Antioxidant and / or Antibacterial of Keang-hleung Paste and its Shelf-life

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ABSTRACTS

Keang-hleung or yellow curry is a traditional popular spicy-sour curry consumed in southern Thailand. In general, the ingredients used in the paste are turmeric rhizome, garlic and chili but some areas may also add galangal rhizome. There were some scientific reports mentioned that many herbs/spices could exhibit free radical scavenging, inhibit bacterial growth, as natural preservative agents. However, those properties may deteriorate during processed. This present study investigated the physicals properties, total phenolic content, antioxidant properties, antibacterial activities, effect of heating temperature and time on those properties of the paste and its ingredients. Moreover, the developed paste was made by addition with garlic and turmeric rhizome and quality changes of the paste with out or with added salt were investigated during storage. It was found that the total phenolic content of garlic, galangal, turmeric, dried chili and Keang-hleung paste extracts determined by the Folin Ciocalteu method were 0.3 ± 0.02, 0.2 ± 0.01, 0.3 ± 0.06, 0.6 ± 0.06 and 0.8 ± 0.02 mg GAE/100 g, respectively. The DPPH (2,2-Diphenyl-1-Picrylhydrazyl) radical scavenging activity of garlic, galangal, turmeric, dried chili and the paste extracts were 0.38 ± 0.07, 0.65 ± 0.41, 1.01 ± 0.34, 0.28 ± 0.65 and 1.77 ± 0.38 mg GAE/100 g, respectively. And ferric reducing power of those samples as mentioned before were 0.03 ± 0.01, 0.05 ± 0.01, 0.07 ± 0.02, 0.07 ± 0.02 and 0.05 ± 0.01 mg GAE/100 g, respectively. For the antibacterial activity, it was found that only garlic and the paste extracts could inhibit *Staphylococcus aureus* and *Bacillus cereus*. The minimal inhibitory concentration (MIC) values of garlic and the paste extracts on *S. aureus* and *B. cereus* were 0.407 and 0.407, 1.306 and 1.306 mg/ml, respectively. The minimal bactericidal concentration (MBC) values of garlic and the paste extracts tested by *S. aureus* and *B. cereus* were 3.259 and 0.815, 5.224 and 2.612 mg/ml, respectively.

The crude extracts of garlic, galangal, turmeric, dried chili and paste were determined for physicals properties, total phenolic content, antioxiant properties, and antibacterial activities after heat treatment. It was found that the physical properties as color and pH values were not significantly difference ($p \geq 0.05$) when compared to unheated sample (control). Antioxidant activities of the crude extracts of garlic, galangal, turmeric and dried chili decreased ($p < 0.05$) while, those activities of crude turmeric and paste extracts increased ($p < 0.05$) as temperature and time increased. Antibacterial activities in the crude of garlic and the paste extracts decreased when temperatures and times increased and disappeared at heating temperature 80^0C for 10 minutes.

The developed Keang-hleung paste with an increase of garlic and turmeric rhizomes from 36% to 39.27% and 19% to 20.73%, respectively was evaluated for antioxidant and antibacterial properties as well as sensory acceptability. The result showed that total phenolic contents of the developed paste increased from 0.85 ± 0.07 to 1.19 ± 0.19 mg GAE/100 g, while DPPH and FRAP activities increased from 1.75 ± 0.51 to 2.22 ± 0.71 mg GAE/100 g and 0.58 ± 0.01 to 0.76 ± 0.02 mg GAE/100 g, respectively. Moreover, MIC and MBC of developed Keang-hleung paste tested by *S. aureus* and *B. cereus* were 0.752 and 1.504, 2.008 and 4.016 mg/ml, respectively, while basic Keang-hleung paste were 1.312 and 2.624, 3.248 and 6.496 mg/ml, respectively.

The developed Keang-hleung paste with addition of salt at 0% and 10% and 20% kept at ambient temperature and 4^0C were monitored for all properties as addressed above. It was found that those properties decreased when time of storage increased. Additionally, there could not determine total phenolic contents and antioxidant activities in paste without and with added 10% salt and stored at 4^0C after 30 days of storage. The paste with added 20% salt stored at 4^0C and at ambient temperature could be detected those properties up to 45 days of storage. However, there were no antibacterial activities in the paste kept at 4^0C and ambient temperature at first week.

The developed Keang-hleung pastes with added 10% and 20 % salts were also evaluated for consumer acceptability as appearance, color, odor, saltiness, spiciness, sourness and overall liking. It was found that the sensory scores of those

attributes were 7.23 ± 0.73, 7.23 ± 0.63, 7.30 ± 0.65, 7.20 ± 0.71, 7.03 ± 0.70, 7.37 ± 0.77 and 7.17 ± 0.75, respectively. Meanwhile, sensory scores of the basic paste were 7.40 ± 0.56, 7.13 ± 0.78, 7.23 ± 0.77, 7.13 ± 0.68, 7.07 ± 0.74, 7.40 ± 0.68 and 7.20 ± 0.66, respectively. In general, a decrease of consumer acceptability on the paste was pronounced when time of storage increased ($p<0.05$) but the sensory acceptability score of all attributes were still more than 6.0.

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LIST OF ABBREVIATIONS AND SYMBOLS

AH	=	Antioxidant
$Asc^{\bullet -}$	=	Tricarbonyl Ascorbate Free Radical
$AscH^{-}$	=	ascorbic acid
AT	=	Ambient Temperature
A_w	=	Water Activity
BP	=	Baird Parker Agar
cfu	=	Colony Forming Unit
DPPH	=	2,2-diphenyl-1-picrylhydrazyl
dw	=	Dried Weight
EC	=	Enzyme Catalase
EMB	=	Eosin Methylene Blue Agar
FRAP	=	Ferric Reducing Antioxidant Power
$FeCl_3$	=	Ferric Chloride
$Fe\ (TPTZ)^{3+}$	=	Tripyridyltriazine
GAE	=	Gallic Acid Equilibrium
GPx	=	Glutathione Peroxidase
GSH	=	Growth Stimulating Hormone
GST	=	Glutathione-*S*-Transferase
HNO_2	=	Nitrous Acid
HOCI	=	Hypochlorous Acid
H_2O_2	=	Hydrogen Peroxide
LLDPE	=	Linear Low Density Polyethylene
LST	=	Lauryl Sulphate Ttryptose Broth
MBC	=	Minimum Bactericidal Concentration
MIC	=	Minimum Inhibitory Concentration
MYP	=	Mannitol Egg Yolk Polymyxin Agar
NA	=	Nutrient Agar
NB	=	Nutrient Broth
$NO_2^{\bullet}$	=	Nitrogen Dioxide Radical

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

$^{I}O_2$	=	Singlet Oxygen
$O_2^{\bullet -}$	=	Superoxide
$OH^{\bullet}$	=	Hydroxyl radical
$ONOO^{-}$	=	Peroxynitrite
PDA	=	Potato Dextrose Agar
$RO^{\bullet}$	=	Alkoxyl Radical
$RO_2^{\bullet}$	=	Peroxyl Radical
ROS	=	Reactive Oxygen Species
Se-OH	=	Selenoles
SOD	=	Superoxide Dismutase
TPTZ	=	2,4,6-Tris (1-Pyridyl)-5-Triazine
UV-vis	=	Ultraviolet and Visible Spectometer
XO	=	Xanthine Oxidase
MR-VP	=	Methyl Red – Voges Proskauer

CHAPTER 1

INTRODUCTION

1.1 Introduction

Free radicals are unstable highly reactive and energized molecules having unpaired electron such as superoxide, hydroxyl, peroxyl and alkoxyl. Those compounds produced by sunlight, ultraviolet light, ionizing radiation, chemical reactions and metabolic processes. They are continuously produced in the human body and they are controlled by endogenous enzymes (superoxide dismutase, glutathione peroxidase, catalase). When there is an over production of these species, an exposure to external oxidant substances or a failure in the defense mechanisms, thereafter damaging of valuable bio-molecules (DNA, lipids, proteins) may occur (Aruoma, 1998). This damage has been associated with an increased risk of cardiovascular disease, cancer and other chronic diseases. In recent years human health has assumed an unprecedented important status. Increased interests in nutrition, fitness and beauty have exaggerated concerns over diet and human health. A new diet health paradigm is more emphasis on the positive aspects of diet.

Recently phytochemicals in fruits vegetables, herbs, and spices have attracted a great deal of attention mainly concentrated on their role in preventing or delaying chronic diseases caused as a result of oxidative stress (Kaur and Kapoor, 2001). Additionally, various compounds found in plants, herbs, and spices have also been shown to possess antimicrobial functions and serve as a source of antimicrobial agents against food borne pathogens (Deans and Ritchie, 1987). Therefore, plant-derived food additive has grown and gained popularity because it assumed to be high quality, nutritious and long shelf-life food products with no preservative agents (Oonmetta-aree *et al.,* 2005).

Traditional Thai food has a very distinctive characteristic because of the special combination of herbs and spices in Thai food preparation (Chaisawadi *et al.,* 2005). Additionally, Thai native herbs are becoming more widely used at a

commercial scale in the food industry, mainly for their flavoring (Voravuthikunchai *et al.,* 2006) aroma, or piquancy and color properties to foods (Srinivasan, 2005). Keang-hleung is a traditional popular spicy-sour curry consumed in southern Thailand. In general, the ingredients used in the paste are turmeric rhizome, garlic and chili; however, some areas may also add galangal rhizome. Some of these ingredients are classified as natural preservative agents because their active compounds provide free radical scavenging such as curcumin (difeuryloylmethane) from turmeric rhizome (Jayaprakasha *et al.,* 2006; Ruby *et al.,* 1995; Cousins *et al.,* 2006; Ahsan *et al.,* 1999). The principle antimicrobial compound, allicin, of garlic *(Allium sativum*) exhibits antibacterial activity against a wide range of Gram-negative and Gram-positive bacteria (Bakri and Douglas, 2005; Siripongvutikorn *et al*., 2005; Jonkers *et al.,* 1999; Ankri and Mirelman, 1999). In Thailand, galangal (*Alpinia galangal*) is used for medical purposes, such as for carminative, stomachic, antispasmodic, antichloristic and antibacterial drugs (Mayachiew and Devahastin, 2008), and as an antioxidation (Mayachiew and Devahastin, 2008; Ruby *et al.,* 1995; Juntachote and Berghofer, 2005; Zaeoung *et al.,* 2005; Jayaprakasha *et al.,* 2006; Kumar *et al.,* 2006). Capsaicin is a compound in chili that could inhibit the growth of *Helicobacter pylori* (Jones *et al.,* 1997).

Many researchers studied individual herb/spice as food additives, but a few scientific data has been reported about using of combination of herbs/spices as natural preservatives and functional foods particularly Keang-hleung pastes. Therefore, the objectives of this study were to determine the antimicrobial and antioxidant activities of Keang-hleung paste and its ingredients before and after heat treatment as well as quality changes of the paste without and with added salt during storage.

1.2 Review of Literature

1.2.1 Free radicals.

Free radicals are unstable highly reactive and energized molecules having unpaired electron. Free radicals react quickly with other compound, trying to capture the electrons needed to gain stability. General free radicals attack the nearest stable molecules, "stealing" its electron. When the molecules that has been attacked and loses its electrons it becomes a free radical itself, beginning a chain reaction. Once the process is started, it can cascade, initiating lipid peroxidation which results in destabilization and disintegration of the cell membranes or oxidation of other cellular components like protein and DNA, finally resulting in the disruption of cells (Halliwell *et al*., 1995). Oxidation caused by free radicals sets reduced capabilities to combat ageing and serious illness, including cancer, kidney damage, atherosclerosis and heart diseases (Ames, 1983).

1.2.2 Type of free radicals.

1.2.2.1 Reactive Oxygen Species (ROS) is a collective term often used by biologists to include oxygen radicals (superoxide [$O_2^{\bullet-}$], hydroxyl [$OH^{\bullet}$], peroxyl [$RO_2^{\bullet}$] and alkoxyl [$RO^{\bullet}$]). Also, certain other non radicals those are either potential oxidizing agent and/or are easily converted into radicals, such as hypochlorous acid (HOCI), ozone (O_3), peroxynitrite ($ONOO^-$) singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2).

1.2.2.2 Reactive Nitrogen Species (RNS) is a term becoming popular to encompass nitrogen dioxide radical ($NO_2^{\bullet}$), peroxynitrite, nitrous acid (HNO_2) and related species.

1.2.3 Sources of free radicals.

ROS can be produced from both endogenous and exogenoussubstances. Potential endogenous sources include mitochondria, cytochrome P450 metabolism, peroxisomes, and inflammatory cell activation (Inoue *et al.,* 2003). Mitochondria have long been known to generate significant quantities of hydrogen peroxide. The hydrogen peroxide molecule does not contain an unpaired

electron and thus is not a radical species. Under physiological conditions, the production of hydrogen peroxide is estimated to account for about approximately 2% of the total oxygen uptake by the organism. However, it is difficult to detect the occurrence of the superoxide radical in intact mitochondria, most probably in consequence of the presence of high SOD activity therein.

Generation of the superoxide radical by mitochondria was first reported more than three decades ago by Loschen *et al*., 1971. After the determination of the ratios of the mitochondrial generation of superoxide to that of hydrogen peroxide, the former was considered as the stoichiometric precursor for the latter. Ubisemiquinone has been proposed as the main reductant of oxygen in mitochondrial membranes (Inoue *et al.,* 2003). Mitochondria generate approximately 2–3 nmol of superoxide/minute per mg of protein, the ubiquitous presence of which indicates it to be the most important physiological source of this radical in living organisms (Inoue *et al*., 2003). Since mitochondria are the major site of free radical generation, they are highly enriched with antioxidants including GSH and enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), which are present on both sides of their membranes in order to minimise oxidative stress in the organelle (Cadenas and Davies, 2000). Superoxide radicals formed on both sides of mitochondrial inner membranes are efficiently detoxified initially to hydrogen peroxide and then to water by Cu, Zn-SOD (SOD1, localised in the intermembrane space) and Mn-SOD (SOD2, localized in the matrix).

Besides mitochondria, there are other cellular sources of superoxide radical, for example xanthine oxidase (XO), a highly versatile enzyme that is widely distributed among species (from bacteria to man) and within the various tissues of mammals (Li and Jackson, 2002). Xanthine oxidase is an important source of oxygen-free radicals. It is a member of a group of enzymes known as molybdenum iron-sulphur flavin hydroxylases and catalyzes the hydroxylation of purines. In particular, XO catalyzes the reaction of hypoxanthine to xanthine and xanthine to uric acid. In both steps, molecular oxygen is reduced, forming the superoxide anion in the first step and hydrogen peroxide in the second (Valko *et al*., 2004). Additional endogenous sources of cellular reactive oxygen species are neutrophils, eosinophils and macrophages. Activated macrophages initiate an increase in oxygen uptake that gives

rise to a variety of reactive oxygen species, including superoxide anion, nitric oxide and hydrogen peroxide (Conner and Grisham, 1996).

Cytochrome P450 has also been proposed as a source of reactive oxygen species. Through the induction of cytochrome P450 enzymes, the possibility for the production of reactive oxygen species, in particular, superoxide anion and hydrogen peroxide, emerges following the breakdown or uncoupling of the P450 catalytic cycle. In addition, microsomes and peroxisomes are sources of ROS. Microsomes are responsible for the 80% H_2O_2 concentration produced in vivo at hyperoxia sites (Gupta *et al.,* 1997). Peroxisomes are known to produce H_2O_2, but not $O_2^{\bullet-}$, under physiologic conditions. Although the liver is the primary organ where peroxisomal contribution to the overall H_2O_2 production is significant, other organs that contain peroxisomes are also exposed to these $H_2O_2^{-}$ generating mechanisms. Peroxisomal oxidation of fatty acids has recently been recognised as a potentially important source of H_2O_2 production as a result of prolonged starvation. The release of the biologically active molecules such as cytokines and others, from activated Kupffer cells (the resident macrophage of the liver) has been implicated in hepatotoxicological and hepatocarcinogenic events. Recent results indicate that there is a close link between products released form activated Kupffer cells and the tumour promotion stage of the carcinogenesis process (Klaunig and Kamendulis, 2004).

Reactive oxygen species can be produced by a host of exogenous processes. Environmental agents including non-genotoxic carcinogens can directly generate or indirectly induce reactive oxygen species in cells. The induction of oxidative stress and damage has been observed following exposure to various xenobiotics. These involve chlorinated compounds, metal (redox and non-redox) ions, radiation and barbiturates. For example 2-butoxyethanol is known to produce ROS indirectly, which causes cancer in mice (Klaunig *et al*., 1997).

1.2.4 Antioxidants.

Antioxidant is any substance that present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (Halliwell, 1990; Halliwell *et al.,* 1987). Antioxidants neutralize free radicals by donating one of their own electrons, ending the

electron-stealing reaction. The antioxidants do not themselves become free radicals by donating electrons because they are stable in either form. Thus they may well be defined as the substances that are capable of quenching or stabilizing free radicals (Kuar and Kapoor, 2001).

