ANTIOXIDANT AND ANTIMUTAGENIC ACTIVITY OF EXTRACTS FROM PERICARP AND SEED OF *ZANTHOXYLUM LIMONELLA* ALSTON

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ฤทธิ์ต้านออกซิเดชันและฤทธิ์ต้านการก่อกลายพันธุ์ของสารสกัดจากผนังผลและเมล็ดมะแขว่น

น.ส.ปฐมาวัลย์ วิภูษณพันธุ์

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Thesis Title	ANTIOXIDANT AND ANTIMUTAGENIC ACTIVITY OF EXTRACTS FROM PERICARP AND SEED OF *ZANTHOXYLUM LIMONELLA* ALSTON
By	Miss Patamawan Wipoosanapan
Field of Study	Food Chemistry and Medical Nutrition
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ปฐมาวัลย์ วิภูษณพันธุ์ : ฤทธิ์ต้านออกซิเดชันและฤทธิ์ต้านการก่อกลายพันธุ์ของสารสกัดจากผนังผลและเมล็ดมะแขว่น. (
ANTIOXIDANT AND ANTIMUTAGENIC ACTIVITY OF EXTRACTS FROM PERICARP AND SEED OF *ZANTHOXYLUM LIMONELLA* ALSTON) อ.ที่ปรึกษาหลัก : ผศ. ภญ. ดร.ลินนา ทองยงค์

การศึกษานี้เป็นการวิเคราะห์ฤทธิ์ต้านออกซิเดชันและฤทธิ์ต้านการก่อกลายพันธุ์ของสารสกัดจากผนังผลและสารสกัดจากเมล็ดมะแขว่น (*Zanthoxylum limonella*) โดยทำการวิเคราะห์หาปริมาณสารประกอบฟีนอลิกและทดสอบฤทธิ์ต้านออกซิเดชันด้วยวิธีการทำลายอนุมูลอิสระดีพีพีเอช และวิเคราะห์ความสามารถในการรีดิวซ์เฟอร์ริกของสารต้านออกซิเดชัน ผลการศึกษาพบว่าสารสกัดผนังผลมีฤทธิ์ต้านออกซิเดชันมากกว่าสารสกัดเมล็ด ทำการทดสอบการก่อกลายพันธุ์โดยตรงของสารสกัดทั้งในสภาวะที่มีและไม่มีไนไตรท์ในการทำปฏิกิริยาต่อเชื้อ *S. typhimurium* สายพันธุ์ TA98 และ TA100 โดยวิธีทดสอบเอมส์ ส่วนการทดสอบผลของสารสกัดในการปรับเปลี่ยนฤทธิ์การก่อกลายพันธุ์ ทำโดยการวัดผลของสารสกัดต่อการก่อตัวของสารก่อกลายพันธุ์มาตรฐานที่เกิดระหว่างปฏิกิริยาของ 1-อะมิโนพัยรีนและไนไตรท์ (antiforming) และผลของสารสกัดต่อสารก่อกลายพันธุ์มาตรฐานที่เกิดขึ้นแล้วจากปฏิกิริยาดังกล่าว (antimutagen) ผลการทดสอบฤทธิ์ก่อกลายพันธุ์โดยตรงพบว่าสารสกัดเมล็ดมะแขว่นไม่แสดงฤทธิ์ก่อกลายพันธุ์โดยตรงต่อเชื้อ *S. typhimurium* ทั้งสองสายพันธุ์ ทั้งในสภาวะที่มีและไม่มีไนไตรท์ นอกจากนี้สารสกัดเมล็ดยังสามารถยับยั้งการก่อตัวของสารก่อกลายพันธุ์มาตรฐานระหว่างการทำปฏิกิริยาของ 1-อะมิโนพัยรีนและไนไตรท์ แต่อย่างไรก็ตามสารสกัดเมล็ดแสดงผลการเสริมฤทธิ์ก่อกลายพันธุ์ของสารก่อกลายพันธุ์มาตรฐานได้เล็กน้อย สำหรับสารสกัดผนังผลเมื่อทำปฏิกิริยากับไนไตรท์แสดงฤทธิ์การก่อกลายพันธุ์โดยตรงและเสริมการก่อตัวของสารก่อกลายพันธุ์มาตรฐานระหว่างการทำปฏิกิริยาของ 1-อะมิโนพัยรีนและไนไตรท์ต่อเชื้อ *S. typhimurium* สายพันธุ์ TA98 อย่างไรก็ตามสารสกัดผนังผลสามารถยับยั้งการก่อกลายพันธุ์ของสารก่อกลายพันธุ์มาตรฐานที่เกิดขึ้นหลังจากการทำปฏิกิริยาของ 1-อะมิโนพัยรีนและไนไตรท์ต่อเชื้อ *S. typhimurium* สายพันธุ์ TA98 และ TA100 ได้ การศึกษานี้แสดงให้เห็นถึงกลไกที่แตกต่างกันของสารสกัดจากผนังผลและเมล็ดมะแขว่นในการยับยั้งฤทธิ์ก่อกลายพันธุ์ของสารก่อกลายพันธุ์มาตรฐาน แต่อย่างไรก็ตามควรหลีกเลี่ยงการรับประทานผลของมะแขว่นร่วมกับไนไตรท์หรืออาหารที่มีส่วนประกอบของของไนไตรท์เพื่อป้องกันการก่อตัวของสารก่อกลายพันธุ์

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ANTIOXIDANT AND ANTIMUTAGENIC ACTIVITY OF EXTRACTS FROM PERICARP AND SEED OF *ZANTHOXYLUM LIMONELLA* ALSTON. Advisor: Asst. Prof. Linna Tongyonk, D.Sc.

This study was conducted to investigate the antioxidant and antimutagenic activity of *Zanthoxylum limonella* seed extract and that of *Z. limonella* pericarp extract. The total phenolic contents and antioxidant activity assay including DPPH assay and FRAP assay were performed. The results showed that the antioxidant activity of pericarp extract was higher than that of the seed extract. The direct mutagenicity assay was determined, with and without nitrite treatment using the Ames test towards *Salmonella typhimurium* strains TA98 and TA100. The mutagenic modification assay was performed by measuring the interfering effect of the pericarp extract and the seed extract on the formation of standard mutagens during the reaction between 1-aminopyrene and sodium nitrite (antiformation) and on the standard mutagen produced from the same reaction (antimutagen). For the seed extract, there was not direct mutagenic either with or without nitrite treatment on both tester strains, and it could interfere the formation of mutagen by decreasing the mutagenic activity. On the other hand, it was found that the seed extract slightly enhance the mutagenic activity of the standard mutagen. In addition, pericarp extract exhibited direct mutagenic activity after being treated with nitrite and increased the mutagenic activity on the formation of standard mutagen. However, the antimutagenic activity of the pericarp extract on the standard mutagen obtained from 1-aminopyrene reacted with sodium nitrite on *S. typhimurium* strains TA98 and TA100 was observed. The present study revealed the different inhibitory mechanisms of *Z. limonella* seed extract and that of *Z. limonella* pericarp extract on mutagenic activity of standard mutagen. However, the consumption of fruits from *Z. limonella* with nitrite or nitrite containing food products should be avoided to prevent the formation of mutagen.

Field of Study:	Food Chemistry and Medical Nutrition	Student's Signature .....
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Patamawan Wipoosanapan

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVATIONS

ºC	degree Celsius
et al.	et alia (and other)
g	gram
h	hour
mg	milligram
min	minute
ml	milliliter
mM	millimolar
N	normality
µl	microlite
µg	microgram
µM	micromolar
nm	nanometres
rpm	revolutions per minute
SD	standard deviation
DMSO	dimethylsulfoxide
1-AP	1-aminopyrene

CHAPTER I

INTRODUCTION

1.1 Background and rationale

Currently, the relationship between food and cancer has gained attention specifically in some foods that increase the risk of cancer. Eating some certain kinds of foods that contain carcinogenic such as arsenic or heavy metals, polycyclic aromatic hydrocarbons (PAHs), heterocyclic amines (HA) or foods containing nitrates or nitrites can increase the risk of cancer (Kelley and Duggan, 2003). Some studies showed a relationship between nitrate or nitrite exposure and an increase in gastric cancer. Nitrate or nitrite is a nitrogen compound that is currently used as a food additive to assist in the process of meat preservation (Honikel, 2008). When nitrate is consumed, it is converted to nitrite (NO^{2-}) by the bacteria in human oral cavity (Pannala et al., 2003). Then nitrite enters to stomach which has an acidic condition, and may react with amine or amide from some foods. The compounds formed by this reaction are nitroso compounds such as nitrosamines and nitrosamides, etc. These compounds are cancer causing substances (Hernandez-Ramirez et al., 2009). Consequently to prevention of cancer, epidemiological studies have shown that certain nutrients and specific natural phytochemicals in vegetables, fruits and spice play an important role in reducing the risk of various diseases such as cancer, cardiovascular disease, diabetes and Alzheimer's disease, etc. (Meadows, 2012). It has been reported that phytochemicals exhibited biological activities on human health

especially antioxidant, anticancer, antimutagen and antimicrobial activity (Liu, 2003). Accordingly, consuming diets high in phytochemicals may help reduce the risk of cancer (Béliveau and Gingras, 2007).

Antioxidants can act as reducing agents, donating electron to free radicals. Free radicals are unstable reactive compounds which have the potential to damage DNA, proteins and lipids (Phaniendra et al., 2015). Therefore, antioxidants can scavenge free radicals and inhibit oxidation that are linked to decrease risk of cancer development (Borek, 2017). Chinese Cancer Prevention Study (CHICAPS) in 1993 reported the effects of beta-carotene, vitamin E and selenium in healthy Chinese women and men, who have risk for gastric cancer. The results of the study showed a significant positive effect of antioxidant to reduce the incidence of gastric cancer (Blot et al., 1993). The same researcher continued this study for 10-year follow-up and found that antioxidants were able to reduce the risk of death from gastric cancer (Qiao et al., 2009).

The *Rutaceae* family comprises almost 1,600 species of trees, shrubs, and climbers distributed throughout the temperate and tropical regions of the world especially in Asia. Most of plants in the *Rutaceae* are aromatic plants whose leaves, fruits or seeds contain a complex mixture of volatile aroma compounds. *Zanthoxylum limonella* aston (*Z. limonella*) is a spice in the family *Rutaceae*. It is considered as the economic crop of the provinces in Northern Thailand. Normally, fruits of *Z. limonella* can be divided into two parts, the pericarp and the seed. Fruits

of *Z. limonella* are used as flavoring agent for many kinds of food. Moreover, *Z. limonella* also have herbal properties as a Thai folk medicine for fever, dizziness, diuretic and flatulence (Supabphol and Tangjitjareonkun, 2015). However, Wongsrisom et al. (2014) found that the pericarp is the part that contains more essential oil and pleasant odor than the seed. The biological activity of different part from *Z. limonella* has been reported but neither of these studies investigated biological activity of the pericarp and that of the seed from *Z. limonella* (Naik, 2015; Nanasombat and Wimuttigosol, 2011; Tangjitjaroenkun et al., 2012).

This study was conducted to investigate the biological activities of *Z. limonella* pericarp and that of the *Z. limonella* seed extract. These activities included antioxidant, mutagenic and antimutagenic activities. The antioxidant activity of the pericarp and that of the seed extract were measured by determining total phenolic contents, 2,2'- diphenyl-1-picrylhydrazyl (DPPH) assay and ferric reducing antioxidant power (FRAP) assay. Furthermore, the direct mutagenicity assay of the pericarp and that of the seed extract was performed with and without nitrite treatment on *Salmonella typhimurium* strain TA98 and TA100 using the Ames test. The mutagenicity modification of the pericarp and that of the seed extract was evaluated by measuring the interfering effect of the extract on the formation of standard mutagens during the reaction between 1-aminopyrene and sodium nitrite (anti-forming) and on the standard mutagen produced from 1-aminopyrene treated with sodium nitrite (anti-mutagen).

1.2 Objectives of the study

1.2.1 To investigate the antioxidant activity of *Z. limonella* pericarp extract and that of the *Z. limonella* seed extract.

1.2.2 To investigate direct mutagenic activity of *Z. limonella* pericarp extract and that of the *Z. limonella* seed extract with or without nitrite treatment using the Ames test.

1.2.3 To investigate the modification effect of *Z. limonella* pericarp extract and that of the *Z. limonella* seed extract on the mutagenicity of sodium nitrite treated 1-AP using the Ames test.

1.3 Benefits of the study

This study has provided preliminary information on antioxidant activity and antimutagenic activity of *Z. limonella* pericarp extract and that of the *Z. limonella* seed extract. This information can be used as a guide to develop herbal medicines or complementary products for further research.

CHAPTER II

LITERATURE REVIEW

2.1 Mah-Khwaen (*Zanthoxylum limonella* Alston)

Mah-Khwaen or *Zanthoxylum limonella Alston* is a spice plant in the family *Rutaceae*. Plants in this family can produce essential oil from the glands on the leaves or thorns; therefore, it has created a unique smell and taste. *Z. limonella* is widely distributed in Southeast Asia such as India, Sri Lanka, Thailand, Myanmar and Philippines etc. In Thailand, *Z. limonella* can be grown in tropical areas, mainly in the Northern area. Consequently, it is considered as the economic crop of the provinces in the north, including Nan, Phayao and Mae Hong Son. *Z. limonella* is medium-sized tree. The stems are reddish and have many thorns around branches. The leaves are paripinnate and long, 30 - 40 cm. The flowers are grouped in an inflorescence and the colour is white. The fruits are divided into pericarps and seeds, 0.3-0.5 cm in diameter. Pericarps are green tuning dark black when they are ripe. Seeds have shiny black color. Moreover, the fruits have a pleasant smell like coriander seeds and taste slightly spicy (Figure 1) (Supabphol and Tangjitjareonkun, 2015).

Normally, people in the Northern area use fruits of *Z. limonella* to add flavor and smell for many kinds of food such as curry, spicy salad, and fried chicken etc. In addition, the different parts of them also have herbal properties as a Thai folk medicine. For example, the stems are used to reduce fever, dizziness, diuretic, and flatulence. The leaves and fruits are used as anti-infection and anti-inflammation.

Furthermore, the essential oil from *Z. limonella* is used as an insect repellent (Charoensup et al., 2016; Wongsrisom et al., 2014).

Figure 1 Dry fruit of *Zanthoxylum limonella* aston

2.2 Phytochemical constituents of *Z. limonella*

Phytochemical substances of *Z. limonella* including monoterpene (sabinene, limonene and terpinen-4-ol), triterpenoid, coumarin, alkaloid, and phenolic compound are found in different parts such as stems, barks and fruits (Tangjitjaroenkun, 2012). The fruits of *Z. limonella* are sources of essential oil, which main chemical component is monoterpene such as limonene (31.09%), terpinen-4-ol (13.94%) and sabinene (9.13%) (Supabphol and Tangjitjareonkun, 2015). A variety of phytochemical constituents found in different parts of *Z. limonella* are shown in Table 1.

