRC 401	1.43	-	1.39	-
NPRC 402	-	-	1.05	-
NPRC 403	1.58	-	-	1.14
NPRC 404	-	-	-	1.90
NPRC 405	1.39	-	1.75	-
NPRC 406	-	-	1.29	-
NPRC 407	-	-	-	-
NPRC 408	-	-	-	-
NPRC 409	1.59	-	1.45	-
NPRC 410	1.39	1.25	-	-
NPRC 411	1.65	1.52	1.48	-
NPRC 412	1.32	1.36	1.56	-
NPRC 413	1.32	-	1.73	-
NPRC 414	1.47	-	1.32	-
NPRC 415	1.37	-	-	-
NPRC 416	1.52	-	1.88	-
NPRC 417	-	-	1.36	-
NPRC 418	-	1.43	1.48	-
NPRC 419	-	-	-	-
NPRC 420	1.42	-	1.07	1.48
NPRC 421	1.34	-	1.24	-
NPRC 422	1.17	-	1.82	-
NPRC 423	1.56	-	1.50	-
NPRC 424	-	-	1.13	1.56
NPRC 425	-	-	-	1.42

Appendix 3 (Continued)

Isolates	Haemolysin	Lecithinase	Lipase	Protease
NPRC 426	-	1.62	1.13	-
NPRC 427	-	-	1.38	-
NPRC 428	-	-	1.32	-
NPRC 429	1.24	1.38	1.15	1.61
NPRC 430	1.67	-	1.20	1.55
NPRC 431	1.54	-	-	-
NPRC 432	1.33	-	1.52	1.69
NPRC 433	1.39	-	1.49	1.49
NPRC 434	1.31	-	1.62	1.71
NPRC 435	1.59	-	1.63	-
NPRC 436	1.31	-	1.38	-
NPRC 437	1.43	1.49	-	1.42
NPRC 438	1.39	1.25	1.26	-
NPRC 439	-	-	-	-
NPRC 440	-	-	-	1.77
NPRC 441	-	-	1.51	-
NPRC 442	-	1.54	-	-
NPRC 443	1.68	-	1.74	1.99
NPRC 444	1.23	-	1.45	-
NPRC 445	-	-	1.25	-
NPRC 446	1.70	-	1.79	-
NPRC 447	-	-	1.59	-
NPRC 448	-	-	1.52	-
NPRC 449	1.51	-	1.45	1.65
NPRC 450	1.64	-	1.74	1.39
NPRC 451	1.44	-	1.71	1.71
NPRC 452	1.22	-	1.43	1.49
NPRC 453	-	-	1.46	-

Isolates	Haemolysin	Lecithinase	Lipase	Protease
NPRC 454	1.38	-	1.74	1.48
NPRC 455	-	-	1.47	1.53
NPRC 456	-	-	1.58	-
NPRC 457	-	-	1.52	-
NPRC 458	1.58	-	1.64	-
NPRC 459	1.12	-	1.94	1.37
NPRC 460	-	-	-	-
NPRC 461	-	-	1.81	1.46
NPRC 462	-	-	-	1.33
NPRC 463	-	-	1.72	1.18
NPRC 464	-	-	-	-
NPRC 465	1.29	-	2.06	-
NPRC 466	1.14	-	1.85	-
NPRC 467	-	-	1.57	-
NPRC 468	-	-	2.02	-
NPRC 469	-	-	-	1.86
NPRC 470	1.11	-	1.68	-
NPRC 471	1.67	-	1.66	1.78
NPRC 472	-	1.34	1.42	-
NPRC 473	1.38	-	1.73	-
NPRC 474	1.61	-	1.82	-
NPRC 475	-	-	1.56	-
NPRC 476	1.77	-	1.52	-
NPRC 477	1.60	-	1.47	-
NPRC 478	-	-	1.78	1.65
NPRC 479	1.46	-	1.69	-
NPRC 480	1.07	-	1.96	1.80
NPRC 481	1.06	-	1.64	1.51
NPRC 482	1.24	-	1.58	-
NPRC 483	-	-	1.67	-

Isolates	Haemolysin	Lecithinase	Lipase	Protease
NPRC 484	1.35	-	1.58	-
NPRC 485	-	-	-	1.87
NPRC 486	1.13	-	1.37	-
NPRC 487	1.18	-	1.52	-
NPRC 488	1.15	-	1.49	1.38
NPRC 489	1.25	-	1.75	1.58
NPRC 490	-	-	1.46	-
NPRC 491	-	-	1.48	-
NPRC 492	1.11	-	1.86	1.37
NPRC 493	-	-	1.93	-
NPRC 494	-	-	1.66	-
NPRC 495	1.52	-	1.45	1.52
NPRC 496	1.55	-	1.61	1.64
NPRC 497	-	-	-	-
NPRC 498	-	-	1.54	-
NPRC 499	-	-	1.63	-
NPRC 500	-	-	-	1.75
NPRC 501	-	-	1.64	-
NPRC 502	1.75	-	1.66	-
NPRC 503	-	-	1.61	-
NPRC 504	1.66	-	1.86	1.58
NPRC 505	1.67	-	1.21	1.73
NPRC 506	1.55	-	1.67	1.75

Reference strains	Haemolysin	Lecithinase	Lipase	Protease
ATCC 25923	1.43	-	2.00	1.57
ATCC 23235	1.35	-	1.50	1.44
ATCC 27664	1.51	-	1.61	1.50

Appendix 4. Sensory score sheet

Title: Sensory test for home made salad dressing or cooked pork

Name……. Date…….

Instruction: Please kindly test the food samples below from left to rght and score each attributes listed below according to your feelings or perception. This is a 9-point hedonic scale score for sensory attributes, where 1 = dislike extremely, 9 = like extremely, while the limit of acceptability was 5 = neither like or dislike. You can decide to use other numbers between 1-4, and 6-8 to express your score.

	Scores			
Sample codes	229	268	295	246
Attributes				
Appearance				
Colour				
Viscousity				
Off flavour/Rancidity				
Texture				
Taste				
Overall acceptability				

Remarks……

……

……

……

Thank you.

VITAE

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Student ID 5010230018

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B. Tech. (Food & Industrial Microbiology)	Federal University of Technology, Akure, Nigeria	1998
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1. Voravuthikunchai, S.P., **Ifesan, B.O.T**., Mahabusarakam, W. and Hamtasin, C. 2008. Antistaphylococcal activity of semi-purified fractions from *Eleutherine americana.* Clin. Microbiol. Infect. 14 (Suppl. 7): P580.
2. **Ifesan, B.O.T**, Hamtasin, C., Mahabusarakam, W. and Voravuthikunchai, S.P. 2009. Inhibitory Effect of *Eleutherine americana* Merr. Extract on *Staphylococcus aureus* Isolated from food. J. Food Sci. 74:31-36.

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1. Voravuthikunchai S.P., **Ifesan B**., Chusri S. and Saising J. 2008. Bioactive natural products as anti-staphylococal infections. *In:* Gupta V.K. *ed*., Bioactive Natural Products: Bioresource Management and Utilization. Studium Press LLC, Houston, Texas, USA. (*In press*).
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