1.2.5 Type of antioxidants.

1.2.5.1 Primary antioxidants: Primary antioxidants can inhibit or retard oxidation by scavenging free radicals by donation of hydrogen atoms or electrons, which converts them to more stable products.

1.2.5.2 Secondary antioxidants: Secondary antioxidants function by many mechanisms, including binding of metal ions, scavenging oxygen, converting hydroperoxides to non-radical species, absorbing UV radiation or deactivating singlet oxygen (Gordon, 1990, 2001).

1.2.6 Source of antioxidants (Valko *et al.*, 2006).

1.2.6.1 Enzymatic antioxidants.

(a) Superoxide dismutase (SOD): One of the most effective intracellular enzymatic antioxidants is superoxide dismutase (SOD) (EC 1.15.1.1). Superoxide dismutase is the antioxidant enzyme that catalyzes the dismutation of $O_2^{\bullet-}$ to O_2 and to the less-reactive species H_2O_2.

(b) Catalase: Catalase (EC 1.11.1.6): is an enzyme present in the cells of plants, animals and aerobic (oxygen requiring) bacteria (Mates *et al.,* 1999). Catalase is located in a cell organelle called the peroxisome. The enzyme very efficiently promotes the conversion of hydrogen peroxide to water and molecular oxygen. Catalase has one of the highest turnover rates for all enzymes: one molecule of catalase can convert approximately 6 million molecules of hydrogen peroxide to water and oxygen each minute:

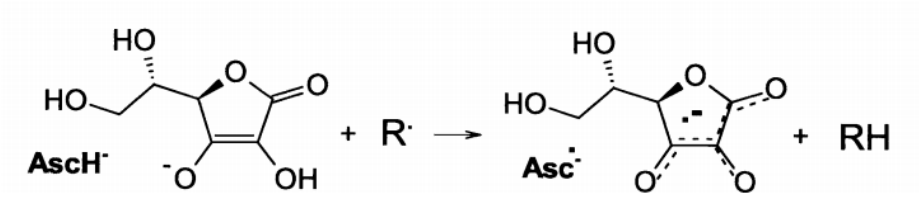
$$2\ H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

The significantly decreased capacity of a variety of tumors for detoxifying hydrogen peroxide is linked to a decreased level of catalase.

(c) Glutathione peroxidase: There are two forms of enzyme glutathione peroxidase, one of which is selenium-independent (glutathione-*S*-transferase, GST, EC 2.5.1.18), another one is selenium-dependent (GPx, EC 1.11.1.19) (Mates *et al.,* 1999). These two enzymes differ in the number of subunits, the bonding nature of the selenium at the active centre and their catalytic mechanisms. Humans have four different Se-dependent glutathione peroxidases (Mates *et al.,* 1999). All GPx enzymes are known to add two electrons to reduce peroxides by forming selenoles (Se-OH) (Mates *et al.,* 1999).

1.2.6.2 Non-enzymatic antioxidants.

(a) Vitamin C: Vitamin C (ascorbic acid) (Fig. 1) is a very important, andpowerful, antioxidant that works in aqueous environments of the body, such as is present in the lungs and in the lens of the eye. Its primary antioxidant partners are Vitamin E and the carotenoids, as well as working along with the antioxidant enzymes. $AscH^-$ is a donor antioxidant and reacts with radicals to produce the resonance stabilized tricarbonyl ascorbate free radical $Asc^{\bullet -}$ (shown by the dashed lines in the illustration of Fig.1) (Valko *et al*., 2004).

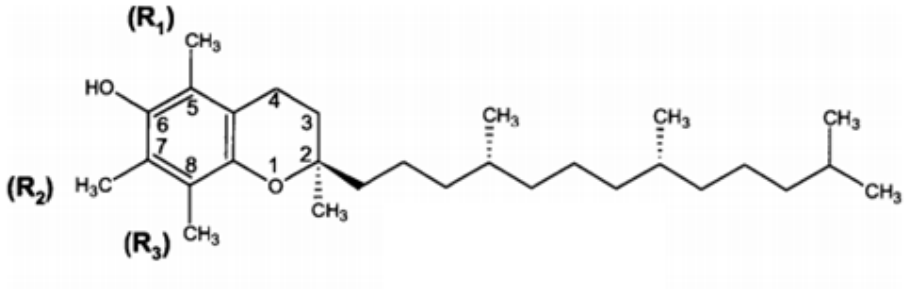


(a) (b)

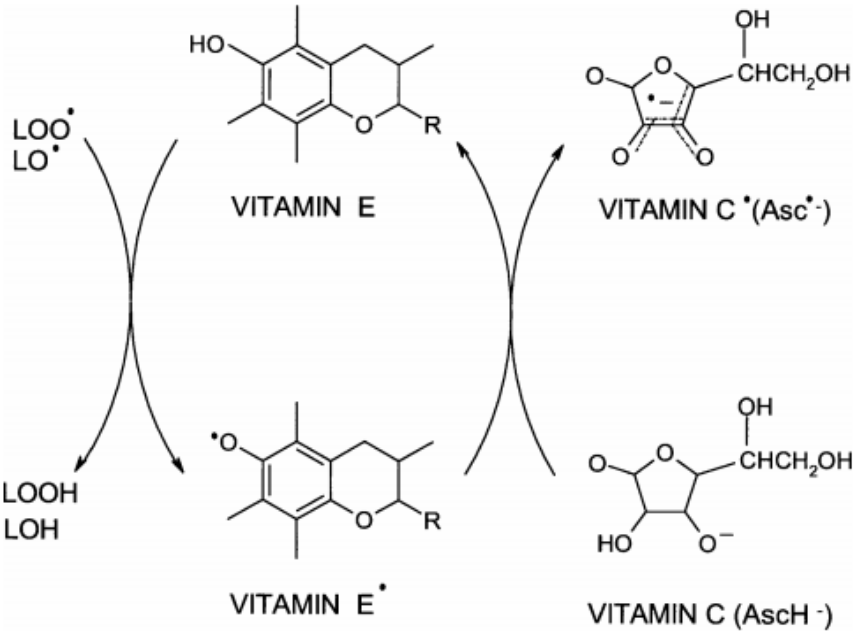
Figure 1 : Ascorbic acid reacts with radicals (a) and ascorbate free radical (b). **Source :** Valko *et al*. (2004)

(b) Vitamin E: Vitamin E is a fat-soluble vitamin that exists in eight different forms. α-tocopherol (Fig. 2a) is the most active form of vitamin E in humans and is a powerful biological antioxidant which is considered to be the major membrane bound antioxidant employed by the cell (Burton and Ingold, 1989). Its main antioxidant function is protection against lipid peroxidation (Pryor, 2000).

As mentioned above, ascorbic acid is regarded as the major aqueous phase antioxidant. Recent evidence suggests that α-tocopherol and ascorbic acid function together (Fig. 2b) in a cyclic-type of process. During the antioxidant reaction, α-tocopherol is converted to α-tocopherol radical by the donation of labile hydrogen to a lipid or lipid peroxyl radical. The α-tocopherol radical can thus be reduced to the original α-tocopherol form by ascorbic acid (Kojo, 2004).



(a)



(b)

Figure 2 : Structure of α - tocopherol (a) and reaction of vitamin E with peroxyl free radicals and regeneration of vitamin E radical (tocopheroxyl radical) through one-electron oxidation of vitamin C (b)

Source : Valko *et al*. (2004)

(c) Carotenoids: The antioxidant activity of carotenoids such as β-carotene (Fig. 3) arises primarily as a consequence of the ability of the conjugated double-bonded structure to delocalized unpaired electrons (Mortensen *et al.,* 2001).

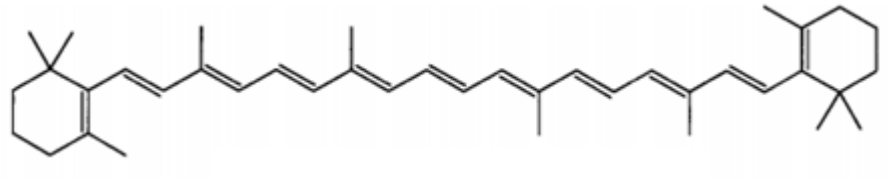


Figure 3 : Structure of all-trans- β-carotene.
Source : Valko *et al*. (2004)

(d) Flavonoids: Flavonoids are a large group of phenolic plant constituents. Phenolic compounds acting as antioxidants may function as terminators of free radical chains and as chelators of redox-active metal ions that are capable of catalysing lipid peroxidation (Schroeter *et al.,* 2002). Flavonoids are ideal scavengers of peroxyl radicals due to their favourable reduction potentials relative to alkyl peroxyl radicals and thus, in principle, they are effective inhibitors of lipid peroxidation. Of particular importance is the hydrogen (electron) donating ability of a flavonoid molecule which acts to scavenge a reactive radical species, and is primarily associated with the presence of a B-ring catechol group (dihydroxylated B-ring) (Schroeter *et al.,* 2002). Structure of flavonoids such as quercetin and flavonol-quercetin were shown in Fig. 4 and 5, respectively.

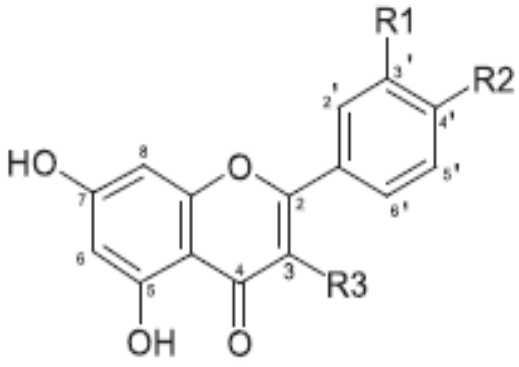


Figure 4 : Structure of quercetin which is one type of flavonoid.
Source : Erlund (2004)

The mechanism of antioxidant to retard or inhibit free radicals is showed as below

$$ROO^{\cdot} + AH \longrightarrow ROOH + A^{\cdot}$$
$$RO^{\cdot} + AH \longrightarrow ROH + A^{\cdot}$$
$$ROO^{\cdot} + A^{\cdot} \longrightarrow ROOA$$
$$RO^{\cdot} + A^{\cdot} \longrightarrow ROA$$

AH is an antioxidant

A· is an antioxidant radical

ROO· is peroxyl radical

RO· is alkoxyl radical

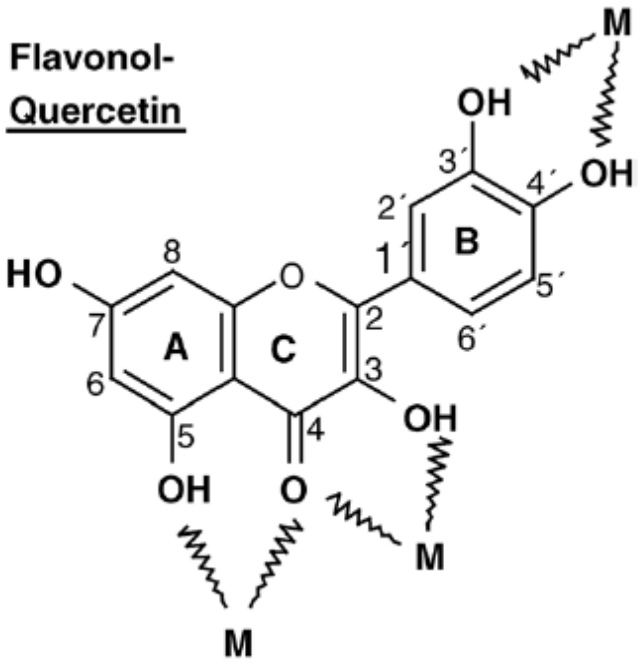


Figure 5 : Structure of flavonoid quercetin (M: coordinated metal ion).

Source : Valko *et al*. (2004)

(e) Selenium: Selenium appears to function as an antimutagenic agent, preventing the malignant transformation of normal cells. These protective effects of Se seem to be primarily associated with its presence in the glutathione peroxidases (GSH-Pxs) and thioredoxin reductase, which are known to protect DNA

and other cellular components from oxidative damage (Trueba *et al.,* 2004; Schrauzer, 2000).

1.2.7 Mechanisms of antibacterial action (Oonmetta-aree, 2005).

Antimicrobial activity varies greatly between different types of microorganisms and it might differ between different strains of the same species. The ability of each antimicrobial agent to penetrate to the bacterial cells is due to the composition and structure of the cell and outer walls of the microorganisms (structure of bacteria was shown in Fig. 6). The fundamental divisions of the bacterial cells, i.e. cell wall, cytoplasmic membrane and cytoplasm, occur in all species. The basic structure and composition of the cytoplasm and the cytoplasmic membrane are largely conserved between different types of bacteria although subtle differences may occur. In terms of susceptibility to antimicrobial agents, the structure and composition of the outer envelope are more interesting as they differ widely between microorganisms. It is generally accepted that the bacterial outer envelope is responsible for the different microbial responses to biocide challenges. For example, the outer membrane of Gram negative bacteria acts as permeability barriers and is responsible for the intrinsic resistance of the microorganisms to antimicrobial compounds (Gilbert *et al.,* 1990; Nikaido and Vaara, 1985). Cytoplasmic membrane composed essentially of a phospholipid bilayer with embedded proteins. It is semi permeable and regulates the transfer of solutes and metabolites in and out of the cell cytoplasm. It is also associated with several important enzymes involved in various cell metabolic functions (Salton and Owen, 1976; Singer and Nicholson, 1972). The cytoplasmic membrane is often considered as the major target site for antimicrobial agents (Fig. 7). Damage to the membrane can take several forms: (i) physical disruption of the membrane; (ii) dissipation of the proton motive force (PMF); (iii) inhibition of membrane-associated enzyme activity; (iv) interference with cell wall synthesis; (v) protein synthesis inhibition; (vi) interference with nucleic acid synthesis; and (vii) inhibition metabolic pathway (Tenover, 2006). Bacterial cytoplasm contains the cell nucleic acid, ribosomes and various enzymes with different functions. These cell components are probably not primary target sites, since an antimicrobial agent has to penetrate within the cell to reach cytoplasmic constituents.

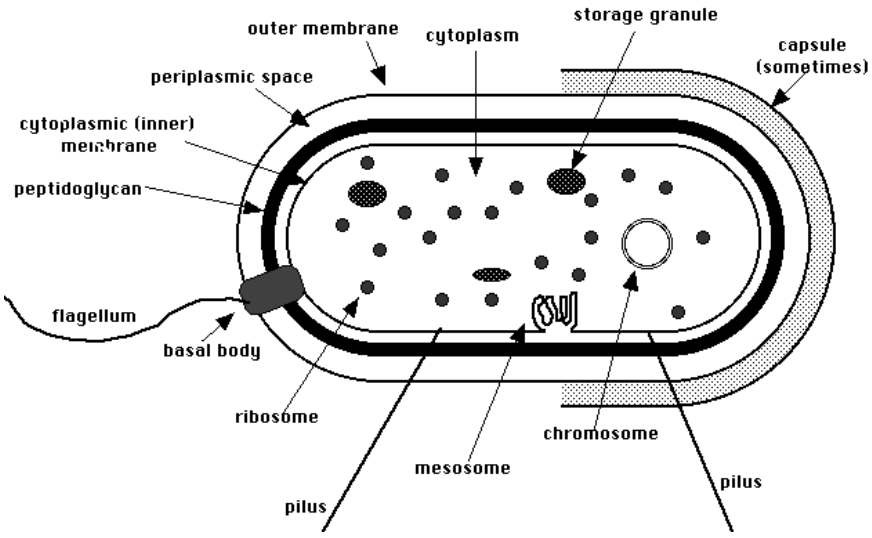


Figure 6 : Structure of bacteria

Source : http://www.micro.siu.edu/micr425/425Notes/01-Introduction.html

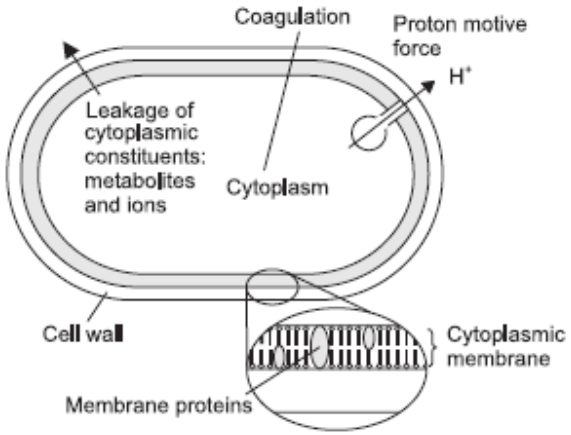


Figure 7 : Locations and mechanisms in the bacterial cell thought to be sites of action for essential oil components: degradation of the cell wall; damage to cytoplasmic membrane; damage to membrane proteins; leakage of cell contents; coagulation of cytoplasm and depletion of the proton motive force.