Table 1 Phytochemical constituents of *Z. limonella*

Plant part	Compound	Chemical category
Stem	Limonellone	Quinolone alkaloid
	(-)-Asarinin	Lignan
	Dihydroalatamide	Aromatic amide
	(-)-Tembamide	
	Dictamnine	Furoquinoline alkaloid
	N-Nornitidine	Benzophenanthridine alkaloid
Stem bark	Lupeol	Triterpene
	Xanthoxyletin	Pyronocoumarin
	Osthol	O-methylated coumarin
	Scopoletin	Hydroxycoumarin
	Rutaecapine	Quinazolinocarboline alkaloid
Fruit	Xanthoxyline	Phenolic compound
	Limonene	Monoterpene hydrocarbons
	Sabinene	
	3-Carene	
	α-Pinene	
	β-Pinene	
	γ-Terpinene	
	α-Terpinene	
	α-Terpineol	Oxygenated monoterpene
	Terpinen-4-ol	
	p-Cymene	Alkylbenzene monoterpene

Terpenenes are isoprene compounds that contain cyclic unsaturated hydrocarbons in the structure (Figure 2). These compounds can be oxidized to the secondary metabolites and are divided into a subunit by the number of carbon atom and isoprene subunits (Table 2) (Grabmann, 2005).

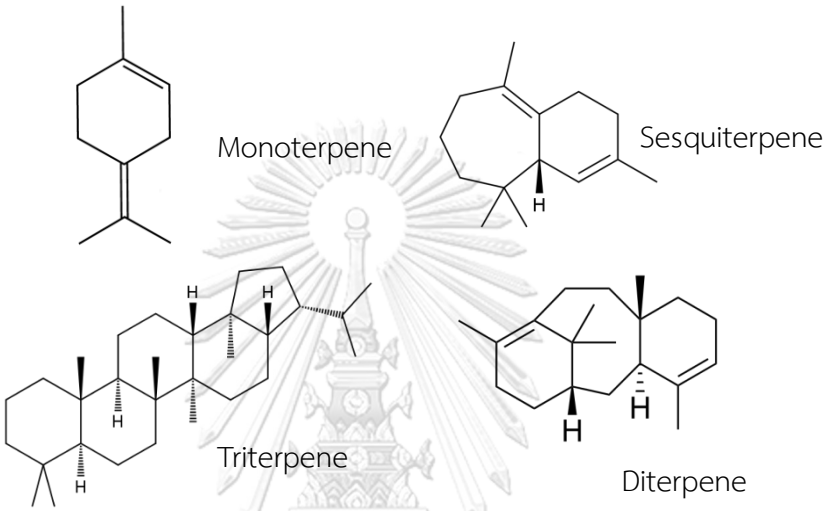


Figure 2 structure of terpenenes
(Cho et al., 2017)

Table 2 Classes of terpenenes (Grabmann, 2005)

Terpenoids	**Number of C-atoms**	**Number of isoprene subunits**
Monoterpene	10	2
Sesquiterpene	15	3
Diterpene	20	4
Triterpene	30	6
Tetraterpene	40	8
Polyterpene	> 40	> 8

Monoterpenes are a class of terpenenes that contain of two isoprene subunits and ten carbon atoms in their structures. Monoterpenes are the main compound of natural essential oils for example fruits, herbs, or spices etc. These compounds promote to the aroma and flavor of essential oil from plant extract (Tavera, 1999). Several studies reported their many biological effects on human health, including antioxidant and antimutagenic activity etc. (Burgos and Gómez-Serranillos, 2012; Grabmann, 2005; Li and Liu, 2009). Antioxidant capacity of an essential oil depends on its composition. Secondary metabolites with conjugated double bonds usually exhibit considerable antioxidative properties (Dhifi et al., 2016). Li and Liu (2009) studied antioxidant activity of α- and ɣ-terpinene. The results showed that α- and ɣ-terpinene had protective effect on the oxidation of DNA. In addition, the oxygenated monoterpenes (linalool, menthol and camphanone) showed good free radical scavenging properties by inhibiting chain reaction of free radicals (Burgos and Gómez-Serranillos, 2012).

The effect of monoterpene against mutagenicity was reported in various studies (Aicha et al., 2011; Antonella et al., 2013; Erdemgil et al., 2008). Antonella et al. (2013) found that limonene and its metabolites can inhibit the chemically-induced mutagenicity on *S. typhimurium* strain TA98 and TA100. Beta-Pinene, alpha-terpinene, limonene and p-cymene of essential oil from field wormwood (*Artemisia campestris*) had no direct mutagenicity. Moreover, it also exhibited antimutagenicity against the carcinogen benzo(a)pyrene; (B[a] P) on *S. typhimurium*

strain TA97 and TA98 (Aicha et al., 2011). Erdemgil et al. (2008) investigated antimutagenic activity of essential oil from russian sage. The essential oil that consists mainly of monoterpene such as limonene, α-globulol and trans-caryophyllene, etc. exhibited antimutagenic activity with or without S9 fraction against 2-aminofluorene and daunomicina on *S. typhimurium strain* TA98 and TA100. Moreover, monoterpenes were reported about their anticancer properties (Gautam et al., 2014; Qi et al., 2018). Jia et al. (2013) reported that limonene could induce apoptosis of colon cancer (LS174T) cells by inhibiting the Akt activation and activating the intrinsic mitochondrial apoptosis signaling pathway in LS174T cells. Maltzman et al. (1991) suggested the anticarcinogenic activity of limonene by stimulating phase I and phase II detoxification during the initiation stage of cancer cell. However, the clinical trials of anticarcigenicity in human are required (Crowell, 1999; Wagner and Elmadfa, 2003).

2.3 Biological activity of *Z. limonella*

2.3.1 Antioxidant activity

A free radical is a molecule or an atom with unpaired electron which makes the substance highly unstable and sensitive to exchange electron with other molecules such as hydroxyl radical (OH•), superoxide radical (O_2•$^-$), oxygen radical ($O_2^{••}$), peroxyl radical (ROO•) and nitric oxide (NO•) etc. (Phaniendra et al., 2015). The molecules that lose electrons are new free radicals by chain reactions (Figure 3).

Initiation:

$LH + R\bullet \longrightarrow R\bullet + RH$

Propagation:

$L\bullet + O_2 \longrightarrow LOO\bullet$

$LOO\bullet + LH \longrightarrow LOOH + L\bullet$

Branching:

$LOOH \longrightarrow LO\bullet + HO\bullet$

$2LOOH \longrightarrow LOO\bullet + LO\bullet + H_2O$

Figure 3 Free radicals chain reaction (Pisoschi et al., 2016)

Free radicals can damage biological molecules including lipid membrane, protein, enzyme, RNA, and DNA etc. Therefore, the imbalance between free radicals and antioxidants can induce oxidative stress and implicate in various pathological conditions such as cardiovascular disease, cancer, neurological disorders and other diseases (Phaniendra et al., 2015). In addition, this stimulation of DNA damage can be linked to the mutation and carcinogenesis of organisms, nevertheless; this process is inhibited by antioxidants (Pham-Huy et al., 2008).

Antioxidant is a compound that can prevent and inhibit oxidative reaction, which involves the exchange of electrons from one substance to an oxidizer. It can inhibit oxidative stress and cell damage by scavenging free radicals. Accordingly, antioxidants play important roles in anticancer properties by preventing DNA damage and improving immunity (National cancer institute, 2017). Normally, a balance

between free radicals and antioxidants can be maintained by endogenous antioxidant enzymes such as catalase, glutathione peroxidase and superoxide dismutase etc. Furthermore, the body can also obtain exogenous antioxidants from food consumption. Vegetables, fruits and spice are major sources of exogenous antioxidants (Lobo et al., 2010).

Antioxidant activity of different parts from *Z. limonella* have been investigated. Tangjitjaroenkun (2012) evaluated the antioxidant activity of extract and essential oil from stems and leaves of *Z. limenella* using DPPH assay and trolox equivalent antioxidant capacity assay. It was found that the antioxidant activity of the extract from stems of *Z. limenella* was higher than those of essential oils and leaves extract. Nanasombat and Wimuttigosol (2011) examined the antioxidant activity of essential oil from fruits of *Z. limonella* and other spices in Thailand. The results showed that essential oil from fruits of *Z. limonella* had strong antioxidant activity. Moreover, a recent study investigated antioxidant activity and fatty acid composition of the essential of fruits from *Z. limonella* using DPPH assay and GC-FID, respectively. The essential oil of fruits exhibited significant free radical scavenging activity, and it contained monounsaturated fatty acid (42%) and saturated fatty acid (35%) (Naik, 2015).

2.3.2 Antibacterial activity

Several studies reported the antimicrobial activity of essential oil from *Z. limonella* (Nanasombat and Wimuttigosol, 2011; Tangjitjaroenkun et al., 2012;

Wongsrisom et al., 2014). Nanasombat and Wimuttigosol (2011) determined antimicrobial activity of essential oil from spices in Thailand using disk diffusion assay and minimum inhibitory concentrations (MICs). The result showed that essential oil of fruits from *Z. limonella* exhibited antimicrobial activity against *Bacillus cereus* and *Staphylococcus aureus*. Furthermore, Tangjitjaroenkun et al. (2012) reported that the main compounds of the essential oil of fruits from *Z. limonella* are monoterpenes (88.3%) such as sabinene (42.7%), limonene (39.1%) and terpinen-4-ol (5.4%). Sabinene and terpinen-4-ol which were pure compounds exhibited antibacterial activity against *Bacilus subtillis*, *S. aureus* (MSSA) and *Escherichia coli,* but antibacterial activity of limonene was not observed. Recently, Wongkatiya et al. (2018) found strong antibacterial activity of essential oil of fruit from *Z. limonella* on *B. cereus*, *S. aureus* and *E. coli.* The results indicated that essential oil of *Z. limonella* is broad spectrum antibacterial activity to gram positive and negative bacteria.

2.3.3 Anticancer activity

Studies on anticancer activity of *Z. limonella* are limited. Murakamia et al. (1995) measured antitumor activity of *Z. limonella* fruit extract by the *in vitro* 12-otetradecanoylphorbol-13-acetate induced Epstein-Barr virus early antigen (EBV-EA) activation assay in lymphoid cell line. The results showed that *Z. limonella* fruit extract exhibited strongly inhibitory effect. Accordingly, *Z. limonella* fruit extract might contain cancer chemopreventive agents (Murakamia et al., 1995). However, the

anticancer activities of *Z. limonella* is required more investigation (Charoensup et al., 2016).

2.4 Gastic cancer and nitrite

Gastic cancer is the fourth most common cancer in the world and is also the second leading cause of cancer death (Rahman et al., 2014). Fifty percent of new patients are found in developing countries. The high risk areas of gastric cancer are Eastern Asia (China and Japan), Eastern Europe, Central and South America, but low-risk areas of gastric cancer include South Asia, North Africa, North America, Australia, and New Zealand (Sitarz et al., 2018). Currently, the incidence of gastric cancer has decreased over the past few years, which may be due to the improvement of public health and food consumption, for example, increasing consumption of fresh fruits and vegetables (Muñoz and Franceschi, 1997). These represent to the importance of consumption behavior which is one of the risk factors for gastric cancer.

The relationship between diet and cancer risk has been more interesting. Frequently consumption of some diets that contain polycyclic aromatic hydrocarbons (PAHs) or heterocyclic amine (HA), such as grilled food and simmering food may increase the risk of cancer (Karimi et al., 2014). Nitrate and nitrite are commonly used as food additives for preserving food and extending shelf life of food. They are often added into processed meat such as sausages, ham, chinese sausage and sour pork (Basak et al., 2017; Cantwell and Elliott, 2017). Moreover, several studies found the relationship between nitrate or nitrite exposure and

increasing of gastric cancer risk (Jakszyn and González, 2006; Larsson et al., 2006; Song et al., 2015). When nitrate is ingested into the body, oral bacteria can convert nitrate to nitrite. After passing through the stomach, nitrite reacts with amine or amide from some kinds of food by nitrosation. The products of this reaction are N-nitroso compounds such as nitrosamines and nitrosamides that are carcinogens (Figure 4) (Hernandez-Ramirez et al., 2009).

Figure 4 Equation of nitrosation (Hernandez-Ramirez et al., 2009)

Several studies investigated the mutagenicity of some foods that reacted with nitrites using bacterial assay (Marquardt et al., 1977; Munzner and Wever, 1984; Namiki et al., 1984; Wakabayashi et al., 1983). Marquardt et al. (1977) reported that raw fishes, beans and beef soup treated with nitrite in acidic conditions showed mutagenicity to *S. typhimurium* strain TA1535 in the presence of activating enzyme (S9 mix). Similarly, Munzner and Wever (1984) found mutagenicity of beef soup extract treated with nitrite on *S. typhimurium* strain TA98, TA100 and TA1538 in the absence and the presence of activating enzyme. Moreover, Wakabayashi et al. (1983) and Namiki et al. (1984) reported that eight brands of soy sauce and some spices treated with nitrite showed strong mutagenicity to *S. typhimurium* in the absence of

activating enzyme. These data indicated that some compounds of diet could produce mutagens after treated with nitrite (Kangsadalampai, 1994).

2.5 Antioxidant assay

Antioxidants can inhibit free radical reaction by several mechanisms such as free radical scavenging, metal chelating, singlet oxygen quenching and enzyme inhibition etc. Therefore, antioxidant capacity should be evaluated by more than one method. The example of the free radicals scavenging of antioxidant is shown in Figure 5.

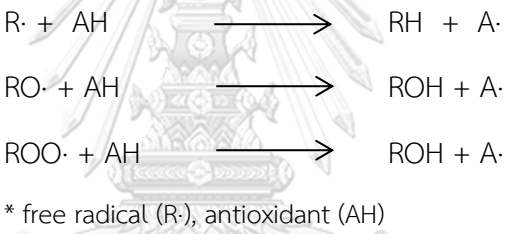


Figure 5 The free radicals scavenging of antioxidant

(Pisoschi and Negulescu, 2012)

Total antioxidant capacity (TAC) assays are divided into two major types based on mechanisms, hydrogen atom transfer (HAT) and single-electron transfer (SET). The HAT methods determine capability of antioxidant to neutralize free radicals by hydrogen atom donation. The reactions of these methods are pH independent and quite rapid. The SET methods detect the capacity of a potential antioxidant to transfer one electron to reduce any compounds (Prior et al., 2005).

These methods are dependent on pH but slower than the HAT-based methods. Methods for total antioxidant capacity determination are shown in Table 3.

Table 3 The HAT and SET methods used to evaluate total antioxidant capacity

Hydrogen atom transfer methods (HAT)
- Oxygen radical absorbance capacity (ORAC)
- Lipid peroxidation inhibition capacity (LPIC)
- Total radical trapping antioxidant parameter (TRAP)
- Inhibited oxygen uptake (IOC)
- Scavenging of H_2O_2 radicals
- ABTS radical scavenging
- Scavenging of super oxide radical formation by alkaline (SASA)
Singlet-electron transfer methods (SET)
- Trolox equivalent antioxidant capacity (TEAC) decolourization
- Ferric reducing antioxidant power (FRAP)
- DPPH free radical scavenging (DPPH)
- Copper (II) reduction capacity

2.5.1 2,2'- Diphenyl-1-picrylhydrazyl (DPPH) assay

2,2'-Diphenyl-1-picrylhydrazyl (DPPH) assay is the basis of a common antioxidant capacity assay. This assay is based on the measurement of free radical scavenging. The DPPH radical (DPPH·) is a stable nitrogen radical which has deep purple color and can be detected by measuring the absorbance around 515-517 nm. When DPPH solution reacts with an antioxidant, it is decolorized to yellow (Figure 6). Although the DPPH assay is usually classified as SET reaction, these indicator radicals

may be neutralized either by electron transfers or by radical quenching via hydrogen atom transfer (Prior et al., 2005). The advantages of this method are simple and rapid; this method needs only a UV-visible spectrophotometer to be performed. It can be analyzed with both hydrophilic and organic solvents (Alam et al., 2013).