Source : Burt (2004).

1.2.8 Influence factors of antioxidation and antibacterial.

Periods: some bioactive compound is rapidly degradation during storage for example the half-life of allicin at room temperature is 2-16 hours; however, in crushed garlic (or in garlic juice) it is 2.4 days (Lawson, 1998). The curcuminoids decomposed very rapidly (more than 90% within 12 hours) when serum was omitted, but were more stable in the presence of serum (Pfeiffer *et al.,* 2003).

Temperature: In general, bioactive compounds were heat labile. Kim *et al.* (2002) who reported the antimicrobial activity of garlic decreased as the heating temperature increased. However, Gorinstein *et al.* (2006) who found total antioxidant potentials (determined by $ABTS^{\cdot+}$ radical cation) in a boiling garlic at 100^0C for 20 minutes and its bioactivity comparable with the raw sample. The antioxidant activity (inhibition of lipid peroxidation) of spice extracts (garlic, ginger, onion, mint, cloves, cinnamon and pepper) were retained even after boiling for 30 minutes at 100°C (Shobana and Naidu, 2000). Juntachote and Berghofer (2004) concluded that holy basil and galangal extracts were heat stability at 80^0C for 1 hour. This antioxidant activity was measured on coupled oxidation of β-carotene and linoleic acid, DPPH scavenging activity, superoxide anion scavenging activity, reducing power, chelating activity on Fe^{2+} and inhibition of lipoxygenase activity.

pH: Juntachote and Berghofer (2004) who reported that the antioxidant activity of holy basil and galangal extracts at neutral pH was higher than at acidic pH ranges. The antioxidative activity determined by DPPH radical scavenging and superoxide anion scavenging of Hsian-tsao (*Mesona procumbens* Hemsl.) extracts decreased when the alkaline concentration was increased (Yen and Huang, 2000).

Light: The curcuminoids are readily decomposed when exposed to bright light (Schieffer, 2002).

1.3 Objectives

The objectives of the thesis were:

1. To evaluate the antimicrobial and antioxidant properties of Keang-hleung paste and its ingredients.
2. To study the effect of temperature and time on antimicrobial and antioxidant properties of Keang-hleung paste and its ingredients.
3. To improve antimicrobial and antioxidation properties of Keang-hleung paste with increasing the spices level.
4. To study the effect of sodium chloride and storage temperature on quality of developed Keang-hleung paste.

CHAPTER 2

MATERIALS AND METHODS

2.1 Raw materials

Turmeric rhizomes (*Curcuma longa*), garlic bulb (*Allium sativum*), dried chili (*Capsicum frutescense*) and galangal rhizomes (*Alpinia galanga*) were purchased from a local market in Hat-Yai, Thailand. To control quality of raw materials used in this experiment, the galangal and turmeric rhizomes were 1 year while chili was 3-4 months of age and obtained from same farmers.

2.2 Chemicals and Reagents

- Folin-Ciocalteu reagent
- 2,2-diphenyl-1-picrylhydrazyl
- Acetate buffer
- 2,4,6-tris (1-pyridyl)-5-triazine (TPTZ)
- Ferric chloride
- HCl
- 0.85% NaCl
- 10 % sodium carbonate
- Gallic acid

All chemicals and reagents were of analytical grade and obtained from Sigma Chemical Co. (St. Louis, MQ, USA) and Merck (Darmstadt, Germany).

2.3 Test organisms

- *Staphylococcus aureus*
- *Bacillus cereus*

Both test bacteria were obtained from the Department of Medical Sciences, Ministry of Public Health, Thailand.

2.4 Media

- Brian Heart Infusion Broth (BHI)
- Plate Count Agar (PCA)
- Mueller Hinton Agar (MHA)
- Eosin Methylene Blue Agar (EMB)
- Nutrient Agar (NA)
- Rabbit Plasma
- Lactose Broth
- Motility Medium
- Mannitol Egg Yolk Polymyxin Agar (MYP)
- Phenol Red Glucose Broth
- Nutrient Broth (NB)
- Methyl Red – Voges Proskauer Medium (MR-VP Medium)
- Tyrosine Agar
- Potato Dextrose Agar (PDA)
- Baird Parker Agar (BP)
- Lauryl Sulphate Tryptose (LST) Broth
- *Escherichia coli* Broth (EC)

All media were of analytical grade and obtained from Merck (Darmstadt, Germany) and Difco (Becton, Dickinson and Company, USA)

2.5 Equipments and Instruments

All equipments were shown in Table 1.

Table 1. List of equipments, model and company/country.

Equipments	**Model**	**Company/country**
Balance	Adventure	Ohaus, Co. USA
Spectrophotometer	Jasco V-530	Servo Co., Ltd, Japan
Blender	Moulinex, TYPE 276	France
Water bath	YCW-010	Panpolytech Co., Ltd, Thailand
Laminar flow	Laminar Air Flow	Flexlab Official Equipment Manufacturing, Co., Ltd. Thailand
Incubator	Incucell	MMM Medcenter Enrichtungen GmbH, Germany
Autoclave	LAC-5060s	Daihan Labtech, Co., Ltd, Korea
pH meter	Mettler Toledo FE20/EL20	International Inc, USA
Hunters tristimulus colorimeter	Hunter Lab	Hunter Associates Laboratory, Inc, USA
Centrifuge	Sorvall T3/RT3 Centrifuge Series	Thermo Fisher Scientific, Inc, Malaysia

2.6 Methods

2.6.1 Study of antibacterial and antioxidant properties of Keang - hleung paste and its ingredients.

The basic recipe of Keang-hleung paste consisted of

- Galangal rhizomes 9%
- Turmeric rhizomes 19%
- Dried chili 36%
- Garlic bulb 36%

This recipe was selected from a famous shop consumed in the Narathiwat province and neighbor province as production approximately 100 Kg/day.

2.6.1.1 All spices were sorted, trimmed and washed thoroughly to remove dust and dirt, and weighed according to the recipe as mentioned above. Then all ingredients were ground with a blender (Moulinex, TYPE 276, France) to make a fine paste as 60 mesh. A 100-g of the paste was extracted with 300 ml of distilled water twice then combined extract was filtered through a filter paper (ϕ125 mm, Cat. no. 1001 125 (Whatman Schleicher and Schuell, England) and kept at 4^0C until used.

2.6.1.2 The extracts were determined for physical, chemical such as total phenolic content and antioxidant properties as well as antibacterial activities according to methods as described below.

Physical qualities

pH values : The extracts were cooled at ambient temperature before subjected to determine with digital pH meter (Mettler Toledo FE20/EL20) as slightly modified by Bozkurt and Erkmen (2002).

Color values : Color values of the extracts were measured using a color meter (Hunter lab Universal Software). The color values were expressed as CIE Lab* coordinates where L^* represents the luminosity (0 = black; 100 = white), a^* the redness ($a^* > 0$) or greenness ($a^* < 0$) and b^* the blueness ($b^* > 0$) or yellowness ($b^* < 0$).

Chemical properties

Total phenolic content : Total phenolic content of extracts was determined by using Folin–Ciocalteu assay with slightly modifications (Zhou and Yu, 2006). The reaction mixture contained 1 ml of the extract, 0.5 ml of the Folin-Ciocalteu reagent, 1 ml of 10 g/100 ml sodium carbonate and 7.5 ml of distilled water. After 45 minutes of reaction at ambient temperature, the absorbance at 765 nm was measured using a UV-visible spectrophotometer (Jasco V-530, Japan Servo Co., Ltd.). A calibration curve was prepared using standard gallic acid (0.016, 0.008, 0.004, 0.002 and 0.001 mg/ml, r^2= 0.995). The results were expressed on a dry weight basis (dw) as mg gallic acid equivalents (GAE) per 100 g of dry sample.

Antioxidant activities

Free radical scavenging ($DPPH^{\cdot}$) assay : Free radical scavenging was determined by using the free radical generator DPPH (2,2-Diphenyl-1-Picrylhydrazyl) assay based on slightly modifications (Yen and Hsieh, 1997). An aliquot (1 ml) of the serially diluted extract samples was mixed to which 1 ml of 500 µM DPPH solution was added. The mixture was thoroughly mixed using a vortex and kept in the dark for 30 minutes. The absorbance, using a spectrophotometer, was then measured at 518 nm against a blank of ethanol without DPPH. The results were expressed on a dry weight basis as mg GAE/100 g dry sample.

Ferric reducing/antioxidant power (FRAP) assay : A FRAP assay was performed using a modified method of Benzie and Strain (1996). Briefly, a 150 µl aliquot of properly diluted extract was mixed with 2850 µl FRAP reagent and incubated at 37^0C for 4 minutes. The absorbance was then determined at 593 nm against a blank that was prepared using distilled water. FRAP was freshly prepared by mixing 2.5 ml of a 10 mM 2,4,6-Tris (1-Pyridyl)-5-Triazine (TPTZ) solution in 40 mM HCl with 2.5 ml of 20 mM $FeCl_3$ $6H_2O$ and 25 ml of 0.3 M acetate buffer at a pH of 3.6. A calibration curve was prepared using different concentrations of gallic acid (0.016, 0.008, 0.004, 0.002 and 0.001 mg/ml, r^2 = 0.997). FRAP values were expressed on a dry weight basis as mg GAE/100 g dry sample.

Antibacterial activities

Minimum inhibitory concentration (MIC) : One loopful of *S. aureus* and *B. cereus* was individually cultured into 5 ml of BHI and incubated in an incubator at 37^0C for 15 hours. Each bacterium was diluted with 0.85% sterile sodium chloride to achieve a final concentration of approximately 10^6 cfu/ml. One-ml of the bacterial suspension at 10^6 cfu/ml was transferred into 1 ml of Muller Hinton Broth (MHB) then 1 ml of a series of two-fold dilutions of each extract was added. The MIC of the extracts was regarded as the lowest concentration of extracts that did not permit any turbidity of the tested microorganism (Lorian, 1995; Lennette *et al.,* 1991).

Minimum bactericidal concentration (MBC) : All the tubes used in the MIC studies that did not show any turbidity with the bacteria and the last tubes with turbidity were determined for MBC. An aliquot of the suspension (0.1 ml) was spread onto Muller Hinton Agar (MHA) and incubated at 35^0C for 15 hours. The MBC was the lowest concentration in which the initial inoculums were killed at the least one log cycle or more (Lorian, 1995; Lennette *et al.,* 1991).

2.6.2 Effect of heating temperature and time on antioxidant and antibacterial properties of Keang-hleung paste and its ingredients.

Keang-hleung paste and its ingredients extract prepared as section 2.6.1.1 was divided and heated at 80, 90 and 100^0C for 10, 20 and 30 minutes in a water bath (Adihan Lab tech Co., Ltd, Korea) and then allowed to cool at ambient temperature. Non-heated Keang-hleung paste and its ingredient extracts were used as control samples. Thereafter the control and heated samples were subjected to determine the total phenolic content, and the antioxidant and antibacterial activities as mentioned in section 2.6.1.2. Then the results were evaluated for antioxidant and antibacterial properties to formulate a new formula having higher in both activities.

2.6.3 Development of Keang-hleung paste for improving antibacterial and antioxidant properties.

The spices that exhibited heat stability for antibacterial and antioxidant properties were picked up to formulate to be a developed Keang-hleung paste. The developed Keang-hleung paste was added with turmeric rhizomes and garlic bulb on

the same ratio from 55% in basic Keang-hleung paste to 60% (D_1) and 65% (D_2) respectively, as shown in Table 2.

Table 2. The ratio of spices in each treatment.

Treatment	Ingredient (%)			
	Garlic	**Galangal rhizomes**	**Turmeric rhizomes**	**Dried chili**
Basic formula	36	9	19	36
D1	39.27	8	20.73	32
D2	42.54	7	22.46	28

D_1 and D_2 as well as basic paste (control) were subjected to determine for physical, chemical, microbiological qualities and consumer acceptability. Sensory evaluation was evaluated in form of the paste and the soup by thirty panels who regularly consume Keang-hleung curry. The paste was evaluated for appearance, color, odor and liking. While the soup was evaluated in term of appearance, color, odor, saltiness, spiciness, sourness and overall liking (detail of soup preparation was addressed in Appendix A).

Physical properties were determined as previous described.

Microbiological analyses

Total viable count (TVC), coliforms**,** *Escherichia coli, Staphylococcus aureus,* and *Bacillus cereus* were analyzed as method of Bacteriological Analytical Manual, 2001 (detail was addressed in Appendix B).

Antioxidant and antibacterial properties were determined as previous described.

All of quality parameters of the paste were evaluated and sensory score would use as a key determinant to select the paste.

2.6.4 Effects of sodium chloride and storage temperatures on quality of developed Keang-hleung paste.

The developed Keang-hleung paste was added with sodium chloride, table salt, at 0%, 10% and 20% then put into laminate (LLDPE/Nylon) bag before brought to keep in refrigerator ($4\pm2^{o}C$) and ambient temperature ($28\pm2^{o}C$). Color (*L*, a*, b**), pH, A_w, fiber, moisture content, total viable count, antibacterial properties and antioxidant properties of the paste were determined during storage and sensory evaluation was studied every 14 days for 3 months. However A_w, fiber and moisture content would reported at day 0 and the end of storage (base on total viable count).

Moisture content (AOAC, 1999)

The sample was precisely weighed and put into moisture can then dried at 105°C according to method of AOAC (1999). The sample was dried until its weight was stable and moisture content was calculated according to equation as below:

Moisture content (%) = (Sample weight before incubation - Sample weight after incubation) x 100/ Sample weight before incubation.

Crude fiber content (AOAC, 1999)

The sample 5 g was weighed and put into beaker with 1.25 ml concentrate sulfuric acid, then heated with hot plate equipped with condenser for 30 minutes and filtered with filter membrane. Sample was rinsed with boiled water until without acidic then the fiber was removed into a new beaker containing with 200 ml of 1.25% NaOH. Sample was refluxed and heated on hot plate for 30 minutes then filtrate through a filter membrane. Sample was washed with boiled water until without basic. Sample was rinsed with 10 ml of 95% ethyl alcohol then filtrate through a filter membrane. Fiber as removed into crucible and dried in hot air oven at 105°C for 3 hours then cooled down in a desiccators and weighted. The fiber sample was re-heated for 30 minutes until the weight is stable then burn in the kiln. Crude fiber content was calculated according to formula as shown below

Crude fiber content = [(Sample weight before burn - Sample weight after burn) x 100]/ Initial sample weight

Statistical Analysis

Data were subjected to analysis of variance, and mean comparisons were made using Duncan's new multiple range test. Statistical analyses were carried out using the SPSS statistical software (SPSS, Inc., Chicago, IL).

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Physical properties of non - heated treatment on Keang-hleung paste and its ingredients.

The *L**, *a** and *b** values of each spice were showed in Table 3. The expression of sample color may due to its coloring substances such as curcuminoids (Cousins, 2006), carotenoids (Tsao and Deng, 2004) and chlorophyll etc. (Suhaj, 2006). From the result, lightness of galangal and garlic flesh was higher than other samples particularly chili therefore the paste exhibited more *L** value when compared with chili.

The pH values of Keang-hleung paste and its ingredients have been presented in Table 3. The result showed that galangal rhizome was lowest in pH because of its high acid content. Generally, fresh produces are a source of vitamin C, weak acid and other organic (Kalt *et al.,* 1999; Hounsome *et al.,* 2009). Based on pH value, however, the paste and its ingredient were classified as low acid foods (pH ≥ 4.6) as prone to spoilage easily.

Table 3. Color and pH values of non - heated basic Keang - hleung paste and its ingredients.

Sample	Color			pH
	L*	***a****	***b****	
Garlic bulb	61.87 ±0.35 [b]	-4.74 ±0.28 [e]	18.41 ±0.21 [d]	6.24 ±0.01 [a]
Galangal rhizomes	62.92 ±0.47 [a]	-0.67 ±0.11 [d]	6.15 ±0.24 [e]	4.78 ±0.04 [e]
Turmeric rhizomes	41.15 ±0.37 [c]	18.25 ±0.35 [c]	62.20 ±0.87 [a]	6.12 ±0.02 [b]
Chili (dried pod)	19.39 ±0.35 [e]	24.45 ±0.77 [b]	29.05 ±0.78 [c]	5.94 ±0.13 [c]
Keang-hleung paste	36.26 ±0.54 [d]	31.10 ±0.43 [a]	55.81±0.17 [b]	5.72 ±0.02 [d]

Each value is expressed as a mean ± SD (n=3).

a-e means that with different letters within a column are significantly difference ($p<0.05$).

3.2 Antibacterial activities of non-heated basic Keang-hleung paste and its ingredients.

The bacterial loads of the different samples were presented in Table 4. The results showed that dried chili was heavily contaminated with bacteria, while garlic had low bacterial loads. Spices and natural agricultural materials are commonly contaminated with microorganism including bacteria, mold and yeasts (Alemela *et al.,* 2002). However, the number and type of microorganism may differ from harvesting, storage, transport and packaging (Siripongvutikorn *et al.,* 2005). But when the ingredients were made as Keang-hleung paste, the bacterial population was 1.5×10^4 or reduced to around a 2 log cycle when compared with dried chili (Table 4). This may due to the functioning of allicin in garlic, which was similar to the findings of Siripongvutikorn *et al.* (2005). The results further showed that only garlic could provide minimum inhibitory inhibition (MIC) on both *S. sureus* and *B. cereus.* When a higher concentration of garlic extract was applied the killing activity (MBC) occurred as shown in Table 5. The antimicrobial effect of garlic apparently results from the interaction of sulphur compounds, like allicin, with sulphur (thiol) groups of microbial enzymes such as trypsin and other proteases. This leads to an inhibition of microbial growth (Jonkers *et al.,* 1999; Bakri and Douglas, 2005).

Some researchers reported that capsaicin, the main active compound for pungency or heat sensation in hot chili, has an antimicrobial property against *Helicobacter pylori* (Jones *et al.,* 1997). However, there was no antimicrobial effect in dried chili in this study. Since extracted solvent used in this experiment was water then capsaicin compound which has less water solubility was less extracted (Santamaria *et al.,* 2002). Some researchers also found that ethanolic extract of galangal and turmeric rhizome has an antimicrobial activity (Oonmetta-aree *et al.,* 2006; Khattak *et al.,* 2005). This implied that most compounds which have an antimicrobial capacity in galangal and turmeric rhizome could not be well dissolved in water or the polar phase condition. It was also possible that the concentration

and/or purity of active compounds were not high enough to inhibit the test bacteria, and/or the different test organisms (Siripongvutikorn *et al.,* 2005). When the ingredients were blended to become Keang-hleung paste, it showed an even less antimicrobial effect than garlic. This suggests that a certain amount of allicin was derived from garlic. The other authors, they reported that the antimicrobial effect of natural extracts decreased when these extracts were applied to food systems (Shelef, 1984; Stecchini *et al.,* 1993; Pandit and Shelef, 1994).

Table 4. Bacterial load in non - heated basic Keang-hleung paste and its ingredients.

Samples	**Bacteria count (cfu/ml)**
Garlic bulb	2.2×10^2
Galangal rhizomes	3.1×10^4
Turmeric rhizomes	4.1×10^4
Chili (dried pod)	4.3×10^6
Keang-hleung paste	1.5×10^4

Each value was expressed as a mean $\pm$ SD (n=3).

Table 5. Antibacterial activities of non - heated basic Keang - hleung paste and its ingredients.

Spices	**MIC (mg/ml)**		**MBC (mg/ml)**	
	S. aureus	***B. cereus***	***S. aureus***	***B. cereus***
Garlic bulb	0.407	0.409	0.815	3.259
Turmeric rhizomes	-	-	-	-
Galangal rhizomes	-	-	-	-
Chilli (dried pod)	-	-	-	-
Keang-hleung paste	1.306	1.307	2.612	5.224

-: means no activity.

3.3 Total phenolic content and antioxidant activities of non - heated basic Keang - hleung paste and its ingredients.

Some antioxidant activities of plant materials have been derived from phenolic substances as documented by Rice-Evans *et al.* (1996). Table 6 showed the total phenolic content of Keang-hleung paste and its ingredients before heating. The highest level of phenolic content was found in chili, while the lowest content was found in galangal rhizome. Surprisingly, when the ingredients were made to Keang-hleung paste, the total phenolic content increased. This may due to some chemical changes and possessed some new active compounds during the blending step in the paste making process.

DPPH is a free radical compound that has been widely used to determine the free radical scavenging capacity of various samples (Amarowicz *et al.,* 2004; Hatano *et al.,* 1988) because of its stability (in radical form), simplicity and fast assay (Bozin *et al.,* 2008). The DPPH free radical scavenging activity of Keang-hleung paste and its ingredients before heating was presented in Table 6. The results showed that the second highest free radical scavenging activity was found in turmeric rhizome though its total phenolic content was quite low. This was probably due to the high activity of the active compound and/or the synergistic effect of curcuminoid compounds, such as curcumin, demethoxycurcumin and bisdemethoxycurcumin. The active ingredients in turmeric rhizomes are a group of phenolic compounds, including curcumin, which is well known for its strong antioxidant activity (Miquel *et al.,* 2002). However, Jayaprakasha *et al.* (2006) reported that the other two curcuminoids were also effective antioxidants. Moreover, Ruby *et al.* (1995) addressed that curcuminoid compounds found in turmeric rhizomes were potent scavengers of hydroxyl radicals. Darrick *et al.* (2001) also reported that bisdemethoxycurcumin and demethoxycurcumin were good in trapping the DPPH radical.

The lowest free radical scavenging activities were found in garlic and chili, it was found that Keang - hleung paste possessed the highest DPPH free radical - scavenging activity. This may due to synergistic antioxidant activity and similar to the findings of Shobana and Naidu (2000). They reported that spice mix (ginger, onion and garlic; onion and ginger; ginger and garlic) showed accumulative

inhibition of lipid peroxidation. They exhibited a synergistic property when compared with individual ones. It may help to confirm that chemical reaction was performed during grinding, blending or mixing process particularly when there was enough time to stimulate the reaction.

The FRAP assay measures the antioxidant effect of any substance in the reaction medium as its reducing ability (Siddhuraju and Becker, 2007) and is commonly used to study the antioxidant capacity of plant materials (Alothman *et al.,* 2009). The antioxidant potential of Keang-hleung paste and its ingredients was estimated from their ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II) complex. The highest value of FRAP was found in turmeric rhizome and chili (Table 6), while the lowest value was found in garlic. Making Keang-hleung paste did not improve the FRAP activity compared with turmeric and chili. The probable reason for the lower FRAP values could be due to the presence of those compounds not reactive towards FRAP. This could be explained from the basic concept that antioxidants are reducing agents because of their ability to donate a single electron or hydrogen atom for reduction. However, not all reducing agents are antioxidants (Dini *et al.,* 2008). The antioxidant activities of phenolic compounds are mainly due to their redox properties, including free radical scavenging, hydrogen donating and singlet oxygen quenching (Mayachiew and Devahastin, 2008). Pulido *et al.* (2000) reported on the reducing capacity of polyphenols, as determined by a FRAP assay. This seems to depend on the degree of hydroxylation and extent of conjugation of the phenolic compounds. However, the FRAP test can not detect compounds which act by radical quenching (H transfer), particularly thiols and proteins (Cao *et al.,* 1997). Basically, the ferric reducing antioxidant power (FRAP) method is based on the reduction of a ferroin analogue, the Fe^{3+} complex of tripyridyltriazine $Fe(TPTZ)^{3+}$ to the intensely blue-colored Fe^{2+} complex $Fe(TPTZ)^{2+}$ by antioxidants in acidic medium. However, reduction capacity does not necessarily reflex antioxidant activity (Wojdyo *et al.,* 2007).

From the result of DPPH scavenging and FRAP activities in this present work implied that the paste may or may not have an increase of antioxidant properties due to their chemical structure.

Table 6. Total phenolic contents and antioxidation properties of non-heated basic Keang-hleung paste and its ingredients.

Sample	Total phenolic contents (mg GAE/100g)	Scavenging radical (mg GAE/100g)	FRAP (mg GAE/100g)
Garlic bulb	0.30 ± 0.02^{c}	0.38 ± 0.07^{d}	0.03 ± 0.01^{c}
Galangal rhizome	0.20 ± 0.01^{d}	0.65 ± 0.41^{c}	0.05 ± 0.01^{b}
Turmeric rhizome	0.30 ± 0.06^{c}	1.01 ± 0.34^{b}	0.07 ± 0.02^{a}
Chili (dried pod)	0.60 ± 0.06^{b}	0.28 ± 0.65^{d}	0.07 ± 0.02^{a}
Keang-hleung paste	0.80 ± 0.02^{a}	1.77 ± 0.38^{a}	0.05 ± 0.01^{b}

Each value is expressed as a mean $\pm$ SD (n=3).

a-d means that with different letters within a column are significantly difference ($p<0.05$).

3.4 Effect of heating temperature and time on the physical properties of basic Keang - hleung paste and its ingredients.

There were no significant changes in L^*, a^* and b^* values in any sample after heating as presented in Table 7, 8, 9, 10 and 11. It could be explained that galangal rhizome and garlic bulb contained less coloring substance. While pigment in turmeric and dried red chili such as yellow pigment (zeaxanthin, violaxanthin, anteraxanthin, β-cryptoxanthin, β-carotene and curcubixanthin A) and red pigment (capsanthin, capsanthin 5,6-epoxide and capsorubin) carotenoids are high thermoresistant (Ornelas, *et al.,* 2009). Ismail and Revathi (2006) reported slightly changes in color values (L^*, a^* and b^*) in puree of red peppers even treated in a wide range of temperatures. Ornelas *et al.* (2009) also reported that boiling at 96^0C in water for 7-13.5 minutes induced slightly changes in color values (L^*, a^*, and b^*) compared to grilling at 210^0C for 8.8-19 minutes of Mexican peppers.

The pH values of basic Keang-hleung paste and its ingredients increased significantly after heating compared to control (Table 7, 8, 9, 10 and 11). A decrease of pH may cause by heat labile of ascorbic acid property (Patras *et al.,* 2009) and/or other organic acids including some phenolic compounds. Vega-Galvez *et al.*

(2009) reported that an increase in drying temperature caused more ascorbic acid on red pepper particularly in samples dried at 90^0C with a maximum loss of 98.2% ascorbic acid. However, it was found that pH of dried chili was not significantly decreased after heating; this may due to effect of heat resistance of some organic acid (citric acid) not ascorbic acid retaining in the dried chili pod.

Table 7. Effect of heating temperature and time on color and pH values of garlic bulb extracts.

Heating Condition		**color**			**pH**
Temp	**Time (minutes)**	***L****	***a****	***b****	
Control	0	62.82±0.40 [a]	-4.26±0.70 [a]	18.67±0.15 [a]	6.11±0.05 [b]
80 ^{0}C	10	62.50±0.71 [a]	-4.28±0.14 [a]	18.63±0.07 [a]	6.23±0.02 [a]
	20	62.96±0.89 [a]	-4.33±0.47 [a]	18.72±0.14 [a]	6.24±0.04 [a]
	30	62.38±0.47 [a]	-4.35±0.18 [a]	18.66±0.23 [a]	6.22±0.04 [a]
90 ^{0}C	10	62.45±0.81 [a]	-4.33±0.25 [a]	18.87±0.27 [a]	6.22±0.03 [a]
	20	62.86±0.71 [a]	-4.39±0.07 [a]	18.84±0.59 [a]	6.20±0.04 [a]
	30	62.90±0.39 [a]	-4.31±0.25 [a]	18.74±0.59 [a]	6.21±0.03 [a]
100 ^{0}C	10	63.07±0.45 [a]	-4.06±0.20 [a]	18.84±0.62 [a]	6.21±0.04 [a]
	20	62.92±0.34 [a]	-4.13±0.11 [a]	18.62±0.56 [a]	6.22±0.02 [a]
	30	62.75±0.34 [a]	-4.07±0.19 [a]	18.91±0.21 [a]	6.20±0.02 [a]

Each value is expressed as a mean $\pm$ SD (n=3).

a-b means that with different letters within a column are significantly difference ($p<0.05$).

Table 8. Effect of heating temperature and time on color and pH values of galangal rhizome extracts.

Heating condition		color			pH
Temp	**Time (minutes)**	***L****	***a****	***b****	
Control	0	65.19±0.19 [a]	-0.62±0.01 [a]	5.82±0.01 [a]	4.77 ±0.02 [b]
80 ^{0}C	10	64.32±0.18 [a]	-0.60±0.03 [a]	5.84±0.09 [a]	5.14 ±0.02 [a]
	20	64.81±0.68 [a]	-0.67±0.06 [a]	5.84±0.08 [a]	5.18 ±0.04 [a]
	30	64.57±0.65 [a]	-0.61±0.03 [a]	5.84±0.02 [a]	5.17 ±0.02 [a]
90 ^{0}C	10	64.62±0.94 [a]	-0.69±0.04 [a]	5.82±0.03 [a]	5.17 ±0.03 [a]
	20	64.82 ±0.52 [a]	-0.68±0.05 [a]	5.87±0.27 [a]	5.16 ±0.03 [a]
	30	64.56 ±0.53 [a]	-0.66±0.72 [a]	5.85±0.34 [a]	5.15 ±0.01 [a]
100 ^{0}C	10	64.62±0.78 [a]	-0.64±0.03 [a]	5.96±0.28 [a]	5.15 ±0.02 [a]
	20	64.76 ±0.35 [a]	-0.68±0.04 [a]	5.92±0.47 [a]	5.16 ±0.01 [a]
	30	64.99±0.38 [a]	-0.66±0.08 [a]	6.04±0.62 [a]	5.15 ±0.12 [a]

Each value is expressed as a mean $\pm$ SD (n=3).

a-b means that with different letters within a column are significantly difference ($p<0.05$).

Table 9. Effect of heating temperature and time on color and pH values of turmeric rhizome extracts.

Heating condition		**color**			**pH**
Temp	**Time (minutes)**	***L****	***a****	***b****	
Control	0	42.96±0.93 [a]	18.49±0.22 [a]	62.81±0.74 [a]	6.29±0.05 [b]
80 ^{0}C	10	42.47±0.61 [a]	18.26±0.20 [a]	62.86±0.09 [a]	6.39±0.06 [a]
	20	42.84±0.13 [a]	18.24±0.12 [a]	63.01±0.40 [a]	6.38±0.06 [a]
	30	43.22±0.23 [a]	18.34±0.55 [a]	62.83±0.46 [a]	6.44±0.05 [a]
90 ^{0}C	10	42.58±0.09 [a]	18.40±0.16 [a]	63.06±0.05 [a]	6.42±0.04 [a]
	20	42.52±0.17 [a]	18.35±0.42 [a]	62.89±0.06 [a]	6.41±0.05 [a]
	30	42.72±0.32 [a]	18.32±0.32 [a]	63.07±0.18 [a]	6.43±0.02 [a]
100 ^{0}C	10	42.67±0.52 [a]	18.24±0.07 [a]	63.02±0.11 [a]	6.43±0.03 [a]
	20	42.63±0.24 [a]	18.25±0.56 [a]	62.83±0.14 [a]	6.42±0.03 [a]
	30	42.64±0.33 [a]	18.32±0.31 [a]	62.81±0.34 [a]	6.41±0.04 [a]

Each value is expressed as a mean $\pm$ SD (n=3).

a-b means that with different letters within a column are significantly difference ($p<0.05$).

Table 10. Effect of heating temperature and time on color and pH values of dried chili (dried pod) extracts.

Heating condition		**color**			**pH**
Temp	**Time (minutes)**	***L****	***a****	***b****	
Control	0	18.90±0.05 [a]	23.39±0.07 [a]	28.90±0.04 [a]	5.97±0.01 [a]
80 ^{0}C	10	19.08±0.04 [a]	23.50±0.08 [a]	29.28±0.07 [a]	6.03±0.02 [a]
	20	18.98±0.31 [a]	23.50±0.06 [a]	29.18±0.06 [a]	5.99±0.03 [a]
	30	19.06±0.18 [a]	23.75±0.63 [a]	29.04±0.34 [a]	6.03±0.05 [a]
90 ^{0}C	10	19.04±0.20 [a]	23.46±0.47 [a]	28.91±0.45 [a]	6.02±0.06 [a]
	20	18.98±0.23 [a]	23.75±0.47 [a]	28.94±0.24 [a]	5.98±0.03 [a]
	30	18.93±0.18 [a]	23.51±0.56 [a]	28.95±0.09 [a]	6.01±0.02 [a]
100 ^{0}C	10	19.07±0.25 [a]	23.80±0.54 [a]	29.01±0.27 [a]	6.02±0.02 [a]
	20	19.00±0.19 [a]	23.60±0.62 [a]	28.94±0.36 [a]	6.01±0.06 [a]
	30	19.07±0.12 [a]	23.52±0.28 [a]	29.10±0.60 [a]	6.00±0.03 [a]

Each value is expressed as a mean $\pm$ SD (n=3).

a means that within a column is not significantly difference ($p<0.05$).

Table 11. Effect of heating temperature and time on color and pH values of basic Keang - hleung paste extracts.