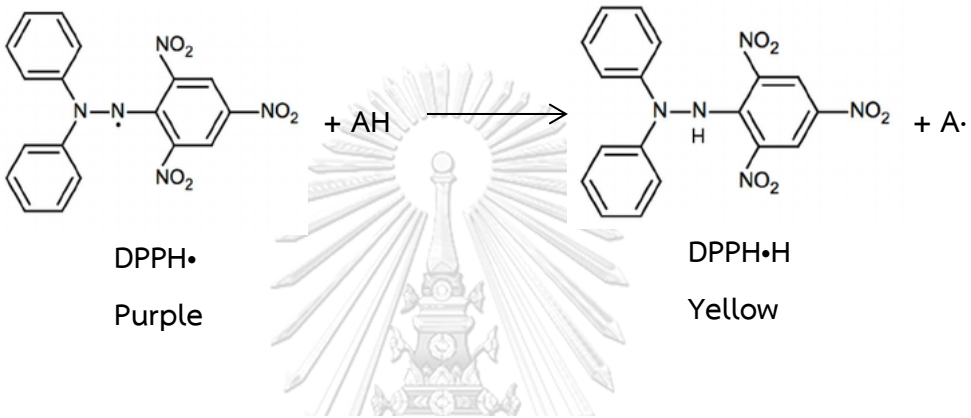
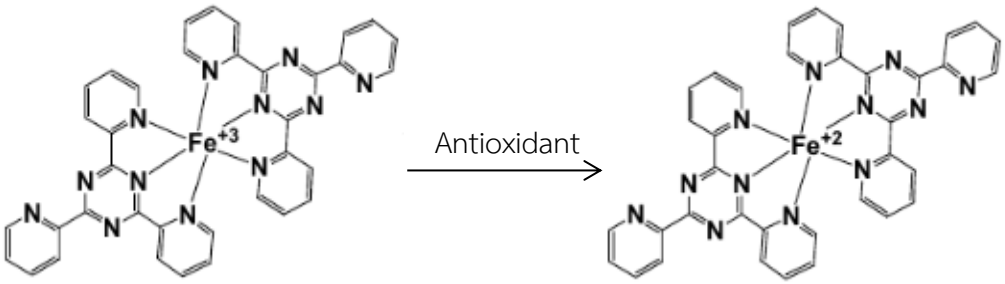


Figure 6 Reaction of DPPH radical with antioxidant

2.5.2 Ferric reducing antioxidant power (FRAP) assay

Ferric reducing antioxidant power (FRAP) assay is the method that determines the antioxidant capacity based on the reduction by the antioxidants. Ferric tripyridyltriazine (Fe^{3+} TPTZ) receives electrons from antioxidants and forms ferrous tripyridyltriazine (Fe^{2+} TPTZ) at low pH (Figure 7). This reduction is determined by measuring the absorbance at 594 nm. This reaction is assumed that the compounds that donate electrons to reduce Fe (II) can donate electrons to free radicals as well (Benzie and Strain, 1996)



Fe^{3+} TPTZ (yellow) Fe^{2+} TPTZ (blue)

Figure 7 The reduction of antioxidant with Fe^{3+} TPTZ (Prior et al., 2005)

2.5.3 Determination of total phenolic contents

The total phenolic contents are determined quantitatively using the Folin–Ciocalteu reagent. The total phenolic contents of antioxidants are expected to associate with antioxidant activity. The principle of this method is based on a chemical reduction of the reagent. The compounds that contain hydroxyl groups react with Folin-Ciocalteu reagent, and then the reagent is reduced and formed phosphotungstic -phosphomolybdic complex (blue solution) by phenolic hydroxyl groups of phenolic componds (Huang et al., 2005).

2.6 Mutagenic activity assay

Mutagens are substances that induce damage or genetic change in cells or organisms including genetic sequence, chromosome structure and chromosome number (Maron and Ames, 1983). Mutagenicity assay is a preliminary test for carcinogenesis. These assays are rapid screening tests for determining mutagenicity of chemicals and can be examined in organisms such as bacteria (*Salmonella*

typhimurium, *Escherichia coli*). However, determination of toxicity in any substances requires several experiments to support the results.

2.6.1 The Ames test

The bacterial reverse mutagenicity test (The Ames test) is wildly used to evaluate the mutagenic activity of chemicals or drugs (Maron and Ames, 1983). The procedure is rapid, convenient and not complicate. The principle of the assay is based on backward or reverse mutation that uses bacteria such as *Salmonella typhimurium* and *Escherichia coli*. The modified strains of *Salmonella typhimurium* that carry mutation gene of histidine synthesis are used. These strains require histidine for growth, so they cannot grow in histidine depleted (His^-) media. If these bacteria are exposed to a mutagen, they will regain the ability to synthesize histidine (His^+). The number of growing colonies of bacteria indicates the number of revertants or the mutagenicity (Figure 8). Furthermore, the genotype of *S. typhimurium* strains were performed by histidine requirement, rfa mutation, R-factor and uvr B mutation to improve their ability to detect mutagen (Ames et al., 1975; Kangsadalampai, 1994). The rfa mutation enhances bacterial cell membrane permeability to permit large chemical molecules. The uvrB mutation is a deletion mutation that involves in biotin synthesis and eliminates excision repair of DNA damage. In addition, the presence of R-factor plasmid (pKM101) makes bacteria strain more sensitive to certain chemicals and mutagens. The genotype of each *Salmonella typhimurium* is shown in Table 4.

Table 4 Characteristics of the genotype of each *Salmonella typhimurium* stain (Maron and Ames, 1983; Mortelmans and Zeiger, 2000).

	Genotype	R-factor	rfa	Uvr B
Framshift mutagen				
TA97	his D 6610	pKM101	Yes	Yes
TA98	his D 3052	pKM101	Yes	Yes
TA1537	his C 3076	No	Yes	Yes
TA1538	his D 3052	No	Yes	Yes
Base-pair substitute mutation				
TA100	his G 46	pKM101	Yes	Yes
TA102	his G 428	pKM101, pAQ1	Yes	Yes
TA104	his G 428	No	Yes	Yes
TA1535	his G 46	No	Yes	Yes

The strains of *Salmonella typhimurium* that used in the present study were TA98 and TA100 that represent to frameshift mutation and base-pair substitution mutation, respectively (Mortelmans and Zeiger, 2000). Mutagens are divided into two categories: direct mutagen and indirect mutagen. Indirect mutagens require enzyme activation to exhibit the mutagenic activity (De serres and Hollaender, 1980). Normally, the Ames test usually adds S-9 mix (rat liver enzyme) in the test model to determine both types of mutagens. However, several studies showed that sodium nitrite in acidic condition could convert indirect mutagen to direct mutagen (Llanes and Tannenbaum, 1982; Marqauard et al., 1977).

2.6.2 1-aminopyrene-nitrite mutagenicity model

1-aminopyrene (1-AP) is a derivative form of 1-nitropyrene. Normally, 1-nitropyrene (1-NP) is produced during incomplete combustion processes and is also predominant nitro-polycyclic aromatic hydrocarbons (nitro-PAHs) which can enter to human body via inhalation (Cherng et al., 1996; Howard et al., 1983). 1-NP is a potent direct mutagen ever tested in the Ames test. Moreover, it exhibited carcinogenicity in male rats (Howard et al., 1983). 1-NP is converted to 1-AP by anaerobic bacteria in human gastrointestinal tract. 1-AP is an indirect mutagen which can be transformed into direct-acting mutagen by nitrite. Many studies presented that 1-AP treated with nitrite in acidic condition at 37 ºC for 4 hours exhibited strong mutagenicity on *S. typhimurium* strain TA98 and TA100 (Kangsadalampai, 1994; Kato et al., 1991).

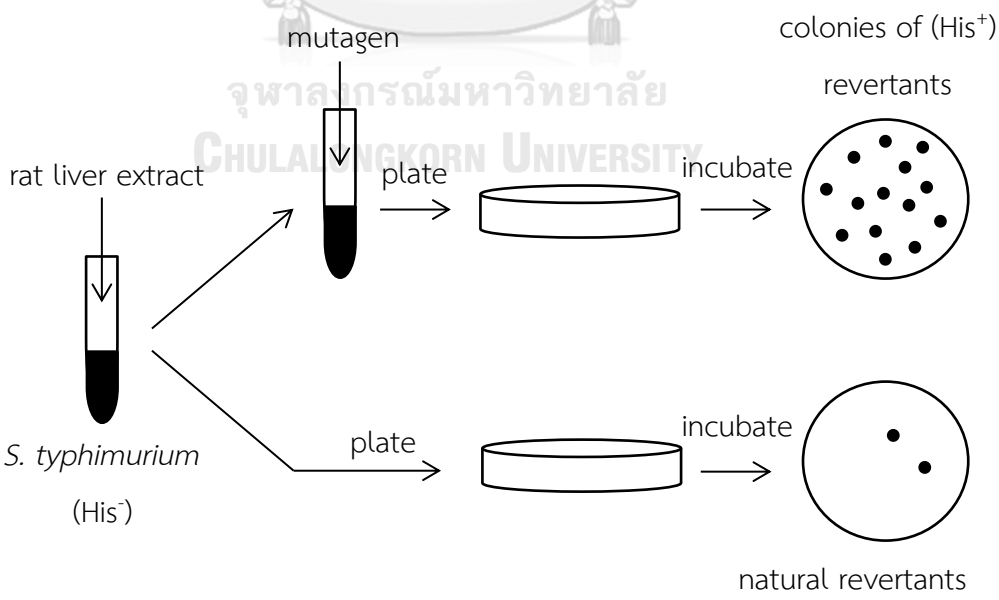


Figure 8 The Ames test

CHAPTER III
MATERIALS AND METHODS

3.1 Chemicals

3.1.1 Antioxidant activity assay

Folin-Ciocalteu reagent was purchased from Fluka Chemika (Buchs, Switzerland). Gallic acid was purchased from Merck (Darmstadt, Germany). Sodium carbonate was supplied from VWR International Ltd (Poole, England). 2,2-diphenyl-1-picrylhydrazyl was purchased from Calbiochem (Darmstadt, Germany). Iron (II) sulfate heptahydrate and sodium acetate were purchased from CARLO ERBA Reagents S.A.S. (Val De Reuil, France). Trolox (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) and Iron (III) chloride were obtained from Sigma-Aldrich Chemie GmbH (St. Louis, MO, USA).

3.1.2 The Ames test

Oxoid nutrient broth No.2 was supplied by Oxoid Ltd. (Hampshire, England). Ampicillin sodium salt was purchased from T.P. Drug laboratories (1969) Co. Ltd (Bangkok, Thailand). 1-aminopyrene, sodium nitrite, D-biotin, L-histidine and ammonium sulfamate were obtained from Sigma Chemical (St. Louis, MO, USA). Agar-agar, magnesium sulfate heptahydrate, sodium chloride, potassium chloride and crystal violet were purchased from (Darmstadt, Germany). Sodium ammonium hydrogen phosphate tetrahydrate and disodium hydrogen phosphate dihydrate were acquired from Sigma-Aldrich Chemie GmbH (St. Louis, MO, USA). Citric acid

monohydrate and sodium dihydrogen phosphate were purchased from VWR International Ltd (Poole, England). D(+)-Glucose and dipotassium hydrogen phosphate anhydrous were obtained from VWR International bvba (Leuven, Belgium). Hydrochloric acid was supplied from VWR International SAS (Fontenay-sous-Bois, France).

3.2 Experimental design

This study was conducted to evaluate the biological activity of pericarp extract and that of seed extract from *Z. limonella*. The antioxidant activity was estimated by total phenolic contents, DPPH radical scavenging activity assay and ferric reducing antioxidant power (FRAP) assay. Furthermore, determination of the mutagenicity activity including direct mutagenicity assay and mutagenicity modification assay was performed. The direct mutagenicity assay was examined, with and without nitrite treatment using the Ames test towards *S. typhimurium* strains TA98 and TA100. The mutagenicity modification assay was performed by measuring the inhibition or enhancement effect of pericarp and that of the seed extract from *Z. limonella* on the formation of mutagen occurred during the reaction between 1-AP and nitrite and on the mutagenic product produced from 1-AP treated with nitrite. The experimental design of this study is shown in Figure 9.

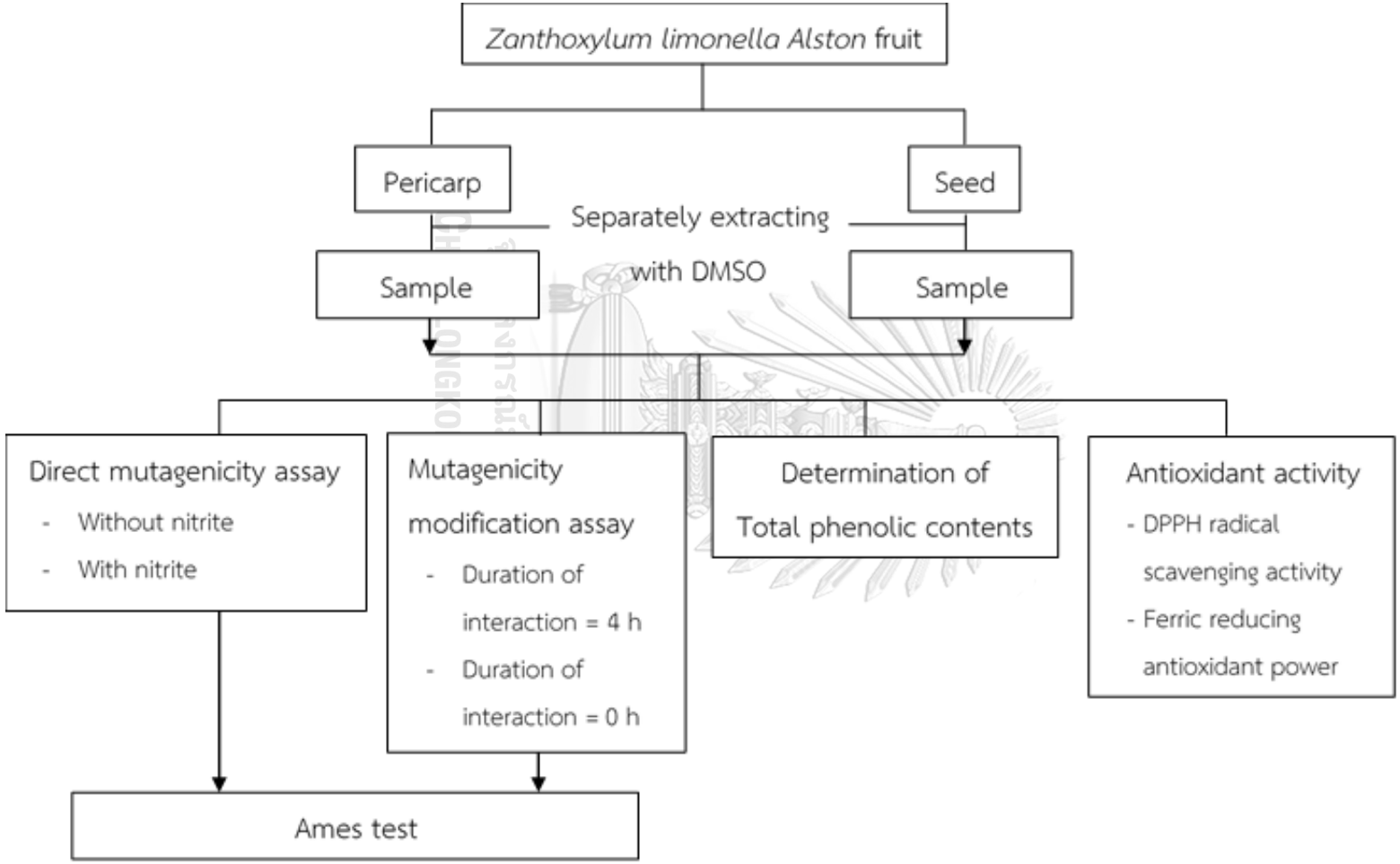


Figure 9 Experimental design

3.3 Sample preparation

Dried fruits of *Z. limonella* (Ma-kwaen) were purchased from a local market in Nan Province, Thailand. In the present study, the two samples, pericarp and seed were determined separately because when this fruit was mature its pericarp was broken, causing the separation of the seed from the pericarp.