Heating condition		color			pH
Temp	**Time (minutes)**	***L****	***a****	***b****	
Control	0	37.98 ±0.37 [a]	32.40 ±0.15 [a]	56.97 ±0.40 [a]	5.56 ±0.01 [b]
80 ^{0}C	10	37.94 ±0.25 [a]	32.46 ±0.51 [a]	57.00 ±0.38 [a]	5.70 ±0.02 [a]
	20	37.81 ±0.37 [a]	32.47 ±0.10 [a]	56.78 ±0.49 [a]	5.72 ±0.02 [a]
	30	38.11 ±0.38 [a]	32.40 ±0.45 [a]	56.85 ±0.46 [a]	5.72 ±0.46 [a]
90 ^{0}C	10	38.06 ±0.42 [a]	32.65 ±0.56 [a]	57.27 ±0.15 [a]	5.74 ±0.02 [a]
	20	37.95 ±0.32 [a]	32.78 ±0.34 [a]	56.88 ±0.43 [a]	5.73 ±0.01 [a]
	30	37.87 ±0.29 [a]	32.53 ±0.15 [a]	56.92 ±0.24 [a]	5.74 ±0.01 [a]
100 ^{0}C	10	38.07 ±0.20 [a]	32.92 ±0.39 [a]	57.38 ±0.17 [a]	5.73 ±0.05 [a]
	20	37.66 ±0.85 [a]	32.48 ±0.37 [a]	56.81 ±0.49 [a]	5.72 ±0.01 [a]
	30	38.02 ±0.37 [a]	32.42 ±0.37 [a]	57.28 ±0.20 [a]	5.73 ±0.04 [a]

Each value is expressed as a mean $\pm$ SD (n=3).

a-b means that with different letters within a column are significantly difference ($p<0.05$).

3.5 Effect of heating temperature and time on antibacterial activities of basic Keang - hleung paste and its ingredients.

Table 12 showed the effect of heating temperature and time on antibacterial activities of garlic extracts. The antibacterial activities of garlic extracts were decreased when temperature increased and there were no activity when the samples were heated at 100^{0}C for 20 minutes. This study similar to the findings of Shashikanth *et al.* (1981) and Sato *et al.* (1990) they reported that the inhibitory component of garlic extracts was completely destroyed by autoclaving or by heating the extract at 100°C for 20 minutes. This may due to allicin is not heat stable or may convert to some form that has no or little antimicrobial activity (Wilknison, 1997; Ankri and Mirelman, 1999). In addition, antibacterial activities of Keang-hleung paste

extracts could remain when the paste was heated at 80^0C for 10 minutes (Table 13). It point out that lower concentration of allicin in the paste seemed to be completely destroyed at lower temperature compared with garlic extract containing high amount of allicin. Moreover, the result showed that *B. cereus* was more resistant to antibacterial activity derived from garlic and the paste. This may due to environmental resistant of spore forming bacteria (Iurlina *et al.,* 2006).

Table 12. Effect of heating temperature and time on antibacterial activities of garlic bulb extracts.

Heating condition		**MIC (mg/ml)**		**MBC (mg/ml)**	
Temp	**Time**	***S. aureus***	***B. cereus***	***S. aureus***	***B. cereus***
Control	0	0.415	0.417	0.830	1.251
80 ^{0}C	10	0.415	0.417	0.830	1.251
	20	0.415	0.417	0.830	1.251
	30	0.415	0.417	0.830	1.251
90 ^{0}C	10	0.830	0.834	1.660	1.668
	20	0.830	0.834	1.660	1.668
	30	0.830	0.834	1.660	1.668
100 ^{0}C	10	1.660	Nd	2.075	Nd
	20	Nd	Nd	Nd	Nd
	30	Nd	Nd	Nd	Nd

Nd = Not detected.

Table 13. Effect of heating temperature and time on antibacterial activities of basic Keang - hleung paste extracts.

Heating condition		**MIC (mg/ml)**		**MBC (mg/ml)**	
Temp	**Time**	***S. aureus***	***B. cereus***	***S. aureus***	***B. cereus***
Control	0	1.308	2.616	1.308	5.232
80 ^{0}C	10	2.616	5.232	2.616	10.464
	20	Nd	Nd	Nd	Nd
	30	Nd	Nd	Nd	Nd
90 ^{0}C	10	Nd	Nd	Nd	Nd
	20	Nd	Nd	Nd	Nd
	30	Nd	Nd	Nd	Nd
100 ^{0}C	10	Nd	Nd	Nd	Nd
	20	Nd	Nd	Nd	Nd
	30	Nd	Nd	Nd	Nd

Nd = Not detected.

3.6 Effect of heating temperature and time on total phenolic contents and antioxidant properties of Keang-hleung paste and its ingredients.

A total phenolic content of garlic, galangal rhizome and dried chili were slightly decreased when increased heating temperature and time as shown in Table 14, 15 and 16, respectively. It point out that these phenolic compounds were not heat stable. While, Jastrzebski *et al.* (2007) who reported that a decrease in the content of total polyphenols of cooked garlic and its antioxidant activities determined by FRAP and DPPH assays were significantly different only after cooking at 100^{0}C after 40 minutes ($p < 0.05$). However, it was accepted that total phenolic content in turmeric rhizome after heat treatment did not destroy as shown in Table 17 that would be described later. Surprisingly, when the ingredients were made into the curry paste, the total phenolic content increased as previously described. Additionally, after heating the total phenolic content of the curry paste tended to increase when heating temperature and time increased (Table 18). An increase of total phenolic content in some plants after heating may be due to the disruption of the plant cell wall, and

therefore bound polyphenolic and flavonoid compounds may be released more easily than those in fresh plants (Peleg *et al.,* 1991). This is similar to the finding of Choi *et al.* (2006) who reported that concentration of free polyphenolic and flavonoid compounds in heated shiitake mushrooms were significantly higher than in raw mushrooms. Guihua *et al.* (2007) also found that the heating process improve phenolic content due to the cleaving of bound esterifies bound and glycosylated, thus leading to the increase of free forms. Another probable reason for an increase of phenolic content in the heated sample is the decrease/inhibition of enzymatic oxidation causing loss in the antioxidant compounds in the raw plant material (Dewanto *et al.,* 2002; Nicoli *et al.,* 1999). On the other hands, the formation of phenolic compounds at high temperatures might be because of the availability of precursors of phenolic molecules by non-enzymatic interconversion between phenolic molecules (Que *et al.,* 2008).

The effect of heating on DPPH radical activity of garlic, galangal rhizome and dried chili was slightly decreased (Table 14, 15 and 16) as temperature and time increased and related to decrease of total phenolic content. This may due to loss of bioactive compounds as previously described. However, free radical scavenging of turmeric rhizome was stable as shown in Table 17. Moreover, it was found that heated basic Keang-hleung showed a slightly increase of free radical scavenging when the heating temperature and heating time increased (Table 18). An interesting observation in basic Keang-hleung was that increased heating temperature did not only lower the antioxidant activity but also showed gradually higher antioxidant activity ($p<0.05$). The high correlation between the mean values of total phenolic content and DPPH in the basic paste was observed as $R^2=0.986$. This indicated that the compound present in Keang-hleung paste was heat resistant and high potent reducing DPPH radicals. This was similar to the finding of Maria *et al.* (2002) who reported that curcuminoids were heat stable. In addition, new active compound formation (Dewanto *et al.,* 2002; Kim *et al.,* 2002) or a release of bound antioxidant during heating process (Shobana and Naidu, 2000) may occur. Another reason may due to breaking down of complex molecule, curcumin to simple molecule as ferulic acid which yield higher antioxidant activity.

Many researchers found that heat treatment could either reduce or retain the DPPH scavenging activity. However, the good correlation between total phenol analysis and the antioxidative assays has been previously reported (Zheng and Wang, 2001). From the present study it can be suggested that greater total phenolic content can be translated into increased DPPH activity. This may be due to curcuminoid, a major active agent in turmeric, which is a strong H^+ donor (Pulla and Lokesh, 1992) and thermally stable (Maria *et al.,* 2002). This confirmed that any food containing turmeric rhizome could bring health benefits and antioxidant properties even when thermally processed.

FRAP of garlic, galangal rhizome and dried chili extracts were slightly decreased (Table 14, 15 and 16) after heat treatment as decreased of total phenolic content may due to loss of bioactive compounds as previously mentioned. Ferric reducing antioxidant power of turmeric rhizome (Table 17) was stable as described above. The FRAP of heated Keang-hleung paste was significantly higher compared with non - heated basic Keang - hleung paste, the control sample (Table 18). A reasonable correlation between the mean values of total phenolic contents and FRAP of R^2=0.552 was observed. This indicated that compounds present in basic Keang-hleung paste provided some reducing ability. It implied that heat treatment could improve both DPPH radical scavenging and FRAP activities. Bentoncelj *et al.* (2007) found a strong relationship between antioxidant capacity evaluated by the FRAP assay and the phenolic content of honey.

Table 14. Effect of heating temperature and time on total phenolic contents and antioxidation properties of garlic bulb extracts.

Heating condition		Total phenolic contents (mg GAE/100g)	Scavenging radical (mg GAE/100g)	FRAP (mg GAE/100g)
Temp	**Time (minutes)**			
Control	0	0.23±0.02 [a]	0.35±0.03 [a]	0.025±0.004 [a]
80 ^{0}C	10	0.22±0.02 [a]	0.34±0.04 [a b]	0.024±0.006 [a]
	20	0.21±0.03 [a]	0.33±0.03 [a b]	0.024±0.004 [a]
	30	0.21±0.04 [a]	0.31±0.06 [a b]	0.023±0.006 [a]
90 ^{0}C	10	0.19±0.02 [a]	0.31±0.03 [a b]	0.023±0.003 [a]
	20	0.19±0.03 [a]	0.30±0.03 [a b]	0.022±0.004 [a]
	30	0.18±0.04 [a]	0.29±0.05 [a b]	0.020±0.003 [a]
100 ^{0}C	10	0.18±0.03 [a]	0.28±0.03 [a b]	0.019±0.004 [a]
	20	0.18±0.06 [a]	0.28±0.01 [b]	0.019±0.003 [a]
	30	0.17±0.04 [a]	0.27±0.04 [b]	0.018±0.003 [a]

Each value is expressed as a mean $\pm$ SD (n=3).

a-b means that with different letters within a column are significantly difference ($p<0.05$).

Table 15. Effect of heating temperature and time on total phenolic contents and antioxidation properties on galangal rhizome extracts.

Heating condition		Total phenolic contents (mg GAE/100g)	Scavenging radical (mg GAE/100g)	FRAP (mg GAE/100g)
Temp	**Time (minutes)**			
Control	0	0.34±0.04 [a]	0.58±0.04 [a]	0.077±0.006 [a]
80 ^{0}C	10	0.32±0.03 [a b]	0.57±0.05 [a b]	0.075±0.007 [a]
	20	0.30±0.05 [a b c]	0.56±0.03 [a b]	0.074±0.005 [a]
	30	0.29±0.05 [a b c]	0.55±0.04 [a b]	0.073±0.007 [a]
90 ^{0}C	10	0.28±0.04 [a b c]	0.54±0.03 [a b]	0.072±0.006 [a]
	20	0.27±0.02 [a b c]	0.53±0.05 [a b]	0.071±0.003 [a]
	30	0.26±0.02 [b c]	0.52±0.04 [a b]	0.070±0.006 [a]
100 ^{0}C	10	0.25±0.03 [b c]	0.50±0.04 [a b]	0.069±0.005 [a]
	20	0.24±0.03 [c]	0.51±0.03 [a b]	0.068±0.006 [a]
	30	0.24±0.03 [c]	0.50±0.05 [b]	0.067±0.005 [a]

Each value is expressed as a mean $\pm$ SD (n=3).

a-c means that with different letters within a column are significantly difference ($p<0.05$).

Table 16. Effect of heaing temperature and time on total phenolic contents and antioxidation properties on chili (dried pod) extracts.

Heating condition		Total phenolic contents (mg GAE/100g)	Scavenging radical (mg GAE/100g)	FRAP (mg GAE/100g)
Temp	**Time (minutes)**			
Control	0	0.50±0.05 [a]	0.29±0.03 [a]	0.065±0.003 [a]
80 ^{0}C	10	0.49±0.03 [a b]	0.28±0.02 [a]	0.065±0.004 [a]
	20	0.46±0.03 [a b c]	0.28±0.04 [a b]	0.065±0.003 [a]
	30	0.45±0.02 [a b c d]	0.27±0.02 [a b]	0.063±0.005 [a b]
90 ^{0}C	10	0.44±0.04 [a b c d]	0.26±0.03 [a b]	0.060±0.004 [a b c]
	20	0.42±0.05 [b c d]	0.25±0.03 [a b]	0.059±0.005 [a b c]
	30	0.41±0.05 [c d]	0.24±0.04 [a b]	0.058±0.004 [a b c]
100 ^{0}C	10	0.40±0.05 [c d]	0.23±0.05 [a b]	0.057±0.008 [a b c]
	20	0.38±0.04 [d]	0.23±0.06 [a b]	0.055±0.005 [b c]
	30	0.38±0.04 [d]	0.22±0.02 [b]	0.054±0.003 [c]

Each value is expressed as a mean $\pm$ SD (n=3).

a-d means that with different letters within a column are significantly difference ($p<0.05$).

Table 17. Effect of heating temperature and time on total phenolic contents and antioxidation properties on turmeric rhizome extracts.

Heating condition		Total phenolic contents (mg GAE/100g)	Scavenging radical (mg GAE/100g)	FRAP (mg GAE/100g)
Temp	**Time (minutes)**			
Control	0	0.31±0.03 [a]	1.16±0.04 [a]	0.050±0.004 [a]
80 ^{0}C	10	0.31±0.02 [a]	1.16±0.02 [a]	0.048±0.003 [a]
	20	0.30±0.04 [a]	1.15±0.03 [a]	0.047±0.005 [a]
	30	0.30±0.05 [a]	1.16±0.05 [a]	0.048±0.003 [a]
90 ^{0}C	10	0.29±0.04 [a]	1.15±0.04 [a]	0.049±0.003 [a]
	20	0.30±0.03 [a]	1.17±0.05 [a]	0.049±0.004 [a]
	30	0.31±0.04 [a]	1.17±0.02 [a]	0.049±0.007 [a]
100 ^{0}C	10	0.29±0.03 [a]	1.15±0.02 [a]	0.047±0.004 [a]
	20	0.29±0.04 [a]	1.17±0.01 [a]	0.050±0.005 [a]
	30	0.30±0.06 [a]	1.17±0.03 [a]	0.048±0.004 [a]

Each value is expressed as a mean $\pm$ SD (n=3).

a means that within a column is not significantly difference ($p \geq 0.05$).

Table 18. Effect of heating temperature and time on total phenolic contents and antioxidation properties on basic Keang-hleung paste extracts.

Heating of Keang-hleung paste		Total phenolic contents (mg GAE/100g)	Scavenging radical (mg GAE/100g)	FRAP (mg GAE/100g)
Temp	Time (minutes)			
Control	0	0.81 ± 0.02^{d}	1.75 ± 0.05^{e}	0.063 ± 0.005^{b}
80 ^{0}C	10	0.82 ± 0.02^{cd}	1.77 ± 0.11^{de}	0.062 ± 0.004^{b}
	20	0.83 ± 0.04^{bc}	1.79 ± 0.03^{cde}	0.065 ± 0.002^{b}
	30	0.85 ± 0.04^{abc}	1.81 ± 0.05^{bcde}	0.079 ± 0.004^{b}
90 ^{0}C	10	0.87 ± 0.04^{abc}	1.82 ± 0.06^{abcd}	0.080 ± 0.006^{a}
	20	0.89 ± 0.07^{abc}	1.85 ± 0.05^{abc}	0.078 ± 0.003^{a}
	30	0.89 ± 0.04^{ab}	1.87 ± 0.07^{abc}	0.078 ± 0.003^{a}
100 ^{0}C	10	0.93 ± 0.02^{ab}	1.92 ± 0.07^{ab}	0.079 ± 0.007^{a}
	20	0.97 ± 0.07^{ab}	1.95 ± 0.02^{ab}	0.077 ± 0.002^{a}
	30	1.06 ± 0.13^{a}	1.98 ± 0.06^{a}	0.079 ± 0.005^{a}

Each value is expressed as a mean $\pm$ SD (n=3).

a-e means that with different letters within a column are significantly difference ($p<0.05$).

3.7 Sensory evaluation of basic and developed Keang-hleung paste and their curry soups.

Sensory scores of basic Keang-hleung and developed Keang-hleung paste (D_1 and $D_{2)}$ and their curry soup were showed in Table 19 and 20, respectively. The results showed that there was no significant difference between score of basic and D_1 paste in terms of appearance, color, odor and overall liking. Additionally, their curry soup were also not significantly different in terms of appearance, color, odor, spiciness and overall liking scores. However, addition of more garlic and turmeric rhizome in D_2 paste reduced sensory acceptability in all attributes compared with basic and D_1 sample. When, curry soup from D_2 was evaluated, it was found that color, odor, appearance and overall liking were significantly lower scoring (Table 20).

This may due to stronger pungent taste and flavor from garlic and turmeric rhizome. Siripongvutikorn (2008) reported that improving antibacterial activity by adding much more garlic caused significantly lower consumer preference compared with basic Tom-Yum soup. It point out that enhancing some activities may alter consumer acceptance that was limitation use of any ingredient in food system as consumer is powerful.

Table 19. Sensory score of basic and developed Keang-hleung paste.

Treatment	**Sensory score**			
	Appearance	**Color**	**Odor**	**Overall liking**
Basic Keang-hleung paste	7.07±0.69 [a]	7.30±0.65 [a]	6.93±0.64 [a]	7.23±0.68 [a]
Developed Keang-hleung paste (D_1)	7.27±0.69 [a]	7.50±0.73 [a]	7.17±0.65 [a]	7.10±0.71 [a]
Developed Keang-hleung paste (D_2)	6.63±0.77 [b]	5.83±0.75 [b]	5.87±0.73 [b]	6.73±0.69 [b]

a-b means that with different letters within a column are significantly difference ($p<0.05$).

Table 20. Sensory score of basic and developed Keang-hleung curry soup.

Treatment	Sensory score						
	Appearance	**Color**	**Odor**	**Sourness**	**Saltiness**	**Spiciness**	**Overall liking**
Basic Keang-hleung paste	7.40±0.56 [a]	7.13±0.78 [a]	7.23±0.77 [a]	7.13±0.68 [a]	7.07±0.74 [a]	7.40±0.68 [a]	7.20±0.66 [a]
Developed Keang-hleung paste (D_1)	7.23±0.73 [a]	7.23±0.63 [a]	7.30±0.65 [a]	7.20±0.71 [a]	7.03±0.70 [a]	7.37±0.77 [a]	7.17±0.75 [a]
Developed Keang-hleung paste (D_2)	5.87±0.73 [b]	5.73±0.69 [b]	5.90±0.71 [b]	7.10±0.71 [a]	6.80±0.61 [a]	7.33±0.66 [a]	5.93±0.74 [b]

a-b means that with different letters within a column are significantly difference ($p<0.05$)

3.8 The Physical properties of basic and developed Keang-hleung paste.

As mentioned in section 3.7, a lower sensory score occurred when the paste was added with higher garlic content (from 36% to 42.54%), therefore only D_1 was selected to evaluate the other qualities.

The color measured as *L**, *a** and *b** values of the basic as control and developed Keang-hleung paste (D_1) were shown in Table 21. But physical properties of D_2 was not determined due to sensory scores in all attributes were lower than scores of basic paste. The result showed that *L** values of the basic and developed Keang-hleung paste were not significantly different. The developed Keang-hleung paste has lower *a** values than control, this may due to lower amount of dried chili. On the other hands, *b** values was not significant.

The pH values of basic and developed Keang-hleung paste were shown in Table 21. The values both treatment were not significant different may due to similar used ingredients.

Table 21. Color and pH values of basic and developed Keang - hleun paste.

Sample	**Color**			**pH**
	L*	***a****	***b****	
Basic Keang-hleung paste	35.93±0.31 [a]	31.80±0.14 [a]	56.15±0.64 [a]	5.85±0.03 [a]
Developed Keang-hleung paste (D_1)	35.90±0.19 [a]	30.66±0.35 [b]	57.05±0.52 [a]	5.87±0.06 [a]

Each value is expressed as a mean ± SD (n=3).

a-b means that with different letters within a column are significantly difference ($p<0.05$).

3.9 Microbiological quality in basic and developed Keang-hleung paste.

The microbiological quality in basic and developed Keang-hleung paste was expressed in Table 22. It was found that TVC values were medium-high but still under standard limit as 10^6 to 10^7 cfu/g (ICMSF, 1986) in both basic and developed Keang-hleung paste. This indicated that a fair sanitary of producer. Based

on TVC value, it may not address that addition more garlic content could retard microorganism growth. However, Siripongvutikorn *et al.* (2008) reported that antibacterial property from Tom-Yum paste would definitely occur after storage 1 day as time consuming of alliinase producing allicin, major antibacterial agent in garlic bulb.

Table 22. Microbiological quality in basic and developed Keang - hleung paste.

Treatment	**Bacteria count (cfu/g)**			
	TVC	**Yeast and Mold**	***E. coli***	***B. cereus***
Basic Keang-hleung paste	1.8×10^4	<10	<3 MPN/g	<30
Developed Keang-hleung paste (D_1)	1.3×10^4	<10	<3 MPN/g	<30

3.10 The antibacterial activities of basic and developed Keang-hleung paste.

The antibacterial activities developed Keang-hleung paste was higher than basic Keang-hleung paste as showed in Table 23. It may due to more garlic content yielding higher allicin in developed Keang-hleung paste when compared with basic one. Additionally, the results revealed that MIC of basic Keang-hleung paste in this section was similar to previous section (Table 23) except MBC property. This may due to variation of spices used in the paste leading to inconstant of active compound content.

Table 23. Antibacterial activities of basic and developed Keang - hleung paste.

Treatment	MIC (mg/ml)		MBC (mg/ml)	
	S. aureus	***B. cereus***	***S. aureus***	***B. cereus***
Basic Keang-hleung paste	1.312	2.624	3.248	6.496
Developed Keang-hleung paste (D_1)	0.752	1.504	2.008	4.016

3.11 Total phenolic contents and antioxidation properties of basic and developed Keang-hleung paste.

The total phenolic contents of basic and developed Keang-hleung paste were showed in Table 24. Increasing of total phenolic contents in developed Keang-hleung was occurred as an increase of 1.72% turmeric rhizome from basic Keang-hleung paste. This may due to cumulative of phenolic and the synergistic property of the active compounds in the paste. Shobana and Naidu (2000) reported that spice extract mixes showed cumulative inhibition of lipid peroxidation indicating synergistic antioxidative activity. This study might contain more than one antioxidant. However, when comparing of Total phenolic content, DPPH scavenging and ferric reducing power of basic Keang-hleung paste were 0.85 ± 0.07, 1.75 ± 0.51 and 0.06 ± 0.01 mg GAE/100g and previous Keang-hleung paste were 0.80 ± 0.02, 1.77 ± 0.38 and 0.05 ± 0.01 mg GAE/100g, respectively there were no difference.

A good free radical scavenging was found in developed Keang-hleung paste as shown in Table 24 compared with control sample. This may due to the high activity of the active compound and/or the synergistic effect of curcuminoid compounds as previously described. Antioxidant activity correlated significantly and positively with total phenolics (Kaur and Kapoor, 2002). Also similar to the findings of Maisuthisakul *et al.* (2007) reported that there was a distinct correlation between studied parameters i.e. total phenolic content, total flavonoid content and antiradical activity in selected Thai indigenous plant parts such as berries and fruits, herbs and vegetables, and chewing plants.

The FRAP activity was increased as total phenolic content increased in developed Keang-hleung paste compared with control sample (Table 24). This study was similar to the findings of Wojdylo *et al.* (2007) reported that a positive relationship between total phenolic contents and FRAP of herbals in Labiatae such as *Salvia officinalis, Origanum vulgare, Marrubium vulgare, Rosmarinus officinalis* and *Melisa officinalis* and Compocitae such as *Artemisia vulgaris, Inula helenium, Silybum marianum, Taraxacum officinale* and *Tanacetum vulgare*.

Table 24. Total phenolic contents of developed Keang-hleung paste.

Sample	Total phenolic contents (mg GAE/100g)	Scavenging radical (mg GAE/100g)	FRAP (mg GAE/100g)
Basic Keang-hleung paste	0.85±0.07 [b]	1.75±0.51 [b]	0.58±0.01 [b]
Developing Keang-hleung paste	1.19±0.19 [a]	2.22±0.71 [a]	0.76±0.02 [a]

Each value is expressed as a mean ± SD (n=3).

a-b means that with different letters within a column are significantly difference ($p<0.05$).

3.12 Physical properties of developed Keang-hleung paste with added salt during storage at 4±2°C.

In general, the company producing curry paste would add some NaCl as preservative agent while, SMEs may not add any NaCl if they could sell the paste within 1-2 days as request of consumer using. Therefore, addition of NaCl into the paste was evaluated in this section.

The value of L^*, a^* and b^* in the developed Keang-hleung paste with 0%, 10% and 20% salts concentration and kept in varied temperature during storage were presented in Table 25. The color values of developed Keang-hleung paste with 0% and 10% salts concentration and kept at ambient temperature were changed only in 3 days. However, the L^*, a^* and b^* values of the developed Keang-hleung paste

with 0% and 10% salts concentration kept in refrigerator decreased during 30 days of storage period. While the developed Keang-hleung paste with added 20% salts concentration kept in refrigerator and at ambient temperature, the L^*, a^* and b^* values decreased during 45 days of storage period. This may probably due to degradation of β-carotene during storage period, since discoloration might result from oxidation of carotenoids (Varoquaux and Wiley, 1994; Lamikanra and Watson, 2000). Ketsa and Pangkool (1994, 1995) reported that the fading of pulp color was most probably due to degradation of β-carotene that contributed to durian color. Moreover, the curcuminoids are readily decomposed when exposed to bright light (Schieffer, 2002).

The pH values during storage time of the Keang-hleung paste were presented in Table 25, the result show that pH values increased at 30 days during storage. This may due to decreasing of ascorbic acid during storage as effect of storage conditions such as temperature, oxygen and light access (Kabasakalis *et al.,* 2000; Zerdin *et al.,* 2003). Klimczak *et al.* (2007) also reported that the decrease in the content of vitamin C upon storage conditions.

Table 25. Effect of added salt on color and pH values of developed Keang - hleung paste during storage at 4±2°C.

Treatment	Storage (weeks)	color			pH
		*L**	*a**	*b**	
control	0	36.06±0.54	32.72±0.45	56.95±0.51	5.89±0.04
	2	ND	ND	ND	ND
	4	ND	ND	ND	ND
Salt 0% 4 ^{0}C	0	35.86±0.14 ^{a}	32.59±0.25 ^{a}	56.99±0.61 ^{a}	5.90±0.03 ^{b}
	2	35.93±0.14 ^{a}	32.69±0.32 ^{a}	57.09±0.41 ^{a}	5.88±0.03 ^{b}
	4	37.76±0.66 ^{b}	29.82±0.32 ^{b}	55.70±0.42 ^{b}	6.75±0.03 ^{a}
Salt 0% AT	0	35.92±0.33	32.66±0.20	57.05±0.72	5.89±0.02
	2	ND	ND	ND	ND
	4	ND	ND	ND	ND
Salt 10% 4 ^{0}C	0	36.05±0.30 ^{b}	32.64±0.10 ^{a}	57.02±0.66 ^{a}	5.90±0.02 ^{b}
	2	35.97±0.43 ^{b}	32.73±0.56 ^{a}	56.91±0.37 ^{a}	6.01±0.05 ^{b}
	4	37.59±0.41 ^{a}	29.89±0.29 ^{b}	55.73±0.49 ^{b}	6.61±0.13 ^{a}
Salt 10% AT	0	36.02±0.29	32.67±0.11	56.85±0.49	5.89±0.04
	2	ND	ND	ND	ND
	4	ND	ND	ND	ND
Salt 20% 4 ^{0}C	0	35.96±0.29 ^{a}	32.64±0.10 ^{a}	57.02±0.57 ^{a}	5.91±0.09 ^{b}
	2	36.02±0.22 ^{a}	32.68±0.45 ^{a}	57.00±0.18 ^{a}	5.87±0.04 ^{b}
	4	35.90±0.23 ^{a}	32.79±0.37 ^{a}	57.04±0.27 ^{a}	6.68±0.08 ^{a}
Salt 20% AT	0	36.07±0.29 ^{b}	32.57±0.01 ^{a}	57.05±0.36 ^{a}	5.91±0.01 ^{b}
	2	35.93±0.19 ^{b}	32.69±0.44 ^{a}	56.92±0.11 ^{a}	5.86±0.02 ^{b}
	4	37.66±0.32 ^{a}	29.77±0.52 ^{b}	55.91±0.51 ^{b}	6.66±0.16 ^{a}

ND = Not determined as end of shelflife evaluated by total viable count (≥ 10^6 cfu/g).

AT = Ambient temperature.

a-b means that with different letters within a column in the same condition are significantly different ($p<0.05$).

3.13 Microbiological quality in developed Keang-hleung paste with added salt during storage at 4±2°C.

Developed Keang-hleung paste with added salts and stored at ambient temperature and 4°C were monitored for TVC, yeasts and moulds, *Staphylococcus aureus*, *Bacillus cereus, Escherichia coli* and coliforms. At the initial stage, TVC of all treatments were in the range of 10^5 log cfu/g and increased as the storage time (Table 26). Compared with previous section, it was found that TVC values of this work were higher approximately a log cycle may due to cross contamination during paste processing from small and medium enterprises (SME) as production process. This present work point out the good manufacturing practice (GMP) is another key success factor for controlling microbiological standard in real situation even laboratoey work is already proved. In addition, TVC of the paste without and with added 10% salts and kept at ambient temperature were more than 10^6 log cfu/g within 3 days. While, TVC of the paste with and without added 10% salts concentration and kept in refrigerator were reach the standard (> 10^6 log cfu/g) within 30 days. However, the paste with added 20% and kept at ambient temperature as well as kept in refrigerator could control TVC values at 10^6 log cfu/g within 45 days. This suggested that storage temperature seemed to play more important role compared with added 10% salts. However, using higher salt concentration as 20% and chilled storage as hurdle have more effect on growth of microorganism. Yeast and mould counts of all treatments were less than 30 cfu/g during storage. Moreover, *S. aureus*, *B. cereus, E. coli* and coliforms were not detected in all treatments throughout the storage period. This might be because of allicin function as previously described and fair sanitation of producer. Generally, washing step would have positive side as removing dust source of microorganism and negative side as added moisture or A_w into sample. And as well known that bacteria prefer more A_w. In addition, washing step may reduce mold and yeast but not bacteria.

Table 26. Microbiological quality in developed Keang-hleung paste with added salt during storage at 4±2°C.

Treatment	Bacteria count (cfu/g)					
	Storage (weeks)	TVC	Yeast and Mold	*E. coli*	*B. cereus*	*S. aureus*
control	0	$1.25x10^5$	10	<3 MPN/g	<30	<30
	2	$\geq 10^6$	ND	ND	ND	ND
	4	$\geq 10^6$	ND	ND	ND	ND
Salt 0% 4 ^{0}C	0	$1.32x10^5$	12	<3 MPN/g	<30	<30
	2	$3.8x10^5$	15	<3 MPN/g	<30	<30
	4	$\geq 10^6$	ND	ND	ND	ND
Salt 0% AT	0	$1.27x10^5$	13	<3 MPN/g	<30	<30
	2	$\geq 10^6$	ND	ND	ND	ND
	4	$\geq 10^6$	ND	ND	ND	ND
Salt 10% 4 ^{0}C	0	$1.25x10^5$	10	<3 MPN/g	<30	<30
	2	$4.5x10^5$	18	<3 MPN/g	<30	<30
	4	$\geq 10^6$	ND	ND	ND	ND
Salt 10% AT	0	$1.30x10^5$	12	<3 MPN/g	<30	<30
	2	$\geq 10^6$	ND	ND	ND	ND
	4	$\geq 10^6$	ND	ND	ND	ND
Salt 20% 4 ^{0}C	0	$1.25x10^5$	<10	<3 MPN/g	<30	<30
	2	$1.33x10^5$	<10	<3 MPN/g	<30	<30
	4	$1.45x10^5$	<10	<3 MPN/g	<30	<30
Salt 20% AT	0	$1.28x10^5$	<10	<3 MPN/g	<30	<30
	2	$1.35x10^5$	<10	<3 MPN/g	<30	<30
	4	$1.56x10^5$	<10	<3 MPN/g	<30	<30

ND = Not determined as end of shelflife evaluated by total viable count ($\geq 10^6$ cfu/g).

AT = Ambient temperature.

3.14 Antibacterial activities of developed Keang-hleung paste with added salt during storage at 4±2°C.

The antibacterial activities of developed Keang-hleung paste during storage have been presented in Table 27 and it was found that their activities were loss during storage. It may due to loss of allicin activity by time as half life phenomenon. Moreover, high normal flora or contaminant in the paste (Table 27) may also interrupt allicin function as interference and diluents. Arora and Kaur (1999) reported that extracted garlic by sterilized distilled water and store at 4°C for 6 days would lose its antibacterial and anti-fungal as 15–29 and 10–25%, respectively. Additionally, they also reported that antibacterial activity of the garlic extract was stable up to 36 hours when stored at 55°C whereas anti-fungal activity was retained only 8 hours under this condition. Based on MIC and MBC values, it showed that addition of 10% salt into the paste did not improve any antibacterial effect except the paste was added with 20% salt. This may due to limitation of salt tolerant of test organism. In addition, it was found that storage temperature did not affect to antibacterial property.

Table 27. Effect of added salt on antibacterial activities of developed Keang-hleung paste during storage at 4±2°C.