The method of Yoshino et al. (1993) was applied for dimethylsulfoxide (DMSO) extraction in this study. The samples were dried at 40 °C for about 4 h and milled with electric grinder. Each part of fruit samples (20 g dry weight) was extracted with 120 ml of DMSO in the ratio of 1:6 w/v and transferred to a centrifuge tube. The tubes were vortexed vigorously for 15 sec and then shaken in a shaking water bath at 30 °C for 30 min. Then the extract was vortexed again for 5 sec and centrifuged at 3000 x g for 30 min at room temperature. The supernatant of each sample extract was collected and sterilized by filtration through a sterile 0.2 µm filter paper before assay. The extraction steps of the pericarp and seed are shown in Figure 10.

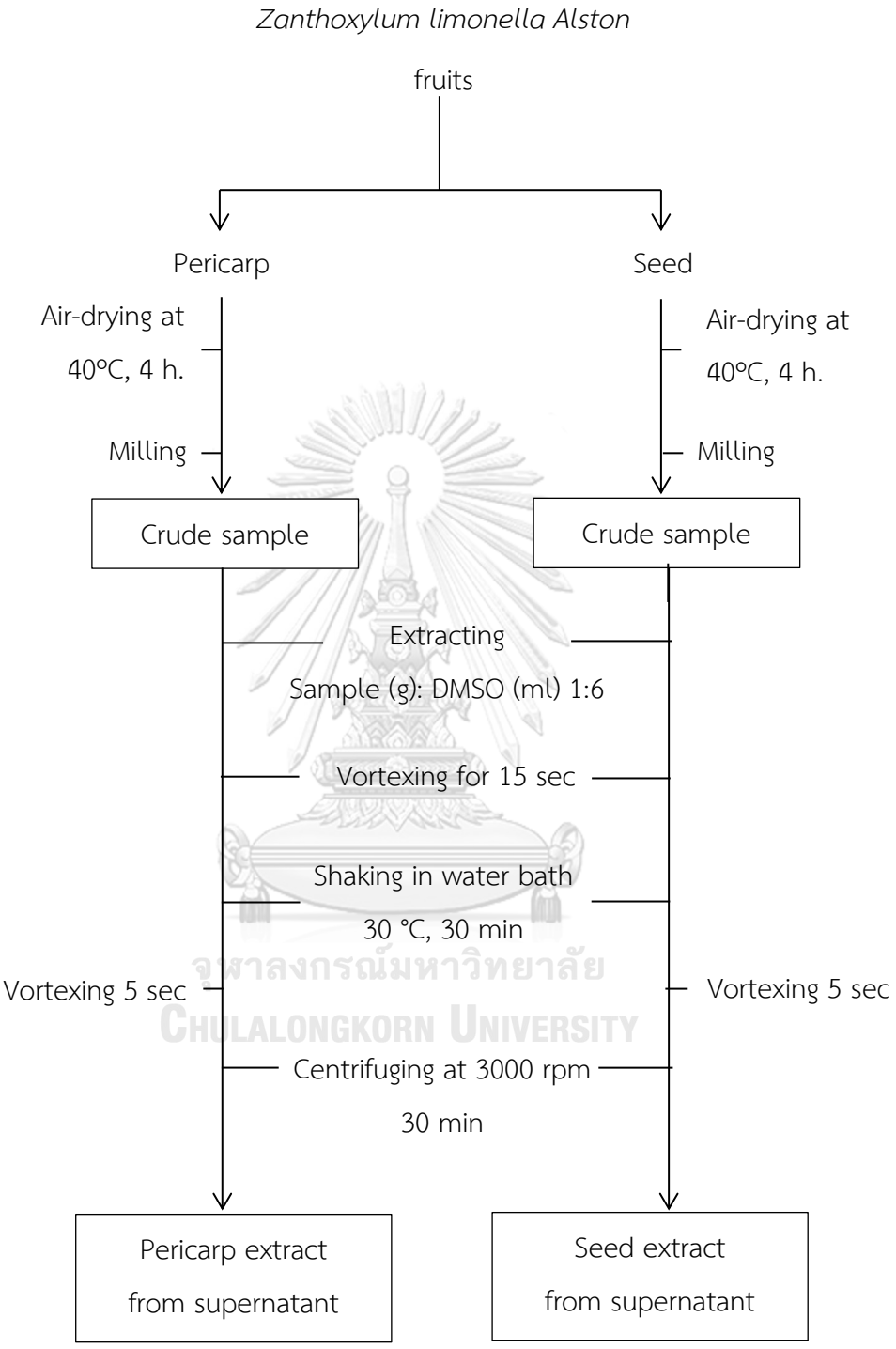


Figure 10 Sample preparation

3.4 Determination of total phenolic contents

The most widely used method for determination of total phenolic contents is Folin-Ciocalteu method, with gallic acid as the standard solution. The protocol was described by Dastmalchi et al. (2007) and Nanasombat and Wimuttigosol (2011). Concisely, 100 µl of each sample extract or gallic acid standard solution (concentration range: 25 - 800 mg/L) was transferred to a volumetric flask (10 ml) that contained 6 ml of distilled water. Then 500 µl Folin-Ciocalteu's reagent and 1.5 ml 20% sodium carbonate solution were added into a volumetric flask. The volume was adjusted to 10 ml with distilled water. After incubation for 30 min at room temperature, the absorbance was measured at 750 nm using spectrophotometer (Thermo Genesys 20, Thermo Scientific, USA). The total phenolic content of each sample was expressed as mg gallic acid equivalents (GAE)/mg dry weight of sample. All measurements were done in triplicate. Data were presented as mean ± SD.

3.5 Antioxidant activity

3.5.1 2,2'- Diphenyl-1-picrylhydrazyl (DPPH) assay

The antioxidant activity of each sample was determined by DPPH assay according to the protocol described by Singh et al. (2008). Briefly, 1 ml of each sample extract or trolox standard solution (concentration range: 0.04 - 0.16 mM in 80% methanol) was mixed with 4 ml of 0.1 mM DPPH (2,2-diphenyl-1-picrylhydrazyl) in methanol. After incubation in the dark for 30 min at room temperature, the

absorbance of the solution was measured at 515 nm using spectrophotometer (Thermo Genesys 20, Thermo Scientific, USA). Trolox equivalent antioxidant capacity of the extract was determined using standard curve of trolox solution (mg trolox/g dry weight sample). The radical scavenging activity was calculated as a percentage of DPPH scavenging activity using the following formula (Kaur et al., 2015).

$$\text{DPPH scavenging activity (\%)} = [1-(A_E/A_D)] \times 100$$

When A_E is the absorbance of sample solution. A_D is the absorbance of control (DPPH only). All measurements were done in triplicate. Data were presented as mean ± SD.

3.5.2 Ferric reducing antioxidant power (FRAP) assay

FRAP assay is based on the redox reaction of ferric tripyridyltriazine complex (Fe^{3+} TPTZ) that is reduced to ferrous tripyridyltriazine complexes (Fe^{2+} TPTZ). The protocol of this method was according to Ladoa et al. (2004). Briefly, 50 µl of each extract or Fe (II) standard solutions (concentration range: 0.0313 - 2 mM) or BHT was mixed with 1.5 ml FRAP reagent (25 mL of 300 mM acetate buffer, 2.5 mL of 10 mM 2,4,6-tri-2-pyridyl-2-triazine in 40 mM HCl and 2.5 mL of 20 mM $FeCl_3.6H_2O$). After incubation in the dark for 5 min at room temperature, the absorbance was measured at 593 nm using spectrophotometer (Thermo Genesys 20, Thermo Scientific, USA). The FRAP value of each extract was calculated using standard curve of Fe (II) standard solutions (mg Fe (II) /g dry weight sample). All measurements were done in triplicate. Data were presented as mean ± SD.

3.6 The Ames test

3.6.1 Bacterial tester strain

Salmonella typhimurium strain TA98 and TA100 represent to frameshift mutation and base-pair substitution mutation, respectively. The tester strains were recommended by Maron and Ames (1983). Both strains were supported by Assoc. Prof. Keaw Kangsadalampai. The tester strains also confirmed genotypes which improve their ability to detect mutagen including rfa mutation, R-factor and uvr B mutation. Before mutagenicity assay, both tester strains were incubated at 37 °C for 16 h in a shaking water bath.

3.6.2 Modified pre-incubation method

The mutagenicity assay was determined using the plate incorporation according to Maron and Ames (1983) and the modified pre-incubation method that was developed by Yahagi et al. (1977). In this study, the test sample was pre-incubated without metabolic activation (S9-mix) toward *S. typhimurium* strains TA98 and TA100.

The reaction mixture (100 µl) was mixed with 500 µl of 0.5 M sterile phosphate buffer (pH 7.4) and 100 µl of each overnight culture in a test tube. Then the tube was incubated at 37 °C for 20 min in shanking water bath (Hotech Instruments Corporation, Taiwan). After pre-incubation, 2 ml of top agar (45 °C) which already contained histidine and biotin was added. The mixture was vortexed and

poured on a minimal glucose agar (MGA) plate. The number of His^+ revertants per plate was counted after plate incubation in an incubator at 37 °C for 48 h.

3.7 Direct mutagenicity of *Z. limonella* extract

3.7.1 With nitrite treatment

The extracts from both parts of *Z. limonella* were evaluated for their direct mutagenicity. Briefly, an aliquot of each sample extract (10, 25 or 50 µl) was transferred to sterilized test tube and its volume was adjusted to 50 µl with DMSO. An appropriate volume of 0.2 N hydrochloric acid was added to adjust pH (3.0-3.5). The solution was mixed with 250 µl of 2 M sodium nitrite (the final volume was adjusted to 1000 µl). Then, the mixture was incubated at 37 ºC for 4 h in a shaking water bath. After the incubation, the reaction was stopped by dipping the tube in the ice bath for 1 min. Then, 250 µl of 2 M ammonium sulfamate was added into the mixture in order to decompose the residual nitrite and the tube was placed in the ice bath again for 10 min. After that the pre-incubation method was conducted following the process described in 3.6.2. The step to determine the direct mutagenicity of *Z.limonella* extract treated with nitrite using the Ames test was shown in Figure 11.

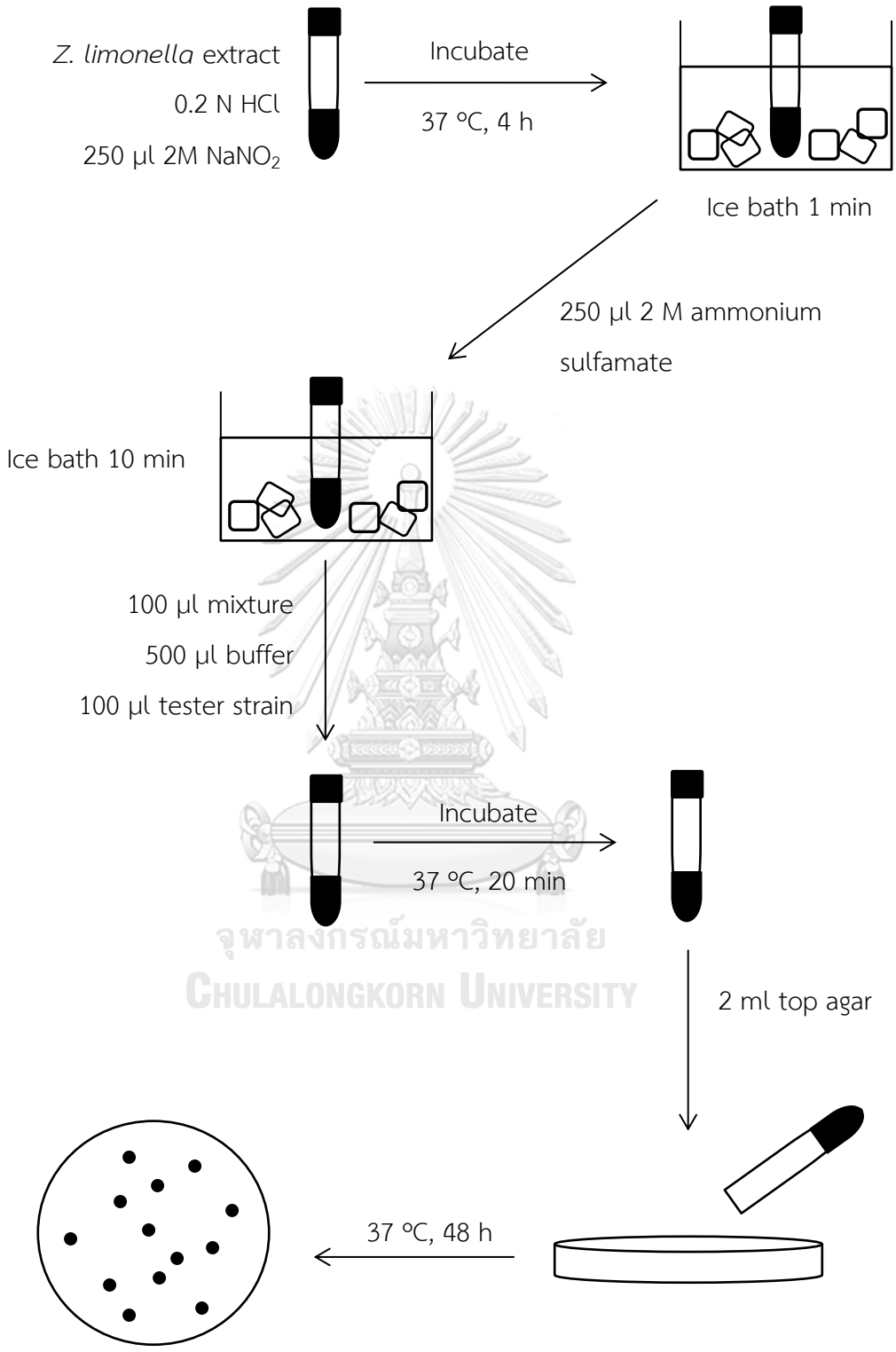


Figure 11 Step to determine the direct mutagenicity of *Z. limonella* with nitrite treatment using Ames test.

3.7.2 Without nitrite treatment

The procedure of the sample extract without nitrite treatment was done as described previously. However, distilled ultrapure water was added instead of 2 M sodium nitrite and 2 M ammonium sulfamate, respectively (Figure 12).