Treatment	Storage (weeks)	MIC (mg/ml)		MBC (mg/ml)	
		S. aureus	***B. cereus***	***S. aureus***	***B. cereus***
control	0	0.898	1.773	3.546	7.092
	2	ND	ND	ND	ND
	4	ND	ND	ND	ND
Salt 0% 4 ^{0}C	0	0.889	1.778	3.556	7.112
	2	No	No	No	No
	4	No	No	No	No
Salt 0% AT	0	0.893	1.786	3.572	7.148
	2	ND	ND	ND	ND
	4	ND	ND	ND	ND
Salt 10% 4 ^{0}C	0	0.895	1.780	3.560	7.120
	2	No	No	No	No
	4	No	No	No	No
Salt 10% AT	0	0.891	1.782	3.564	7.128
	2	ND	ND	ND	ND
	4	ND	ND	ND	ND
Salt 20% 4 ^{0}C	0	0.453	0.906	1.812	3.624
	2	No	No	No	No
	4	No	No	No	No
Salt 20% AT	0	0.458	0.916	1.832	3.664
	2	No	No	No	No
	4	No	No	No	No

ND = Not determined as end of shelflife determined by total viable count ≥ 10^6 cfu/g.

No = No antibacterial activities.

AT = Ambient temperature.

3.15 Total phenolic contents of Developed Keang - hleung paste with added salt during storage at 4±2°C.

The phenolic contents of developed Keang-hleung paste during storage have been presented in Table 28, time and temperature of storage significantly affected the total phenolic content as determined by Folin–Ciocalteu assay. There was a significant decrease in total phenols during storage under the experimental conditions. Similar studied of Klimczak *et al.* (2007) who reported that a significant decrease in total polyphenols (caffeic, p-coumaric, ferulic and sinapic acids) of orange juices stored at during 18, 28 and 38^{0}C for 4 months were 7%, 11% and 20%, respectively. Vallejo *et al.* (2003) also reported that the levels of vitamin C also slightly decreased (2.4% loss) when broccoli was wrapped in low-density polyethylene even stored in a cold room at 1^{0}C for 7 days.

The paste without and with added 10% salt were significantly loss the phenolic contents when stored at ambient temperature only for 3 days. While the paste without and with added 10% salt and kept at 4^{0}C could retain phenolic contents up to 30 days. Moreover, phenolic contents in the paste with added 20% salt and kept at ambient temperature as well as kept at 4^{0}C were detected up to 45 days. It point out that higher salt concentration and low temperature may help to inhibit polyphenol oxidase leading to lower phenolic compound degradation.

The DPPH activity of developed Keang-hleung paste decreased as storage time and temperature increased as presented in Table 28. Similar to study of Del *et al.* (2004) who reported that a slightly decrease in the TEAC (trolox equivalent antioxidant capacity) value measured by DPPH method in orange juice stored at 4^{0}C for 15 days. A decrease in antioxidant activity may be linked to a lower content of phenolic compounds in stored Keang-hleung paste when compared with fresh one. Moreover, DPPH activity of the paste without and with added 10% salt exhibited only in 3 days when kept at ambient temperature. However, developed paste without and with added 10% salts and kept at 4^{0}C could prolong DPPH activity up to 30 days of storage. Based on DPPH activity, the paste with added 20% salt and kept at 4^{0}C as well as the developed paste with added 20% salt and kept at ambient temperature were 45 days that longest compared with other treatments.

The FRAP value of developed Keang-hleung paste during storage have been presented in Table 28. The result showed that a decrease of FRAP caused by time and temperature of storage may due to a decrease of phenolic content as explained above. Similar to finding of Klimczak *et al.* (2007) who reported that FRAP values of orange juices reduced by 23%, 34% and 57% when stored at 18, 28 and 38^0C, respectively for 6 months. Moreover, changes of FRAP values in the pastes were similar pattern as occurred in DPPH activity as mentioned before. Therefore, it could be addressed that a decrease of total phenolic contents was responsible to lower antioxidation activities.

Table 28. Effect of added salt on total phenolic contents and antioxidation properties of developed Keang-hleung paste during storage at 4±2°C.

Treatment	Storage (weeks)	Total phenolic contents (mg GAE/100g)	Scavenging radical (mg GAE/100g)	FRAP (mg GAE/100g)
control	0	1.18±0.21	2.22±0.78	0.743±0.011
	2	ND	ND	ND
	4	ND	ND	ND
Salt 0% 4 ^{0}C	0	1.21±0.13 ^{a}	2.23±0.71 ^{a}	0.075±0.015 ^{a}
	2	1.18±0.11 ^{a}	2.21±0.63 ^{a}	0.073±0.016 ^{a}
	4	0.99±0.06 ^{b}	1.68±0.52 ^{b}	0.054±0.014 ^{b}
Salt 0% AT	0	1.19±0.14	2.20±0.64	0.076±0.015
	2	ND	ND	ND
	4	ND	ND	ND
Salt 10% 4 ^{0}C	0	1.20±0.23 ^{a}	2.20±0.73 ^{a}	0.076±0.013 ^{a}
	2	1.18±0.18 ^{a}	2.18±0.66 ^{a}	0.074±0.016 ^{a}
	4	0.93±0.10 ^{b}	1.64±0.43 ^{b}	0.059±0.012 ^{b}
Salt 10% AT	0	1.20±0.20	2.19±0.73	0.074±0.013
	2	ND	ND	ND
	4	ND	ND	ND
Salt 20% 4 ^{0}C	0	1.18±0.21 ^{a}	2.20±0.71 ^{a}	0.073±0.013 ^{a}
	2	1.20±0.18 ^{a}	2.21±0.73 ^{a}	0.075±0.014 ^{a}
	4	1.24±0.24 ^{a}	2.21±0.72 ^{a}	0.074±0.017 ^{a}
Salt 20% AT	0	1.21±0.19 ^{a}	2.22±0.66 ^{a}	0.076±0.020 ^{a}
	2	1.19±0.16 ^{a}	2.20±0.71 ^{a}	0.075±0.018 ^{a}
	4	0.98±0.11 ^{b}	1.68±0.57 ^{b}	0.058±0.014 ^{b}

ND = Not determined as end of shelflife determined by total viable count (≥ 10^6 cfu/g).

AT = Ambient temperature.

a-b means that with different letters within a column in the same condition are significantly differrent ($p<0.05$).

3.16 Sensory evaluation of developed Keang - hleung paste and their curry soup during storage at 4±2°C.

Sensory score of the paste and the soup was shown in Table 29 and 30. Appearance, color, odor and over liking scores of developed paste without and with added 10% salts and kept in refrigerator were significantly decreased at 30 days of storage. While, scores of those attributes in the paste with added 20% salts and kept both in refrigerator and at ambient temperature were significantly difference at 45 days of storage (Table 29). Also a decrease of appearance, color, odor, spiciness and over liking scores of the curry soup was occurred when increased storage time or temperature. From observation, it was found that key parameter for consumer acceptability was dark color either in the paste and the soup. Loss of yellow-red color may due to enzymatic browning reaction and degradation of color substance of dried chili and turmeric rhizome such as β-carotene and curcumoind pigment, respectively. Another reason for the decrement of acceptability scores was losing of spiciness or hot pungent taste may due to degradation of capsaicin derived from chili. Schweiggert *et al.* (2006) who reported that capsaicinoid degradation was initiated within the first 4 weeks during storage at ambient temperature. Topuz and Ozdemir (2004) also reported that loss of capsaicin was 13.6% during storage at ambient temperature.

Table 29. Sensory score of developed Keang - hleung paste with and without added salt during storage at 4±2°C and ambient temperature.

Treatment	Storage (weeks)	Sensory evaluation			
		Appearance	Color	Odor	Over liking
control	0	7.43±0.68	7.37±0.56	7.40±0.72	7.40±0.62
	2	ND	ND	ND	ND
	4	ND	ND	ND	ND
Salt 0% 4 ^{0}C	0	7.50±0.57 ^{a}	7.27±0.69 ^{a}	7.33±0.66 ^{a}	7.50±0.51 ^{a}
	2	7.33±0.61 ^{a}	7.33±0.61 ^{a}	7.37±0.62 ^{a}	7.33±0.55 ^{a}
	4	6.17±0.70 ^{b}	6.20±0.71 ^{b}	6.10±0.71 ^{b}	6.23±0.73 ^{b}
Salt 0% AT	0	7.47±0.73	7.33±0.66	7.37±0.67	7.30±0.70
	2	ND	ND	ND	ND
	4	ND	ND	ND	ND
Salt 10% 4 ^{0}C	0	7.40±0.68 ^{a}	7.43±0.63 ^{a}	7.23±0.73 ^{a}	7.37±0.62 ^{a}
	2	7.27±0.69 ^{a}	7.37±0.62 ^{a}	7.23±0.73 ^{a}	7.43±0.68 ^{a}
	4	6.13±0.68 ^{b}	6.23±0.73 ^{b}	6.17±0.75 ^{b}	6.30±0.75 ^{b}
Salt 10% AT	0	7.37±0.56	7.43±0.68	7.27±0.69	7.43±0.50
	2	ND	ND	ND	ND
	4	ND	ND	ND	ND
Salt 20% 4 ^{0}C	0	7.37±0.56 ^{a}	7.40±0.62 ^{a}	7.30±0.65 ^{a}	7.47±0.68 ^{a}
	2	7.30±0.70 ^{a}	7.43±0.77 ^{a}	7.17±0.70 ^{a}	7.50±0.78 ^{a}
	4	6.43±0.63 ^{b}	6.37±0.72 ^{b}	6.50±0.51 ^{b}	6.47±0.68 ^{b}
Salt 20% AT	0	7.33±0.61 ^{a}	7.33±0.61 ^{a}	7.30±0.60 ^{a}	7.30±0.65 ^{a}
	2	7.13±0.68 ^{a}	7.40±0.62 ^{a}	7.20±0.71 ^{a}	7.37±0.70 ^{a}
	4	6.17±0.70 ^{b}	6.53±0.68 ^{b}	6.30±0.65 ^{b}	6.43±0.63 ^{b}

ND = Not determined as end of shelf life determined by total viable count (≥ 10^6 cfu/g).

AT = Ambient temperature.

a-b means that with different letters within a column in the same condition are significantly difference ($p<0.05$).

Table 30. Sensory score of developed Keang-hleung curry soup with and without added salt during storage at 4±2°C and ambient temperature.

Treatment	Storage (weeks)	Sensory evaluation						
		Appearance	**Color**	**Odor**	**Over all**	**Salty**	**Spicy**	**Sour**
control	0	7.67±0.66	7.23±0.68	7.43±0.63	7.53±0.68	7.07±0.69	7.70±0.70	7.57±0.63
	2	ND	ND	ND	ND	ND	ND	ND
	4	ND	ND	ND	ND	ND	ND	ND
Salt 0% 4 ^{0}C	0	7.60±0.62 ^{a}	7.43±0.63 ^{a}	7.47±0.68 ^{a}	7.47±0.68 ^{a}	7.00±0.70 ^{a}	7.67±0.66 ^{a}	7.50±0.57 ^{a}
	2	7.57±0.68 ^{a}	7.37±0.67 ^{a}	7.30±0.60 ^{a}	7.40±0.56 ^{a}	7.20±0.71 ^{a}	7.53±0.63 ^{a}	7.40±0.68 ^{a}
	4	6.47±0.63 ^{b}	6.30±0.65 ^{b}	6.37±0.67 ^{b}	6.53±0.68 ^{b}	7.27±0.64 ^{b}	6.33±0.61 ^{b}	7.27±0.64 ^{b}
Salt 0% AT	0	7.53±0.57	7.47±0.63	7.30±0.72	7.40±0.68	7.13±0.68	7.37±0.62	7.50±0.63
	2	ND	ND	ND	ND	ND	ND	ND
	4	ND	ND	ND	ND	ND	ND	ND
Salt 10% 4 ^{0}C	0	7.50±0.51 ^{a}	7.33±0.61 ^{a}	7.33±0.71 ^{a}	7.43±0.63 ^{a}	7.17±0.65 ^{a}	7.50±0.63 ^{a}	7.43±0.68 ^{a}
	2	7.40±0.68 ^{a}	7.47±0.68 ^{a}	7.63±0.67 ^{a}	7.40±0.62 ^{a}	7.17±0.70 ^{a}	7.43±0.68 ^{a}	7.33±0.66 ^{a}
	4	6.33±0.71 ^{b}	6.37±0.67 ^{b}	6.43±0.68 ^{b}	6.30±0.70 ^{b}	7.20±0.66 ^{b}	6.53±0.68 ^{b}	7.20±0.61 ^{b}
Salt 10% AT	0	7.47±0.51	7.37±0.56	7.40±0.62	7.37±0.56	7.23±0.68	7.60±0.56	7.40±0.56
	2	ND	ND	ND	ND	ND	ND	ND
	4	ND	ND	ND	ND	ND	ND	ND

(Continued)

Treatment	Storage (weeks)	Sensory evaluation						
		Appearance	**Color**	**Odor**	**Over all**	**Salty**	**Spicy**	**Sour**
	2	7.43±0.73 [a]	7.13±0.68 [a]	7.47±0.63 [a]	7.23±0.68 [a]	7.27±0.69 [a]	7.57±0.63 [a]	7.37±0.72 [a]
	4	6.53±0.63 [b]	6.50±0.63 [b]	6.60±0.62 [b]	6.57±0.68 [b]	7.23±0.68 [b]	6.60±0.72 [b]	7.27±0.69 [b]
Salt 20% AT	0	7.60±0.68 [a]	7.37±0.62 [a]	7.43±0.57 [a]	7.37±0.62 [a]	7.20±0.55 [a]	7.53±0.63 [a]	7.47±0.68 [a]
	2	7.33±0.66 [a]	7.37±0.67 [a]	7.50±0.63 [a]	7.33±0.61 [a]	7.13±0.68 [a]	7.47±0.63 [a]	7.27±0.69 [a]
	4	6.43±0.68 [b]	6.33±0.66 [b]	6.47±0.63 [b]	6.43±0.57 [b]	6.93±0.69 [b]	6.37±0.67 [b]	7.23±0.68 [b]

ND = Not determined as end of shelf life determined by total viable count ($\geq 10^6$ cfu/g).

AT = Ambient temperature.

a-b means that with different letters within a column in the same condition are significantly difference ($p<0.05$)

3.17 Aw, fiber and moisture content of Keang - hleung paste with added salts during storage.

The moisture, A_w and fiber content of Keang-hleung paste were presented on Table 31. The results showed that moisture content and A_w were not significantly changed during storage may due to effect of packaging (LLDPE/Nylon) as protect permeability of moisture and oxygen. However, addition of 20% salt significantly reduced moisture and A_w content in the paste compared with other pastes.

Table 31. Moisture content, A_w, fiber content of developed Keang-hleung paste with and without added salt during storage at 4±2°C and ambient temperature.

Treatment	Moisture content (%)		A_w		Fiber content (%)	
	Initial	End	Initial	End	Initial	End
Control	61.25±0.55 [a]	61.36±0.65 [a]	0.78±0.04 [a]	0.77±0.04 [a]	2.87±0.10 [a]	2.90±0.06 [a]
Salt 0% 4 ^{0}C	61.31±0.62 [a]	61.25±0.45 [a]	0.79±0.02 [a]	0.82±0.79 [a]	2.83±0.12 [a]	2.87±0.13 [a]
Salt 0% RT	61.28±0.69 [a]	61.34±0.70 [a]	0.76±0.03 [a]	0.79±0.03 [a]	2.84±0.16 [a]	2.83±0.16 [a]
Salt 10% 4 ^{0}C	61.23±0.51 [a]	60.34±0.93 [a]	0.74±0.02 [a]	0.78±0.03 [a]	2.80±0.06 [a]	2.91±0.07 [a]
Salt 10% RT	61.33±0.85 [a]	61.33±0.59 [a]	0.75±0.03 [a]	0.78±0.05 [a]	2.79±0.05 [a]	2.84±0.11 [a]
Salt 20% 4 ^{0}C	59.26±0.59 [b]	59.26±0.45 [b]	0.68±0.01 [b]	0.65±0.02 [b]	2.62±0.03 [b]	2.59±0.05 [b]
Salt 20% RT	59.29±0.63 [b]	59.26±0.25 [b]	0.67±0.02 [b]	0.64±0.03 [b]	2.61±0.04 [b]	2.58±0.06 [b]

Initial is a first day of storage

End is a last day of each storage condition evaluated by total viable count (≥ 10^6 cfu/g); CT = 3 day, Salt 0% 4 ^{0}C = 30 days; Salt 0% RT = 3 days; Salt 10% 4 ^{0}C = 30 days; Salt 10%

RT = 3 days; Salt 20% 4 ^{0}C = 45 days; Salt 20% RT = 30 days

RT = Room temperature

a-b means that with different letters within a column are significantly difference ($p<0.05$)

CHAPTER 4

CONCLUSION

Differences of L^*, a^* and b^* and pH values of Keang-hleung paste and its ingredients were detected as a result of color pigment and organic acid in each spice. Fresh garlic plays the key role for inhibiting both *S. aureus* and *B. cereus*, whereas dried chili, turmeric rhizome and galangal rhizome had no effect. However, after each ingredient was blended together and turned to be the Keang-hleung paste, antibacterial properties were shown. In general, the paste could improve the total phenolic content, free radical scavenging and ferric reducing antioxidant powers compared with its individual ingredient. In summary, the paste has potential use as an antioxidant and antimicrobial as well as a functional food.