3.7.3 Positive control

A standard mutagen or positive control in this study was 1-aminopyrene treated with sodium nitrite in acidic condition. This reaction has been shown mutagenicity on *S. typhimurium* strain TA98 and TA100 in the absence of metabolic activation (Kato et al., 1991).

Briefly, 10 µl or 40 µl of 1-AP (0.075 mg/ml) were treated with nitrite in an acidic condition for *S. typhimurium* strain TA98 and TA100, respectively. The mutagenic product from reaction of 1-AP and nitrite was applied as standard mutagen or positive control of the Ames test. The procedure was done as followed in 3.7.1.

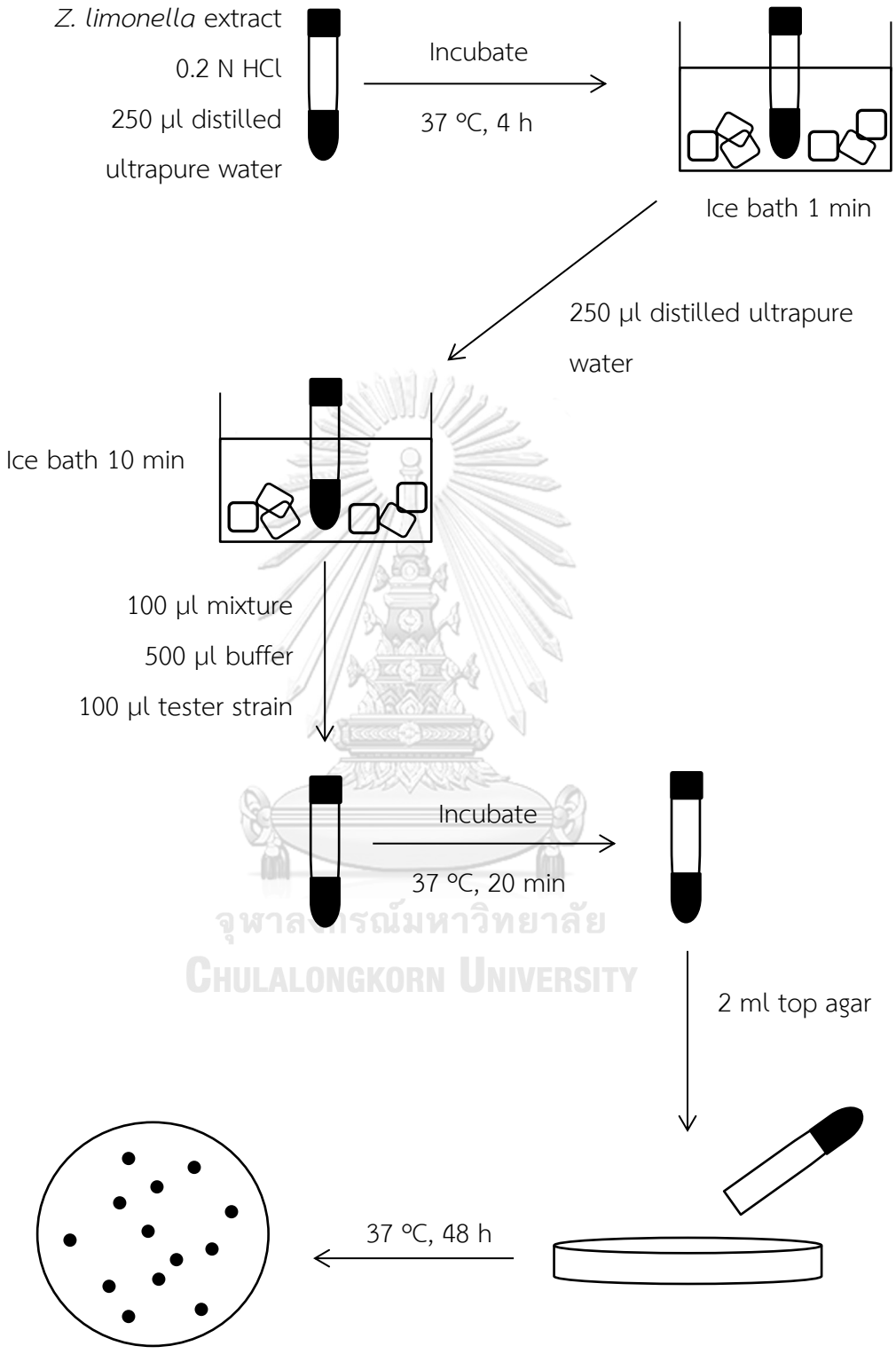


Figure 12 Step to determine the direct mutagenicity of *Z. limonella* without nitrite treatment using Ames test.

3.8 Mutagenicity modification effect of *Z. limonella* extract

The modification effect of each sample on the formation of mutagen during the reaction between 1-AP and sodium nitrite was evaluated using the Ames test (the pre-incubation method). The procedure is shown in Figure 13. Briefly, 0.075 mg/ml of 1-AP (10 µl or 40 µl for *S. typhimurium* strain TA98 or TA100, respectively) was adjusted pH to 3.0-3.5 with 0.2 N hydrochloric acid and added each concentration of sample (10, 25 or 50 µl) before treated with 250 µl of 2 N sodium nitrite in an acidic condition. The mixture was incubated at 37 ºC for 4 h in shaking water bath. After incubation, the reaction was stopped by dipping the tube in the ice bath for 1 min. Then, 250 µl of 2 M ammonium sulfamate was added in the mixture in order to decompose the residual nitrite and the tube was placed in the ice bath again for 10 min. After that the pre-incubation method was conducted following the process described in 3.6.2.

The mutagenicity modification effect of each sample on the mutagenic product produced from 1-AP treated with nitrite was also investigated. The procedure is shown in Figure 14. Briefly, 0.075 mg/ml of 1-AP (10 µl or 40 µl for *S. typhimurium* strain TA98 and TA100, respectively) was adjusted pH to 3.0-3.5 with 0.2 N hydrochloric acid and treated with 250 µl of 2 N sodium nitrite in an acidic condition. The mixture was incubated at 37 ºC for 4 h in shaking water bath. After incubation, the reaction was stopped by dipping the tube in the ice bath for 1 min. 250 µl of 2 M ammonium sulfamate was added into the mixture in order to decompose the

residual nitrite and the tube was placed in the ice bath again for 10 min. Then, the reaction mixture was added with various amounts of sample extracts (10, 25 or 50 µl) and was determined using pre-incubation method as described in 3.6.2.

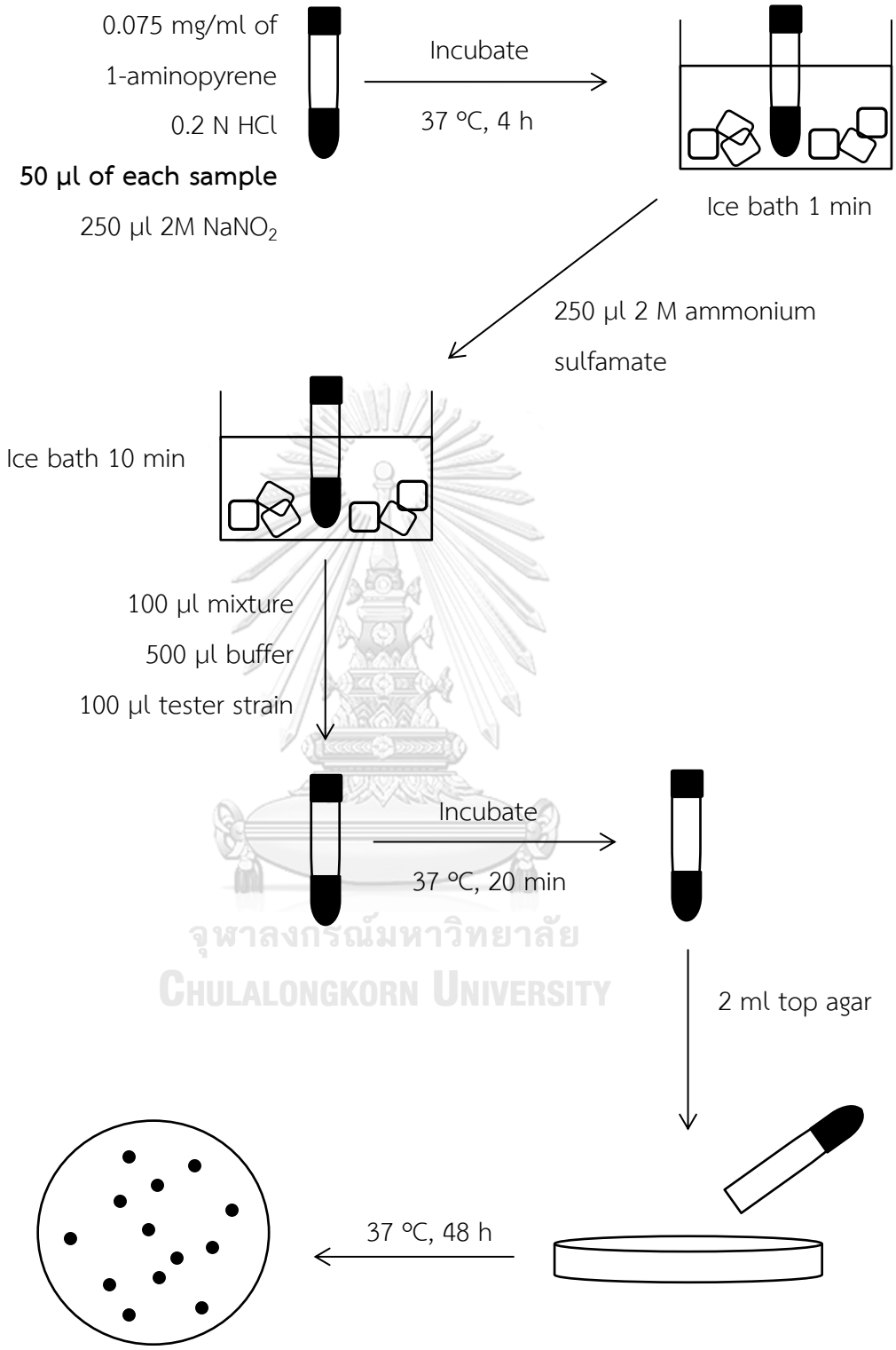


Figure 13 Step of determine the modification effect of each sample on the formation of mutagen during the reaction between 1-aminopyrene and nitrite

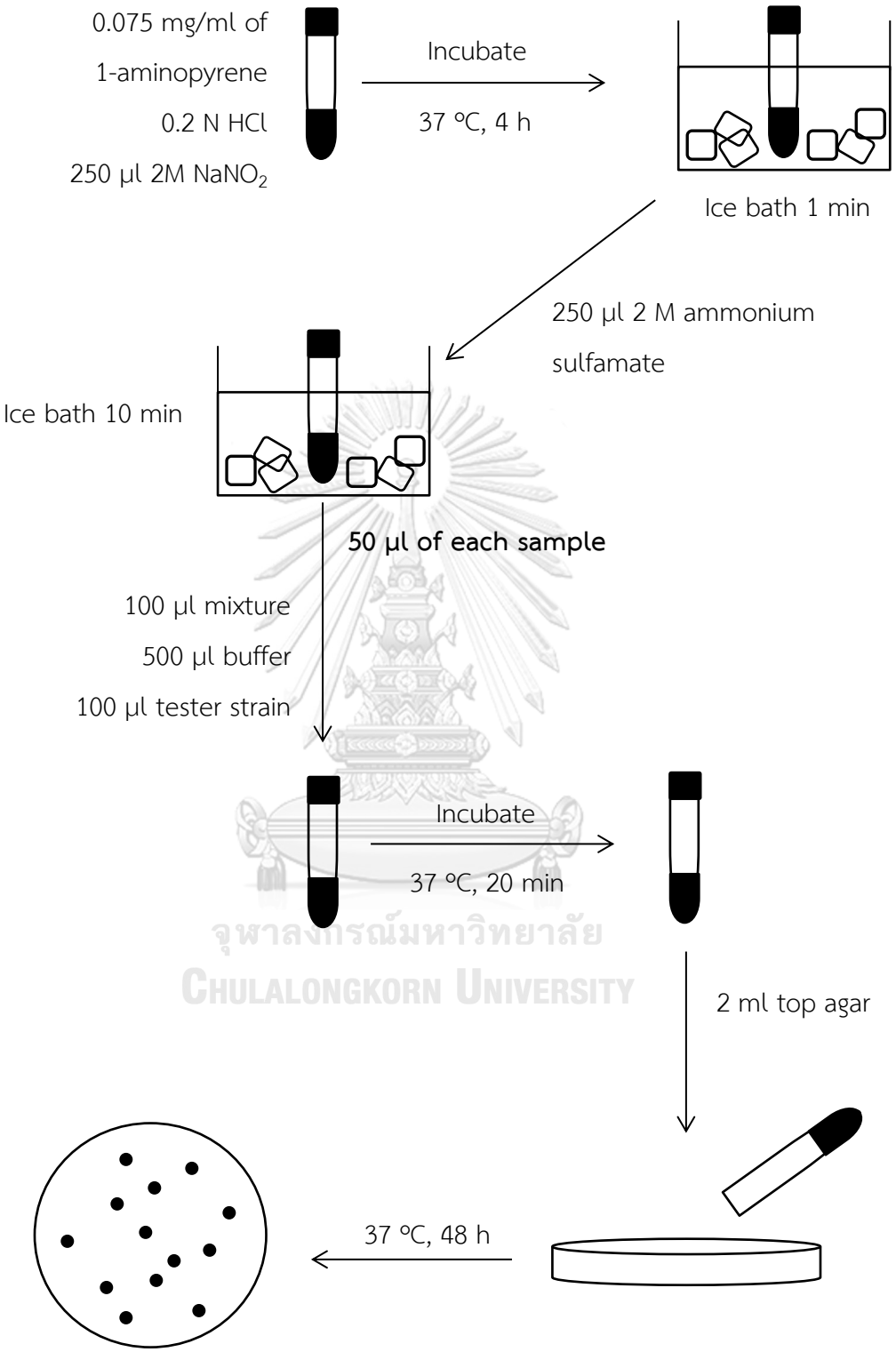


Figure 14 Step of determine the mutagenicity modification effect of each sample on the mutagenic product from 1-aminopyrene treated with nitrite

3.9 Data evaluation

The mutagenicity of each sample was presented as number of histidine revertants per plate. The data were reported as mean with standard deviation of six plates from two different determinations. If the results meet the following criteria, it can be indicated that the sample has mutagenic activity (Blackburn et al., 1986; Santos et al., 2011).

1. The data show dose-reponse relationship manner.
2. There are at least two concentrations of sample that have the number of histidine revertants per plate higher than those of spontaneous revertants (Mutagenicity index (MI) ≥1).
3. There is at least one concentration of sample that has the number of histidine revertants per plate higher than two-fold of those of spontaneous revertants. (Mutagenicity index (MI) ≥2).

Mutagenicity index (MI) was calculated from the average number of histidine revertants per plate of sample divided by the average number of spontaneous revertants.

The modification effect of each sample on the mutagenicity of nitrite treated 1-AP was evaluated by calculating the percentage of modification (Calomme et al., 1995).

$$\% \text{ Modification} = (A-B/A-C) \times 100$$

Where A is the number of histidine revertants per plate induced by nitrite treated standard mutagen 1-AP (standard mutagen), B is the number of histidine revertants per plate induced by mutagen in the present of each sample and C is the number of spontaneous revertants per plate. The percentage of modification were qualitatively ranked according to the following Table 5 (Bunkova et al., 2005).

Table 5 Criteria for evaluation as the inhibition or enhancement of mutagenicity

% Modification	**inhibition or enhancement**
± <20	No effect
± 20-40	Weak activity
± 40-60	Effective or moderate activity
± >60	Strong or potent activity

+ = inhibition effect

- = enhancement effect

CHAPTER IV

RESULTS

4.1 Determination of total phenolic contents

The absorbance values of the extracts of pericarp and that of the seed from *Z. limonella* were calculated to GAE value (mg GAE/g dry sample) from the equation of standard gallic acid solution curve (y = 0.0011x + 0.0008) (Appendix A). The extract of pericarp and that of the seed had total phenolic contents expressed as GAE values of 10.29 ± 0.56 and 1.23 ± 0.19 mg GAE/g dry sample, respectively. The result indicated that the extract of pericarp contained more phenolic compound than that of the seed extract. The result is shown in Table 6.

4.2 Antioxidant activity

4.2.1 2,2'- Diphenyl-1-picrylhydrazyl (DPPH) assay

DPPH assay was used to measure free radical scavenging in order to express the antioxidant activity of the samples. The absorbance values of the extract of pericarp and that of the seed from *Z. limonella* were calculated to trolox equivalent antioxidant capacity (TEAC) of the extract (mg trolox/g dry sample) from the equation of standard trolox solution curve (y = -4.259x + 0.7554) (Appendix A). According to the result presented in Table 6, the TEAC values of the pericarp and that of the seed extracts were 0.547 ± 0.024 and 0.204 ± 0.050, respectively. Table 7 reveals the percentage of DPPH scavenging activities of the extract of pericarp and that of the seed from *Z. limonella* at different concentrations. The result indicated that the

pericarp extract had higher percentage of DPPH scavenging activity than that of the seed extract.

4.2.2 Ferric reducing antioxidant power (FRAP) assay

FRAP assay was used to determine the antioxidant capacity of the extracts of pericarp and that of the seed from their potentiality to reduce ferric tripyridyltriazine (Fe^{3+} TPTZ) complex. Antioxidant activity was revealed as FRAP value (mg Fe (II) /g dry sample). The absorbance value of each extract was calculated to FRAP value from the equation of $FeSO_4 \cdot 7H_2O$ standard curve (y = 0.6827x - 0.0135) (Appendix A). The FRAP values of the pericarp extract and that of the seed extract were 257.91 ± 3.74 and 17.94 ± 1.45, respectively. The results indicated that the extract from pericarp showed higher reducing power than that from the seed. The reducing power (FRAP value) of all extract are presented in Table 6.

Table 6 Antioxidant activity and total phenolic content of extracts from different part of *Z. limonella*

Sample	Total phenolic content (mg GAE/g dry sample)[a]	TEAC value (mg trolox/g dry sample)[b]	FRAP value (mg Fe (II) /g dry sample)[c]
Pericarp	10.29 ± 0.56	0.547 ± 0.024	257.91 ± 3.74
Seed	1.23 ± 0.19	0.204 ± 0.050	17.94 ± 1.45

All values are reported as mean with standard deviation of triplicate determinations.

[a] Total phenolic content was calculated from a standard curve of gallic acid (25-800 mg/l).

[b] TEAC value was calculated from a standard curve of Trolox (0.04-0.16 mM).

[c] FRAP value was calculated from a standard curve of $FeSO_4 \cdot 7H_2O$ (0.0313 -2 mM).

Table 7 The percentage of DPPH scavenging activity of the extracts from different parts of *Z. limonella* at different concentrations

Concentration (g/ml)	Percentage of DPPH scavenging activity	
	Pericarp	Seed
0.25	81.80	39.37
0.125	80.18	34.25
0.0625	78.22	22.44
0.025	69.85	0.00
0.00625	29.66	0.00

4.3 Mutagenic activity

4.3.1 The direct mutagenic activity of *Z. limonella* extract

Table 8 reveals the direct mutagenicity of *Z. limonella* pericarp extract and that of the *Z. limonella* seed extract with or without nitrite treatment on *S. typhimurium* strain TA98. Both sample extracts without nitrite treatment showed no mutagenicity on *S. typhimurium* strain TA98 because the number of histidine revertants per plate of all concentrations of each extract was less than two times of the spontaneous revertants. After being treated with sodium nitrite, 25 and 50 µl/plate of the pericarp extract induced 29±6 and 66±7 histidine revertants per plate, respectively; the values were more than twice of that of spontaneous revertants. These results indicated that an increased number of revertant colonies were related to an increased dose of pericarp extract (dose response relationship). However, the mutagenicity of seed extract with nitrite treatment was not observed.

The direct mutagenic activity of *Z. limonella* pericarp extract and that of the *Z. limonella* seed extract without and with nitrite treatment on *S. typhimurium* strain TA100 are shown in Table 9. Both samples were not direct mutagenic on *S. typhimurium* strain TA100.

Table 8 The direct mutagenic activity of extracts from different parts of *Z. limonella* with or without nitrite treatment on *S. typhimurium* strain TA98

Sample	Amount of extract (µl/plate)	without nitrite treatment		with nitrite treatment	
		Number of His+ revertants/plate[a]	MI[C]	Number of His+ revertants/plate[a]	MI[C]
Pericarp	0[b]	15 ± 3		15 ± 3	
	0	10 ± 3	0.66	9 ± 4	0.60
	10[d]	10 ± 2	0.66	20 ± 2	1.33
	25[e]	10 ± 2	0.66	29 ± 6	1.93
	50[f]	12 ± 4	0.81	**66 ± 7**	4.51
Seed	0[b]	26 ± 1		26 ± 1	
	0	28 ± 2	1.08	27 ± 3	1.04
	10[d]	33 ± 2	1.27	32 ± 5	1.23
	25[e]	32 ± 4	1.23	30 ± 2	1.15
	50[f]	34 ± 3	1.31	31 ± 4	1.19

[a] reported as mean ± standard deviation of six plates from two different determinations.

[b] Spontaneous mutation

[c] Mutagenicity index (MI) is calculated from number of His^+ revertants/plate of sample divides by the average number of spontaneous revertants.

[d] equivalent to 2.5 mg dry sample /plate

[e] equivalent to 6.5 mg dry sample /plate

[f] equivalent to 12.5 mg dry sample /plate

His^+ revertants/plate of *S. typhimurium* strain TA98 induced with the positive mutagen was 1943 ± 111

Table 9 The direct mutagenic activity of extracts from different parts of *Z. limonella* with or without nitrite treatment on *S. typhimurium* strain TA100

Sample	Amount of extract (µl/plate)	without nitrite treatment		with nitrite treatment	
		Number of His+ revertants/plate[a]	MI[c]	Number of His+ revertants/plate[a]	MI[c]
Pericarp	0[b]	136 ± 8		136 ± 8	
	0	128 ± 9	0.94	136 ± 8	0.86
	10[d]	122 ± 9	0.90	157 ± 11	1.15
	25[e]	128 ± 8	0.94	208 ± 16	1.53
	50[f]	77 ± 7	0.57	220 ± 34	1.62
Seed	0[b]	92 ± 3		92 ± 3	
	0	86 ± 10	0.93	88 ± 9	0.96
	10[d]	81 ± 7	0.88	92 ± 10	1.00
	25[e]	92 ± 7	1.00	89 ± 11	0.97
	50[f]	96 ± 9	1.04	91 ± 11	0.99

[a] reported as mean ± standard deviation of six plates from two different determinations.

[b] Spontaneous mutation

[c] Mutagenicity index (MI) is calculated from number of His^+ revertants/plate of sample divides by the average number of spontaneous revertants.

[d] equivalent to 2.5 mg dry sample /plate

[e] equivalent to 6.5 mg dry sample /plate

[f] equivalent to 12.5 mg dry sample /plate

His^+ revertants/plate of *S. typhimurium* strain TA100 induced with the positive mutagen was 821 ± 19

4.3.2 Mutagenicity modification effect of *Z. limonella* extract

This section was divided into two parts. The first part was to investigate the addition of sample extract at the beginning of the reaction between 1-AP and sodium nitrite for evaluating the antiforming activity. Table 10 reveals that both *Z. limonella* pericarp extract and *Z. limonella* seed extract interfered with the formation of standard mutagen on *S. typhimurium* strain TA98. It was found that the pericarp extract increased the formation of standard mutagen when the extract was added at the beginning of the reaction. The percentages of the mutagenic modification of the pericarp extract were 61%, 52% and 47% enhancement for 10, 25 and 50 µl/plate, respectively. On the other hand, the seed extract decreased the formation of standard mutagen. The percentages of modification of the seed extracts were 69%, 64% and 72% inhibition for 10, 25 and 50 µl/plate, respectively.

The second part was to determine the effect of sample extracts on the mutagenicity of standard mutagen that obtained from the reaction between 1-AP and sodium nitrite in order to evaluate the antimutagenicity. Table 10 shows that the lowest amount of the pericarp extract increased the mutagenicity of the standard mutagen but decreased with the other two higher amounts (46% and 84% inhibition for 25 and 50 µl/plate, respectively). On the other hand, the effect of the seed extract on the mutagenicity of standard mutagen was not observed (<-20% enhancement).

Table 10 The modification effect of *Z. limonella* on the mutagenicity of nitrite treated 1-AP on *S. typhimurium* strain TA98

Sample	Amount of extract (µl/plate)	Interaction between sample and standard mutagen			
		Sample presented at the beginning of the formation of standard mutagen		Sample added after the formation of standard mutagen	
		Number of His^+ revertants/plate[a]	%Modification[b]	Number of His^+ revertants/plate[a]	%Modification[b]
Pericarp	0[c]	1966 ± 133		1951 ± 192	
	10[d]	3162 ± 158	-61	2949 ± 207	-52
	25[e]	2980 ± 235	-52	1069 ± 89	+46
	50[f]	2885 ± 207	-47	320 ± 44	+84
Seed	0[c]	1641 ± 152		1590 ± 88	
	10[d]	510 ± 63	+69	1745 ± 160	-10
	25[e]	595 ± 60	+64	1863 ± 163	-17
	50[f]	465 ± 117	+72	1778 ± 128	-12

[a] reported as mean ± standard deviation of six plates of two different experiments.

[b] + or - represents inhibiting or enhancing modification, respectively.

[c] Standard mutagen: 1-aminopyrene interacted with sodium nitrite

[d] equivalent to 2.5 mg dry sample/plate

[e] equivalent to 6.5 mg dry sample/plate

[f] equivalent to 12.5 mg dry sample/plate

No. spontaneous mutation/plate of *S. typhimurium* strain TA98 was 13 ± 2

Table 11 reveals the mutagenicity modification effect of sample extracts on *S. typhimurium* strain TA100. When the pericarp extract was added to the reaction mixture at the beginning of the reaction between 1-AP and sodium nitrite, there was no effect on the mutagenicity of the mutagen. Meanwhile, the seed extract showed weak inhibitory effect (20%, 22% and 29% inhibition for 10, 25 and 50 µl/plate, respectively).

The effect of sample extracts on the mutagenicity of standard mutagen that obtained from the reaction between 1-AP and sodium nitrite on *S. typhimurium* strain TA100 are shown in Table 11. The two higher amounts of the extract of pericarp could inhibit the mutagenicity of the standard mutagens (53% and 56% inhibition for 25 and 50 µl/plate, respectively). On the other hand, the modification effect of the seed extracts was not observed (<-10% enhancements) on *S. typhimurium* strain TA100.

Table 11 The modification effect of *Z. limonella* on the mutagenicity of nitrite treated 1-AP on *S. typhimurium* strain TA100

Sample	Amount of extract (µl/plate)	Interaction between sample and standard mutagen			
		Effect on mutagen formation (duration of presence was 4h)		Effect on mutagenic products (duration of interaction was 0 h)	
		Number of His^+ revertants/plate[a]	%Modification[b]	Number of His^+ revertants/plate[a]	%Modification[b]
Pericarp	0[c]	1154 ± 73		1149 ± 90	
	10[d]	1234 ± 101	-8	1045 ± 128	+10
	25[e]	1288 ± 54	-13	614 ± 77	+53
	50[f]	1351 ± 98	-19	578 ± 49	+56
Seed	0[c]	1270 ± 121		1266 ± 102	
	10[d]	1043 ± 88	+20	1353 ± 131	-8
	25[e]	1015 ± 41	+22	1385 ± 83	-10
	50[f]	938 ± 80	+29	1343 ± 74	-7

[a] reported as mean ± standard deviation of six plates of two different experiments.

[b] + or - represents inhibiting or enhancing modification, respectively.

[c] Standard mutagen: 1-aminopyrene interacted with sodium nitrite

[d] equivalent to 2.5 mg dry sample/plate

[e] equivalent to 6.5 mg dry sample/plate

[f] equivalent to 12.5 mg dry sample/plate

No. spontaneous mutation/plate of *S. typhimurium* strain TA100 was 131 ± 20

CHAPTER V
DISCUSSION

The aims of this study were to determine the antioxidant and antimutagen activities of pericarp extract and that of the seed extract from *Z. limonella*. Normally, *Z. limonella* fruit (including pericarp and seed) have been widely used as a flavoring agent for some kinds of food. Biological activities of different parts of *Z. limonella* were investigated. Many studies reported that essential oil and extract of stems, leaves and fruits of *Z. limonella* showed antioxidant, antimicrobial, antiviral and anticancer activity (Naik, 2015; Nanasombat and Wimuttigosol, 2011; Tangjitjaroenkun, 2012). However, Wongsrisom et al. (2014) and Supabphol and Tangjitjareonkun (2015) reported that the pericarp and the seed of *Z. limonella* contained different phytochemical constituents. Therefore, the purposes of this study were to investigate the antioxidant and anti-mutagen activities of Z. *limonella* pericarp extract, compared with that obtained from *Z. limonella* seed extract.

5.1 Antioxidant activity

In the present study, the total phenolic content of *Z. limonella* pericarp extract and that of the *Z. limonella* seed extract was determined with the Folin-Ciocalteu method. Moreover, the antioxidant activity of each extract was evaluated by two different in *in vitro* assays namely DPPH assay and FRAP assay. Piluzza and Bullitta (2011) suggested that the phenolic content highly contributed to the total

antioxidant capacity of several plant species. The antioxidant properties of phenolic compounds involve their redox properties that allow them to act as reducing agents, hydrogen donor, metal chelators, and singlet oxygen quenchers (Piluzza and Bullitta, 2011). From this study, it was found that the pericarp extract showed a higher total phenolic content than that of the seed extract. So it was not surprising that the pericarp extract exhibited a higher antioxidant activity than that of the seed extract. Wongsrisom et al. (2014) found that *Z. limonella* seed contained lower essential oil than *Z. limonella* pericarp. Supabphol and Tangjitjareonkun (2015) and Wongsrisom et al. (2014) reported that the phytochemical constituents of essential oil of *Z. limonella* fruit were monoterpene hydrocarbons and oxygenated monoterpene such as limonene, sabinene, α-terpineol and α-terpinene etc. This monoterpenes showed antioxidant activity through the direct ROS scavenging pathway and modulating the endogenous antioxidant system (Kadri and Zarai, 2011).