There was no effect of temperature and time on the L^*, a^* and b^* and pH values of the paste and its ingredients. In addition, the total phenolic content, free radical scavenging and ferric reducing antioxidant powers of the paste slightly increased after heating. However, the antibacterial activities of the paste and garlic bulb extracts decreased when the heating temperature and time increased.

The more turmeric rhizome and garlic the more improvement of antioxidant and antibacterial in the paste. However, it was found that the proper formulation for improving antioxidant and antibacterial actives without any doubt from consumer preference was turmeric rhizome: dried chili: garlic bulb: galangal rhizome as 20.72: 32: 39.27: 8, respectively.

The salts concentration and storage temperatures have some effect L^*, a^* and b^*, pH values, total phenolic content, free radical scavenging and ferric reducing antioxidant powers of the developed paste during storage. The antibacterial activities of developed paste were unstable during storage. Though, the consumer acceptability was decreased when increased time, the scores were accepted with higher than 6 in the paste with added 20% salt. The developed paste with added 20% salt could prolong shelflife from less than 7 days (the paste without added salt) to 45 days.

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APPENDIX

APPENDIX A

Keang-hleung paste and curry soup preparation for sensory evaluation.

The dish was made by adding 55 g of the paste into boiled water 900 ml for 2 minutes before adding fish 500 g and seasoned with some ingredients as recipe. The consumer acceptability was evaluated in term of color, odor, appearance, saltiness, spiciness, sourness and overall liking attributes. Samples were placed in closed containers, coded with three-digit random numbers. Panelists evaluated the samples in individual booths in a sensory laboratory. They scored the sample on the basis of 9-point hedonic scale, ranging from 'like extremely = 9' through 'neither like nor dislike = 5' to 'dislike extremely= 1' as described by (Meilgaard *et al.,* 1999).

The recipe of Keang-hleung curry consisted of

- Water 900 ml
- Lemon juice 75 ml
- Shrimp paste (Kapi) 30 g
- Keang-hleung paste 55 g
- Salts 20 g
- Short-body mackerel 500 g

APPENDIX B

Microbiological analysis

Total Viable Count (BAM, 2001).

Twenty-five grams of Keang-hleung paste was blended with 225 ml of sterilized 0.85 % normal saline and kept in the refrigerator for 30 minutes. Serial dilution was made at 10^{-1} to 10^{-5} by using the sterilized 0.85 % normal saline. Appropriate dilution was plated using Plate Count Agar was added (PCA, Merck, Germany). The plates were separately incubated at 35±2 ^{0}C for 48 hours for mesophilic bacteria and incubated at 7 ± 2 ^{0}C for 7 days for psychrophilic bacteria. Microbial counts were recorded as colony forming unit/gram of sample (cfu/g)

Determination of Coliform and *E.coli* (BAM, 2001).

One - ml of serial dilution at 10^{-1}, 10^{-2} and 10^{-3} was carried out according to method of BAM (2001). The results were expressed in most probable number (MPN) using three tubes at each dilution.

Presumptive test for coliforms, fecal coliforms and *E. coli*.

The samples were prepared as previous described. Transfer 1 ml portions to 3 Lauryl tryptose (LST) tubes for each dilution for at least 3 consecutive dilutions. Incubate LST tubes at 35 ± 2°C. Examine tubes and record reactions at 24 hours for gas, i.e., displacement of medium in fermentation vial or effervescence when tubes are gently agitated. Re-incubate gas-negative tubes for an additional 24 hours and examine and record reactions again at 48 hours. Perform confirmed test on all presumptive positive (gas) tubes.

Confirmed test for fecal coliforms and *E. coli*.

From each gassing LST tube from the Presumptive test, transfer a loopful of each suspension to a tube of EC broth (a sterile wooden applicator stick may also be used for these transfers). Incubate EC tubes 24 hours at 45.5 ± 2°C and

examine for gas production. If negative, re-incubate and examine again at 48 hours. The positive result will take to calculate fecal coliform MPN and continue for *E. coli* analysis.

Completed test for *E. coli*.

To perform the completed test for *E. coli*, gently agitate each gassing EC tube and streak for isolation, a loopful to an EMB agar plate and incubate for 18-24 hours at 35±2°C. Examine plates for suspicious *E. coli* colonies. Transfer up to 5 suspicious colonies from each EMB plate to NA slants incubate for 18-24 hours at 35 ± 2°C and use for further testing. Identification of any 1 of the 5 colonies as *E. coli* is sufficient to regard that EC tube as positive; hence, not all 5 isolates may need to be tested. Perform Gram stain. All cultures appearing as Gram-negative, short rods should be tested for the IMViC reactions below and also re-inoculated back into LST to confirm gas production.

Indole production. Inoculate tube of tryptone broth and incubate 24 hours at 35 ± 2°C. Test for indole by adding 0.2-0.3 ml of Kovacs' reagent. Appearance of distinct red color in upper layer is positive test.

Voges-Proskauer (VP)-reactive compounds. Inoculate tube of MR-VP broth and incubate 48 hours at 35 ± 2°C. Transfer 1 ml to 13 x 100 mm tube. Add 0.6 ml α-naphthol solution and 0.2 ml 40% KOH, and shake. Add a few crystals of creatine. Shake and let stand 2 hours. Test is positive if eosin pink color develops.

Methyl red-reactive compounds. After VP test, incubate MR-VP tube additional 48 hours at 35 ± 2°C. Add 5 drops of methyl red solution to each tube. Distinct red color is positive test. Yellow is negative reaction.

Citrate. Lightly inoculate tube of Koser's citrate broth; avoid detectable turbidity. Incubate for 96 hours at 35 ± 2°C. Development of distinct turbidity is positive reaction.

Gas from lactose. Inoculate a tube of LST and incubate 48 hours at 35 ± 2°C. Gas production (displacement of medium from inner vial) or effervescence after gentle agitation is positive reaction.

Interpretation: All cultures that (a) ferment lactose with gas production within 48 hours at 35 ± 2°C, (b) appear as Gram-negative non spore

forming rods and (c) give IMViC patterns of ++-- (biotype 1) or -+-- (biotype 2) are considered to be *E. coli*. Calculate MPN of *E. coli* based on proportion of EC tubes in 3 successive dilutions that contain *E. coli*.

Determination of *Staphylococcus aureus* (BAM, 2001).

The samples were prepared as previous described in total viable count determination. 0.1-ml of serial dilution at 10^{-1}, 10^{-2} and 10^{-3} was plated on Baird-Parker agar (BP). Typical colonies, black and surround with clear zone were picked to check for plasma clot.

Coagulase test.

Transfer suspect *S. aureus* colonies into small tubes containing 0.2-0.3 ml BHI broth and emulsify thoroughly. Inoculate agar slant of suitable maintenance medium, e.g., TSA, with loopful of BHI suspension. Incubate BHI culture suspension and slants 18-24 hours at 35 ± 2°C. Retain slant cultures at room temperature for ancillary or repeat tests in case coagulase test results are questionable. Add 0.5 ml reconstituted coagulase plasma with EDTA to the BHI culture and mix thoroughly. Incubate at 35 ± 2°C and examine periodically over 6 hours for clot formation. Only firm and complete clot that stays in place when tube is tilted or inverted is considered positive for *S. aureus*. Partial clotting, formerly 2+ and 3+ coagulase reactions, must be tested further. Test known positive and negative cultures simultaneously with suspect cultures of unknown coagulase activity. Stain all suspect cultures with Gram reagent and observe microscopically.

Determination of *Bacillus cereus* (BAM, 2001).

Prepare serial dilutions from 10^{-2} to 10^{-6} by transferring 10 ml homogenized sample (1:10 dilution) to 90 ml dilution blank, mixing well with vigorous shaking, and continuing until 10^{-6} dilution is reached. Inoculate duplicate Mannitol Egg Yolk Polymyxin Agar (MYP) plates with each dilution of sample (including 1:10) by spreading 0.1 ml evenly onto surface of each plate with sterile glass spreading rod. If reactions are not clear, incubate plates for additional 24 hours before counting colonies. Select plates that contain an estimated 15-150 eosin pink,

lecithinase-producing colonies. Mark bottom of plates into zones with black felt pen to facilitate counting and count colonies that are typical of *B. cereus*.

Conformation of *B. cereus*.

Pick 5 or more eosin pink, lecithinase-positive colonies from MYP agar plates and transfer to nutrient agar slants. Incubate slants 24 hours at 30 ± 2°C. Prepare Gram-stained smears from slants and examine microscopically. *B. cereus* will appear as large Gram-positive bacilli in short-to-long chains; spores are ellipsoidal, central to subterminal, and do not swell the sporangium. Transfer 3 mm loopful of culture from each slant to 13 x 100 mm tube containing 0.5 ml of sterile phosphate-buffered dilution water and suspend culture in diluent with Vortex mixer. Use suspended cultures to inoculate the following confirmatory media:

Phenol red glucose broth.

Inoculate 3 ml broth with 2 mm loopful of culture. Incubate tubes anaerobically 24 hours at 35 ± 2°C in GasPak anaerobic jar. Shake tubes vigorously and observe for growth as indicated by increased turbidity and color change from red to yellow, which indicates that acid has been produced anaerobically from glucose. A partial color change from red to orange/yellow may occur, even in uninoculated control tubes, due to a pH reduction upon exposure of media to CO_2 formed in GasPak anaerobic jars. Be sure to use appropriate positive and negative controls so that a distinction can be made between positive and "false-positive" reactions.

Nitrate broth.

Inoculate 5 ml broth with 3 mm loopful of culture. Incubate tubes 24 hours at 35 ± 2°C. To test for nitrite, add 0.25 ml each of nitrite test reagents A and C to each culture. An orange color, which develops within 10 minutes, indicates that nitrate has been reduced to nitrite.

Modified VP medium.

Inoculate 5 ml medium with 3 mm loopful of culture and incubate tubes 48 ± 2 hours at 35 ± 2 °C. Test for production of acetylmethyl-carbinol by pipetting 1 ml culture into 16 x 125 mm test tube and adding 0.6 ml alpha-naphthol solution (R89) and 0.2 ml 40% potassium hydroxide (R89). Shake, and add a few

crystals of creatine. Observe results after holding for 1 hours at room temperature. Test is positive if pink or violet color develops.

Tyrosine agar.

Inoculate entire surface of tyrosine agar slant with 3 mm loopful of culture. Incubate slants 48 hours at 35 ± 2°C. Observe for clearing of medium near growth, which indicates that tyrosine has been decomposed. Examine negative slants for obvious signs of growth, and incubate for a total of 7 days before considering as negative.

Lysozyme broth.

Inoculate 2.5 ml of nutrient broth containing 0.001% lysozyme with 2 mm loopful of culture. Also inoculate 2.5 ml of plain nutrient broth as positive control. Incubate tubes 24 hours at 35 ± 2°C. Examine for growth in lysozyme broth and in nutrient broth control. Incubate negative tubes for additional 24 hours before discarding.

MYP agar.

This test may be omitted if test results were clear-cut with original MYP agar plates and there was no interference from other microorganisms which were present. Mark bottom of a plate into 6-8 equal sections with felt marking pen, and label each section. Inoculate premarked 4 cm sq area of MYP agar plate by gently touching surface of agar with 2 mm loopful of culture. (Six or more cultures can be tested in this manner on one plate). Allow inoculum to be fully absorbed before incubating for 24 hours at 35 ± 2°C. Check plates for lecithinase production as indicated by zone of precipitation surrounding growth. Mannitol is not fermented by isolate if growth and surrounding medium are eosin pink. (Yellow color indicates that acid is produced from mannitol.) *B.cereus* colonies are usually lecithinase-positive and mannitol-negative on MYP agar.

Record results obtained with the different confirmatory tests.

Tentatively identify as *B. cereus* those isolates which 1) produce large Gram-positive rods with spores that do not swell the sporangium; 2) produce lecithinase and do not ferment mannitol on MYP agar; 3) grow and produce acid from glucose anaerobically; 4) reduce nitrate to nitrite (a few strains may be negative); 5) produce acetylmethylcarbinol (VP-positive); 6) decompose L-tyrosine; and 7) grow in

the presence of 0.001% lysozyme. These basic characteristics are shared with other members of the *B. cereus* group, including the rhizoid strains *B. mycoides*, the crystalliferous insect pathogen *B. thuringiensis*. And the mammalian pathogen *B. anthracis*. However, these species can usually be differentiated from *B. cereus.* by determining specific characteristics typical of each species or variety. Strains that produce atypical results from these tests require additional analysis before they can be classified as *B. cereus*.

Enumeration of Yeasts and Molds (BAM, 2001).

Antibiotic solutions.

Antibiotics are added to mycological media to inhibit bacterial growth. Chloramphenicol is the antibiotic of choice, because it is stable under autoclave conditions. Therefore, media preparation is easier and faster due to the elimination of the filtration step. The recommended concentration of this antibiotic is 100 mg/liter medium. If bacterial overgrowth is apparent, prepare media by adding 50 mg/liter chloramphenicol before autoclaving and 50 mg/liter filter-sterilized chlortetracycline when the media have been tempered, right before pouring plates.

Prepare stock solution by dissolving 0.1 g chloramphenicol in 40 ml distilled water; add this solution to 960 ml medium mixture before autoclaving. When both chloramphenicol and chlortetracycline are used, add 20 ml of the above chloramphenicol stock solution to 970 ml medium before autoclaving. Then, prepare chlortetracycline stock solution by dissolving 0.5 g antibiotic in 100 ml distilled water and filter sterilize. Use 10 ml of this solution for each 990 ml of autoclaved and tempered medium. Refrigerate in the dark and re-use remaining stock solutions for up to a month. Stock solutions should be brought to room temperature before adding to tempered medium.

Procedures.

Sample preparation. Analyze 25-50 g from each subsample; generally, larger sample sizes increase reproducibility and lower variance compared with small samples. Test individual subsamples or composite according to respective

Compliance Program for the food under analysis. Add appropriate amount of 0.1% peptone water to the weighed sample to achieve 10^{-1} dilution, then homogenize in a stomacher for 2 minutes. Alternatively, blending for 30-60 seconds can be used but is less effective. Make appropriate 1:10 (1+9) dilutions in 0.1% peptone water. Dilutions of 10^{-6} should suffice.

Plating and incubation of sample.

Spread-plate method. Aseptically pipet 0.1 ml of each dilution on pre- poured, solidified DRBC agar plates and spread inoculum with a sterile, bent glass rod. DG18 is preferred when the water activity of the analyzed sample is less than 0.95. Plate each dilution in triplicate.

Pour-plate method. Use sterile cotton-plugged pipet to place 1.0 ml portions of sample dilution into prelabeled 15 x 100 mm Petri plates (plastic or glass), and immediately add 20-25 ml tempered DG18 agar. Mix contents by gently swirling plates clockwise, then counterclockwise, taking care to avoid spillage on dish lid. After adding sample dilution, add agar within 1-2 minutes; otherwise, dilution may begin to adhere to dish bottom (especially if sample is high in starch content and dishes are plastic) and may not mix uniformly. Plate each dilution in triplicate. From preparation of first sample dilution to pouring or surface-plating of final plate, no more than 20 minutes (preferably 10 minutes) should elapse.

Note: Spread plating of diluted sample is considered better than the pour plate method. When the pour plate technique is used, fungal colonies on the surface grow faster and often obscure those underneath the surface, resulting in less accurate enumeration. Surface plating gives a more uniform growth and makes colony isolation easier. DRBC agar should be used for spread plates only. Incubate plates in the dark at 25 ± 2°C. Do not stack plates higher than 3 and do not invert.

Note: Let plates remain undisturbed until counting.

Counting of plates.

Count plates after 5 days of incubation. If there is no growth at 5 days, re-incubate for another 48 hours. Do not count colonies before the end of the incubation period because handling of plates could result in secondary growth from dislodged spores, making final counts invalid. Count plates containing 10-150

colonies. If mainly yeasts are present, plates with 150 colonies are usually countable. However, if substantial amounts of mold are present, depending on the type of mold, the upper countable limit may have to be lowered at the discretion of the analyst. Report results in colony forming units (cfu)/g or cfu/ml based on average count of triplicate set. Round off counts to two significant figures. If third digit is 6 or above, round off to digit above (e.g., 456 = 460); if 4 or below, round off to digit below (e.g., 454 = 450). If third digit is 5, round off to digit below if first 2 digits are an even number (e.g., 445 = 440); round off to digit above if first 2 digits are an odd number (e.g., 455 = 460). When plates from all dilutions have no colonies, report mold and yeast counts (MYC) as less than 1 times the lowest dilution used. Isolate individual colonies on PDA or MA, if further analysis and species identification is necessary.

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