5.2 Direct mutagenicity of *Z. limonella* extract

It was found that *Z. limonella* seed extract, either with or without nitrite treatment, had no direct mutagenicity on both *S. typhimurium* strains. The results showed that *Z. limonella* seed extract did not contain any substances, which could produce a direct mutagen after being treated with nitrite. On the other hand, the direct mutagenic activity of *Z. limonella* pericarp extract after being treated with nitrite on *S. typhimurium* strains 98 was observed. This result was not surprising

because several investigations found the possibility of the mutagenicity of many food products after nitrite treatment. Wakabayashi et al. (1983) reported mutagenicity of pickled vegetables, sun-dried seafood, soy sauces, and fish sauces after treated with nitrite. In addition, many spices including chili, laurel, nutmeg, and pepper presented the mutagenicity on *S. typhimurium* strains TA98 and TA100 after being treated with nitrite (Namiki et al., 1984). Moreover, Stoltz et al. (1984) suggested that several fruits and vegetables containing high antioxidants showed mutagenicity on *Salmonella* tester strains using the Ames test.

The results of total phenolic content from this study showed that pericarp extract contained more phenolic compound than the seed extract. It indicated that the reaction between phenolic compounds and sodium nitrite may formed direct mutagen in acidic condition. This hypothesis was consistent with the study of Ohshima et al. (1989). They found that simple phenolic compounds (including phenol, 3-methoxycatechol, catechol and vanillin) had direct mutagenicity on *S. typhimurium* strains TA98 and TA100 after being treated with sodium nitrite. Accordingly, the consumption of *Z. limonella* pericarp with nitrite or food products that contained nitrite; such as, sausages, ham and bacon, should be avoided to prevent the formation of mutagens.

5.3 Antiforming of *Z. limonella* extract

The result obtained from the addition of each sample extract at the beginning of the reaction between 1-aminopyrene and sodium nitrite exhibited the

interfering effect of *Z. limonella* extract on the formation of standard mutagen. The results showed that the seed extract could decrease the formation of standard mutagen. Normally, the reaction between nitrite and 1-aminopyrene could produce the direct mutagen (nitro-compound) via the nitration reaction (Howard et al., 1983). It indicated that there might be some compounds in the seed extract that could inhibit the nitration of 1-AP. Supabphol and Tangjitjareonkun (2015) reported that there were oil and unsaturated fatty acid accumulated in the seeds of *Z. limonella*. Fatty acids have been reported in previous study that they could react with nitrite under acidic condition to produce nitro-fatty acids (Lima et al., 2002) and could inhibit nitrosation reaction by scavenging nitrite (kikugawa and kato, 1991). Therefore, it was possible that the mechanism to reduce the formation of standard mutagen of the *Z. limonella* seed extract was caused by the scavenging activity of fatty acids that contained in the seeds. However, there is limited information on the phytochemical composition of *Z. limonella* seed; therefore, further experiment to determine the active compounds of the seed is required.

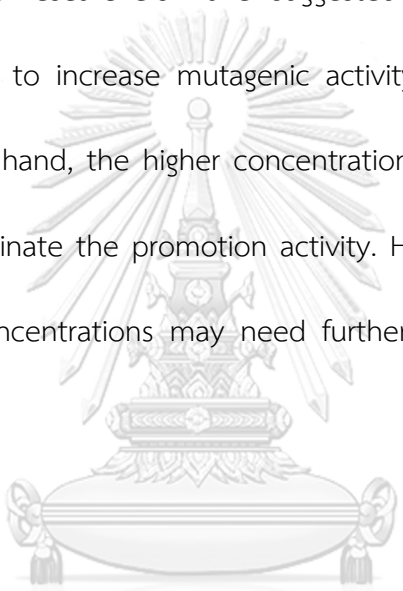
On the other hand, the pericarp extract increased the formation of the standard mutagen during the reaction between 1-AP and sodium nitrite on *S. typhimurium* strain TA98. This result may be caused by the phenolic compounds that contained in the *Z. limonella* pericarp extract. This compounds could react with nitrites and then got new mutagens (Ohshima et al. (1989). In the same system, standard mutagen was generated from the reaction between 1 -AP and nitrite.

Therefore, the pericarp extract increased the mutagenicity of the standard mutagen by a combination of mutagenic activities of the new mutagen and the standard mutagen. This indicated the additive effect. However, consumption of *Z. limonella* fruits may not be harmful to customers because most spices are used in small quantities.

5.4 Antimutagen of *Z. limonella* extract

The addition of *Z. limonella* pericarp extract into the finished reacting solution showed the antimutagenic activity against direct mutagenicity of the newly formed standard mutagen on *S. typhimurium* strains TA98 and TA100. Normally, the mutagenic expression of direct mutagens required bacterial biotransformation to activate their mutagenicity on the bacterial DNA (Arimochi et al., 1998; Mermelstein et al., 1981). Inhibition on bacterial enzymes for metabolic activation of nitro-compounds may be another mechanism of antimutagenicity against standard mutagen. *Z. limonella* pericarp is rich in aromatic compounds which contained monoterpene (Supabphol and Tangjitjareonkun, 2015; Wongsrisom et al., 2014). This assumption is consistent with the previous study that reported the inhibition effect of monoterpene (linalool and beta-caryophyllene) on the mutagenicity of 2-nitrofluorene on *S. typhimurium* strains TA98 and TA100 by inhibiting metabolic enzymes of bacteria (Di Sotto et al., 2008). Therefore, the antimutagenicity of the pericarps extract may involve with the inhibitory effect on metabolic enzymes of bacteria, nitroreductase and O-transferase.

However, the lowest concentration of the pericarp extract increased the mutagenicity of the standard mutagen on *S. typhimurium* strains TA98, but the decreasing effect was found with the other two higher amounts. Similarly, Stavric (1994) reported that the lowest dose of green tea extract significantly stimulated the mutagenicity of some heterocyclic amines (PhIP, Trp-P-1 and Trp-P-2) but decreased with higher dose. The researchers have suggested that there may be enough quantities of enhancer to increase mutagenic activity of mutagens in diluted tea extracts. On the other hand, the higher concentration may contain more inhibitory factors that could eliminate the promotion activity. However, *Z. limonella* pericarp extract at different concentrations may need further investigation to confirm this hypothesis.



CHAPTER VI
CONCLUSION

Z. limonella pericarp extract exhibited higher total phenolic contents and antioxidant activity than that of *Z. limonella* seed extract. The seed extract had no direct mutagenic activity, either with or without nitrite treatment on *S. typhimurium* strains TA98 and TA100. On the other hand, the pericarp extract showed a direct mutagenic activity after being treated with nitrite on *S. typhimurium* strains TA98. In accordance with the mutagenicity modification effect, this study showed the different mechanisms of the effect of *Z. limonella* pericarp extract and that of the *Z. limonella* seed extract on mutagenicity modification of standard mutagen on *S. typhimurium* strains TA98 and TA100. The seed extract inhibited the formation of standard mutagen that occurred during the reaction between 1-AP and sodium nitrite. Moreover, antimutagenicity of pericarp extract on standard mutagen that produced from 1-AP treated with nitrite was observed.

This finding suggested that the consumption of pericarp from *Z. limonella* with nitrite or nitrite containing food products should be avoided to prevent the formation of mutagen. However, consumption of *Z. limonella* fruits may not be harmful to customers because most spices are used in small quantities.

REFERENCES

Aicha, N., Ines, S., Mohamed, B. S., Ines, B., Soumaya, K., Kamel, G., et al. 2011. Chemical composition, mutagenic and antimutagenic activities of essential oils from (Tunisian) *Artemisia campestris* and *Artemisia herba-alba*. J Essent Oil Res. 20(5): 471-477.

Alam, M. N., Bristi, N. J., and Rafiquzzaman, M. 2013. Review on in vivo and in vitro methods evaluation of antioxidant activity. Saudi Pharm J. 21(2): 143-152.

Ames, B. N., McCann, J., and Yamasaki, E. 1975. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutat Res. 31(6): 347-363.

Antonella, D. S., Federico, D., Grazia, S. M., and Gabriela, M. 2013. Antimutagenic and antioxidant activities of some bioflavours from wine. Food Chem Toxicol. 60: 141-146.

Arimochi, H., Kinouchi, T., Kataoka, K., Kuwahara, T., and Ohnishi, Y. 1998. Activation of 1-nitropyrene by nitroreductase increases the DNA adduct level and mutagenicity. J Med Invest. 44(3): 193-198.

Basak, A. K., Chatterjee, T., Ghosh, S. K., and Chakravarty, A. 2017. Impacts of dietary exposure to sodium or potassium salts of nitrate and nitrite on the development of *Drosophila melanogaster*. Interdiscip Toxicol. 10(2).

Béliveau, R., and Gingras, D. 2007. Role of nutrition in preventing cancer. Can Fam Physician. 53: 1905-1911.

Benzie, I. F. F., and Strain, J. J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of ‘‘Antioxidant Power’’: The FRAP assay. Anal. Biochem. 239: 70-76.

Blackburn, R. G., Deitch, A. R., Ceinwen, S. A., and Carl, M. R. 1986. Predicting carcinogenicity of petroleum distillation fractions using a modified *salmonella* mutagenicity assay. Cell Biol Toxicol. 2(1): 63-84.

Blot, W. J., Li, J.-Y., Taylor, P. R., Guo, W., Dawsey, S., Wang, G.-Q., et al. 1993. Nutrition intervention trials in Linxian, China: Supplementation with specific vitamin/mineral combinations, cancer incidence, and disease specific mortality

in the general population. J Natl Cancer Inst. 85(18): 1483–1491.

Borek, C. 2017. Dietary antioxidants and human cancer. JRM. 6(1): 53-61.

Bunkova, R., Marova, I., and Nemec, M. 2005. Antimutagenic properties of green tea. Plant Food Hum Nutr. 60(1): 25-29.

Burgos, G. E., and Gómez-Serranillos, M. P. 2012. Terpene compounds in nature: A review of their potential antioxidant activity. Curr Med Chem. 19: 5319-5341.

Calomme, M., Pieters, L., Vlietinck, A., and Berghe, V. D. 1995. Inhibition of bacterial mutagenesis by citrus flavonoids. Planta Medica. 62: 222—226.

Cantwell, M., and Elliott, C. 2017. Nitrates, nitrites and nitrosamines from processed meat intake and colorectal cancer risk. J Clin Nutr Diet. 3(4).

Charoensup, R., Duangyod, T., Phuneerub, P., and Singharachai, C. 2016. Pharmacognostic specification of *Zanthoxylum limonella* (Dennst.) Alston: Fruits and seeds in Thailand. J Adv Pharm Technol Res. 7(4): 134-138.

Cherng, S. H., Lin, S. T., and Lee, H. 1996. Modulatory effects of polycyclic aromatic hydrocarbons on the mutagenicity of 1-nitropyrene: a structure-activity relationship study Mutat Res. 367 177-185

Cho, K. S., Lim, Y. R., Lee, K., Lee, J., Lee, J. H., and Lee, I. S. 2017. Terpenes from Forests and Human Health. Toxicol Res. 33(2): 97-106.

Crowell, P. L. 1999. Prevention and therapy of cancer by dietary monoterpenes. J Nutr. 129: 775S–778S.

Dastmalchi, K., Damien Dorman, H. J., Laakso, I., and Hiltunen, R. 2007. Chemical composition and antioxidative activity of Moldavian balm (*Dracocephalum moldavica* L.) extracts. LWT. 40(9): 1655-1663.

De serres, F. J., and Hollaender, A. 1980. Chemical mutagens: principles and methods for their detection. New york: National Institute of Environmental Health Sciences.

Dhifi, W., Bellili, S., Jazi, S., Bahloul, N., and Mnif, W. 2016. Essential oils' chemical characterization and investigation of some biological activities: A critical review. Medicines. 3(4).

Di Sotto, A., Evandri, M. G., and Mazzanti, G. 2008. Antimutagenic and mutagenic activities of some terpenes in the bacterial reverse mutation assay. Mutat Res.

653(1-2): 130-133.

Erdemgil, F. Z., Ilhan, S., Korkmaz, F., Kaplan, C., Mercangöz, A., Arfan, M., et al. 2008. Chemical composition and biological activity of the essential oil of *Perovskia atriplicifolia* from Pakistan. Pharm Biol. 45(4): 324-331.

Gautam, N., Mantha, A. K., and Mittal, S. 2014. Essential oils and their constituents as anticancer agents: a mechanistic view. Biomed Res Int. 2014: 154106.

Grabmann, J. 2005. Terpenoids as plant antioxidants. Vitam Horm. 72: 505-535.

Hernandez-Ramirez, R. U., Galvan-Portillo, M. V., Ward, M. H., Agudo, A., Gonzalez, C. A., Onate-Ocana, L. F., et al. 2009. Dietary intake of polyphenols, nitrate and nitrite and gastric cancer risk in Mexico City. Int J Cancer. 125(6): 1424-1430.

Honikel, K. O. 2008. The use and control of nitrate and nitrite for the processing of meat products. Meat Sci. 78(1-2): 68-76.

Howard, P. C., Beland, F. A., and Cerniglia, C. E. 1983. Reduction of the carcinogen 1-nitropyrene to 1-aminopyrene by rat intestinal bacteria. Carcinogenesis. 4(8): 985-990.

Huang, D., Ou, B., and Prior, R. 2005. The chemistry behind antioxidant capacity assays. J Agric Food Chem. 53: 1841-1856.

Jakszyn, P., and González, C. A. 2006. Nitrosamine and related food intake and gastric and oesophageal cancer risk: A systematic review of the epidemiological evidence. World J Gastroenterol. 12(27): 4296-4303.

Jia, S. S., Xi, G. P., Zhang, M., Chen, Y. B., Lei, B., Dong, X. S., et al. 2013. Induction of apoptosis by D-limonene is mediated by inactivation of Akt in LS174T human colon cancer cells. Oncol Rep. 29(1): 349-354.

Kadri, A., and Zarai, Z. 2011. Chemical constituents and antioxidant properties of *Rosmarinus officinalis* L. essential oil cultivated from the South-Western of Tunisia. J Med Plants Res. 5(29).

Kangsadalampai, K. 1994. Toxicology: Principle and Application for Food and Nutrition. Nakhon Pathom: The Institute of Nutrition.

Karimi, P., Islami, F., Anandasabapathy, S., Freedman, N. D., and Kamangar, F. 2014. Gastric cancer: descriptive epidemiology, risk factors, screening, and prevention. Cancer Epidemiol Biomarkers Prev. 23(5): 700-713.

Kato, T., Tadokoro, N., Tsutsui, M., and Kikugawa, K. 1991. Transformation of arylamines into direct-acting mutagens by reaction with nitrite. Mutat Res. 249: 243-254.

Kaur, R., Kaur, U., and Walia, H. 2015. Evaluation of free radical scavenging activities of aqueous extracts of fruits of *Ziziphus mauritiana* and *Eriobotrya japonica* through in vitro antioxidant assays. GJRR. 2(1): 30-36.

Kelley, J. R., and Duggan, J. M. 2003. Gastric cancer epidemiology and risk factors. J Clin Epidemiol. 56: 1–9.

kikugawa, K., and kato, T. 1991. Mutagens in food: Detection and preventrion, H. Hayatsu, ed. CRC Press, Boca Raton, Florida.

Ladoa, C., Thenb, M. r., Vargac, I., keb, E. v. S., and Szentmiha´ lyid, K. r. 2004. Antioxidant property of volatile oils determined by the ferric reducing ability. Z Naturforsch. 56c: 354-358.

Larsson, S. C., Bergkvist, L., and Wolk, A. 2006. Processed meat consumption, dietary nitrosamines and stomach cancer risk in a cohort of Swedish women. Int J Cancer. 119(4): 915-919.

Li, G. X., and Liu, Z. Q. 2009. Unusual antioxidant behavior of alpha- and gamma-terpinene in protecting methyl linoleate, DNA, and erythrocyte. J Agric Food Chem. 57(9): 3943-3948.

Lima, E. S., Mascio, P. D., Rubbo, H., and D.S.P, A. 2002. Characterization of linoleic acid nitration in human blood plasma by mass spectrometry. Biochem. 41: 10717-10722.

Liu, R. H. 2003. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. Am J Clin Nutr. 78: 517S–520S.

Llanes, P. B. G., and Tannenbaum, S. R. 1982. Formation of an activated N-nitroso compound in nitrite-treated fava beans (Viciafaba). Carcinogenesis. 3: 1379-1384.

Lobo, V., Patil, A., Phatak, A., and Chandra, N. 2010. Free radicals, antioxidants and functional foods: Impact on human health. Pharmacogn Rev. 4(8): 118-126.

Maltzman, H. T., Christou, M., Gould, N. M., and Jefcoate, R. C. 1991. Effects of monoterpenoids on in vivo DMBA-DNA adduct formation and on phase I hepatic metabolizing enzymes. J Carcinog. 12(11): 2081-2087.

Maron, D. M., and Ames, B. N. 1983. Revised methods for the *Salmonella* mutagenicity

test. Mutat Res. 113: 173-215.

Marqauard, H., Rufino, F., and Weisburger, J. H. 1977. Mutagenic activity of nitrite-treated foods: human stomach cancer may be related to dietary factors. Science. 196: 1000-1001.

Marquardt, H., Rufino, F., and Weisburger, J. H. 1977. On the aetiology of gastric cancer: Mutagenicity of food extracts after incubation with nitrite. Fd cosmet toxicol. 15: 97-100.

Meadows, G. G. 2012. Diet, nutrients, phytochemicals, and cancer metastasis suppressor genes. Cancer Metastasis Rev. 31(3-4): 441-454.

Mermelstein, R., Kiriazides, K. D., Butler, M., McCOY, C. E., and Rosenkranz, S. H. 1981. The extraordinary mutagenicity of nitropyrenes in bacteria. Mutat Res. 89: 187-196.

Mortelmans, K., and Zeiger, E. 2000. The Ames Salmonella/microsome mutagenicity assay. Mutat Res. 455: 29–60.

Muñoz, N., and Franceschi, S. 1997. Epidemiology of gastric cancer and perspectives for prevention. Salud Publica Mex. 39(4): 318–330.

Munzner, R., and Wever, J. 1984. Mutagenic activity in the stomach and small intestine of rats after oral administration of nitrosated beef extract. Cancer lett. 25: 225-230.

Murakamia, A., Jiwajindab, S., Koshimizuc, K., and Ohigashia, H. 1995. Screening for in vitro anti-tumor promoting activities of edible plants from Thailand. Cancer J. 95: 139-I 146

Naik, R. 2015. GC-FID analysis of fatty acids and biological activity of *Zanthoxylum rhetsa* seed oil. Orient J Chem. 31(4): 1929-1935.

Namiki, K., Yamanaka, M., Osawa, T., and Namiki, M. 1984. Mutagen formation by nitrite-spice reactions. J. Agric. Food Chem. 32(4): 948-952.

Nanasombat, S., and Wimuttigosol, P. 2011. Antimicrobial and antioxidant activity of spice essential oils. Food Sci. Biotechnol. 20(1): 45-53.

National cancer institute. 2017. Antioxidants and Cancer Prevention. [online]. Available from: https://www.cancer.gov/about-cancer/causes-prevention/risk/diet/antioxidants-fact-sheet#r6. [2017, May 12].

Ohshima, H., Friesen, M., Malaveille, C., Brouet, I., Hautefeuille, A., and Bartsch, H. 1989. Formation of direct-acting genotoxic substances in nitrosated smoked fish and meat products: Identification of simple phenolic precursors and phenyldiazonium ions as reactive products. Fd Chem Toxic. 27(3): 193-203.

Pannala, A. S., Mani, A. R., Spencer, J. P. E., Skinner, V., Bruckdorfer, K. R., Moore, K. P., et al. 2003. The effect of dietary nitrate on salivary, plasma, and urinary nitrate metabolism in humans. Free Radic Biol Med. 34(5): 576-584.

Pham-Huy, A. L., He, H., and Pham-Huy, C. 2008. Free radicals, antioxidants in disease and health. Int J Biomed Sci. 4(2): 89-96.

Phaniendra, A., Jestadi, D. B., and Periyasamy, L. 2015. Free radicals: properties, sources, targets, and their implication in various diseases. Indian J Clin Biochem. 30(1): 11-26.

Piluzza, G., and Bullitta, S. 2011. Correlations between phenolic content and antioxidant properties in twenty-four plant species of traditional ethnoveterinary use in the Mediterranean area. Pharm Biol. 49(3): 240-247.

Pisoschi, A. M., and Negulescu, G. P. 2012. Methods for total antioxidant activity determination: a review. Biochem Anal Biochem. 1(1).

Prior, R., Wu, X., and Schaich, K. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem. 53(10): 4290-4302.

Qi, F., Yan, Q., Zheng, Z., Liu, J., Chen, Y., and Zhang, G. 2018. Geraniol and geranyl acetate induce potent anticancer effects in colon cancer Colo-205 cells by inducing apoptosis, DNA damage and cell cycle arrest. JBUON. 23(2): 347.

Qiao, Y. L., Dawsey, S. M., Kamangar, F., Fan, J. H., Abnet, C. C., Sun, X. D., et al. 2009. Total and cancer mortality after supplementation with vitamins and minerals: follow-up of the Linxian general population nutrition intervention trial. J Natl Cancer Inst. 101(7): 507-518.

Rahman, R., Asombang, A. W., and Ibdah, J. A. 2014. Characteristics of gastric cancer in Asia. World J Gastroenterol. 20(16): 4483-4490.

Santos, J. L., Bosquesi, P. L., Almeida, A. E., Chin, C. M., and Varanda, E. A. 2011. Mutagenic and genotoxic effect of hydroxyurea. Int J Biomed Sci. 7(4): 263-267.

Singh, G., Kapoor, I. P., Singh, P., de Heluani, C. S., de Lampasona, M. P., and Catalan, C. A. 2008. Chemistry, antioxidant and antimicrobial investigations on essential oil and oleoresins of *Zingiber officinale*. Food Chem Toxicol. 46(10): 3295-3302.

Sitarz, R., Skierucha, M., Mielko, J., Offerhaus, G. J. A., Maciejewski, R., and Polkowski, W. P. 2018. Gastric cancer: epidemiology, prevention, classification, and treatment. Cancer Manag Res. 10: 239-248.

Song, P., Wu, L., and Guan, W. 2015. Dietary nitrates, nitrites, and nitrosamines intake and the risk of gastric cancer: a meta-analysis. Nutrients. 7(12): 9872-9895.

Stavric, B. 1994. Biological significance of trace levels of mutagenic heterocyclic aromatic amines in human diet: a critical review. Fd Chem. Toxic. 32(10): 977-994.

Stoltz, D. R., Stavric, B., Stapley, R., Klassen, R., Bendall, R., and Krewski, D. 1984 Mutagenicity screening of foods. II. Results with fruits and vegetables. Environ Mutagen. 6(3): 343-354.

Supabphol, R., and Tangjitjareonkun, J. 2015. Chemical constituents and biological activities of *Zanthoxylum limonella* (Rutaceae): a review. Trop J Pharm Res. 13(12): 2119.

Tangjitjaroenkun, J. 2012. Antioxidant effect of *Zanthoxylum limonella* Alston. J Med Plants Res. 6(8).

Tangjitjaroenkun, J., Chavasiri, W., Thunyaharn, S., and Yompakdee, C. 2012. Bactericidal effects and time–kill studies of the essential oil from the fruits of *Zanthoxylum limonella* on multi-drug resistant bacteria. JEOR. 24(4): 363-370.

Tavera, L. H. 1999. Monoterpenes in essential oils. Biosynthesis and properties. Adv Exp Med Biol. 464: 49-62.

Wagner, K. H., and Elmadfa, I. 2003. Biological relevance of terpenoids. Overview focusing on mono-, di- and tetraterpenes. Ann Nutr Metab. 47(3-4): 95-106.

Wakabayashi, K., Ochiai, M., Saito, H., Tsuda, M., Suwa, Y., Nagao, M., et al. 1983. Presence of 1-methyl-1,2,3,4-tetrahydro-,8-carboline-3-carboxylic acid, a precursor of a mutagenic nitroso compound, in soy sauce. Proc. Natl. Acad.Sci. 80: 2912-2916.

Wongkatiya, N., Akekawatchai, C., Sanguansermsri, P., fraser, L. H., Pratoomsoot, C., and

Sanguansermsri, D. 2018. Chemical compositions and biological properties of essential oils from *Zanthoxylum rhetsa* (Roxb.) DC and *Zanthoxylum limonella* Alston. Afr J Tradit Complement Altern Med. 15(2): 12-18.

Wongsrisom, N., Jinata, J., Manosan, B., Kuntakhoo, J., Wankuan, S., and Sriyam, S. 2014. Anti-bacterial activities of essential oils from Mah-Khwuaen (*Zanthoxylum limonella Alston*). KMUTT R&D Journal. 37(1): 3-5.

Yahagi, T., Nagao, M., Seino, Y., Matsushima, T., Sugimura, T., and Okada, M. 1977. Mutagenicities of N-nitrosamines on *salmonella*. Mutat Res. 48: 121-130.

APPENDICES

APPENDIX A

PREPARATION OF REAGENTS FOR ANTIOXIDANT ASSAY

Total phenolic content Reagent:

Chemicals

1. Folin-Ciocalteu reagent
2. 20% sodium carbonate solution
3. 800 mg/l Gallic acid

   Standard Gallic acid was run in triplicate using several concentrations (800, 400, 200, 100, 50, and 25 mg/l)

DPPH Reagent:

Chemicals

1. 0.1 mM DPPH (2,2-diphenyl-1-picrylhydrazyl) in 80% methanol
2. 0.32 mM Trolox in 80% methanol

   Standard Trolox was run in triplicate using several concentrations (0.32, 0.24, 0.16, 0.08, 0.06, and 0.04 mM)

FRAP Reagent:

Chemicals

1. 300 mM Acetate buffer (pH 3.6)

   (1.6 g sodium acetate plus 8 ml glacial acetic acid and made up to 500 ml with distilled water.)

2. 10 mM TPTZ (2, 4, 6-tri [2-pyridyl]-s-triazine) solution in 40 mM HCl

3. 40 mM HCl

   (0.71 ml HCl made up to 200 ml with distilled water.)

4. 20 mM Ferric chloride

   (0.054 g $FeCl_3.6H_2O$ made up to 10 ml with distilled water.)

5. FRAP reagent (25 mL of 300 mM acetate buffer, 2.5 mL of 10 mM 2,4,6-tri-2-pyridyl-2-triazine and 2.5 mL of 20 mM $FeCl_3.6H_2O$)

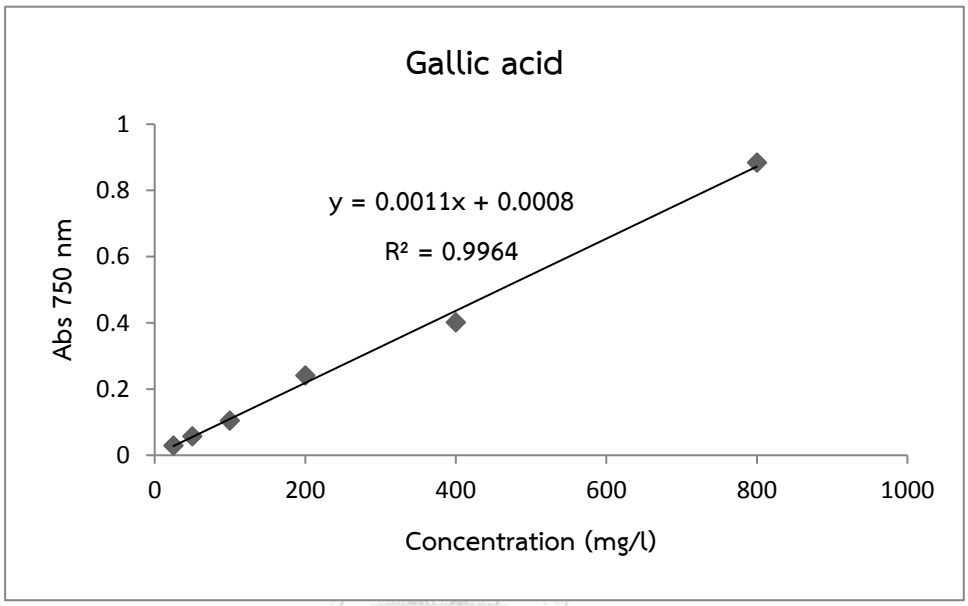
6. 2M ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$)

   Standard was run in triplicate using several concentrations (2, 1, 0.5, 0.25, 0.125, 0.0625, and 0.0313 mM)

Standard curve for total phenolic contents and DPPH assay and FRAP assay

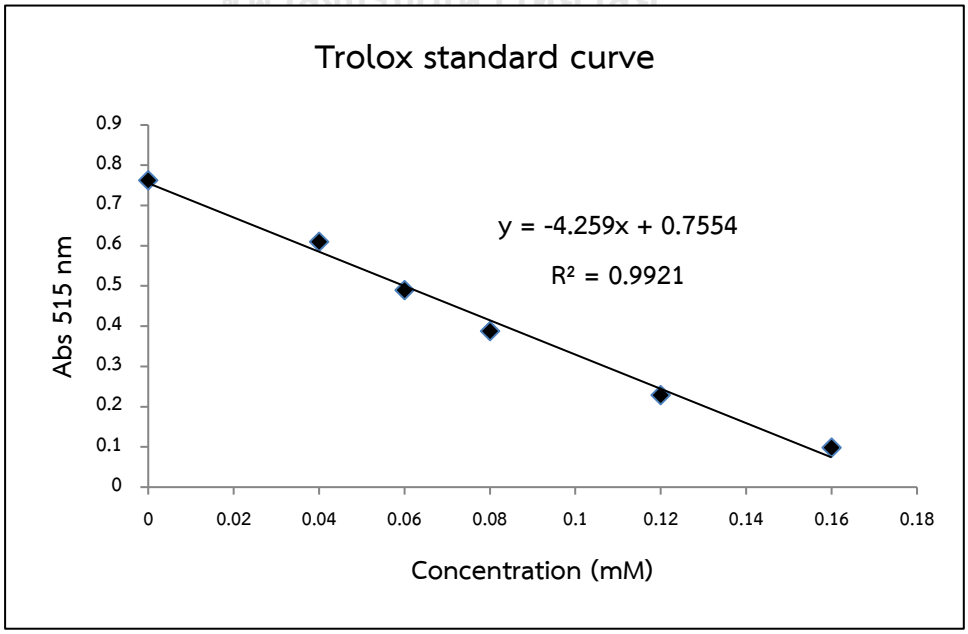
1. Total phenolic content

Standard curve for gallic acid



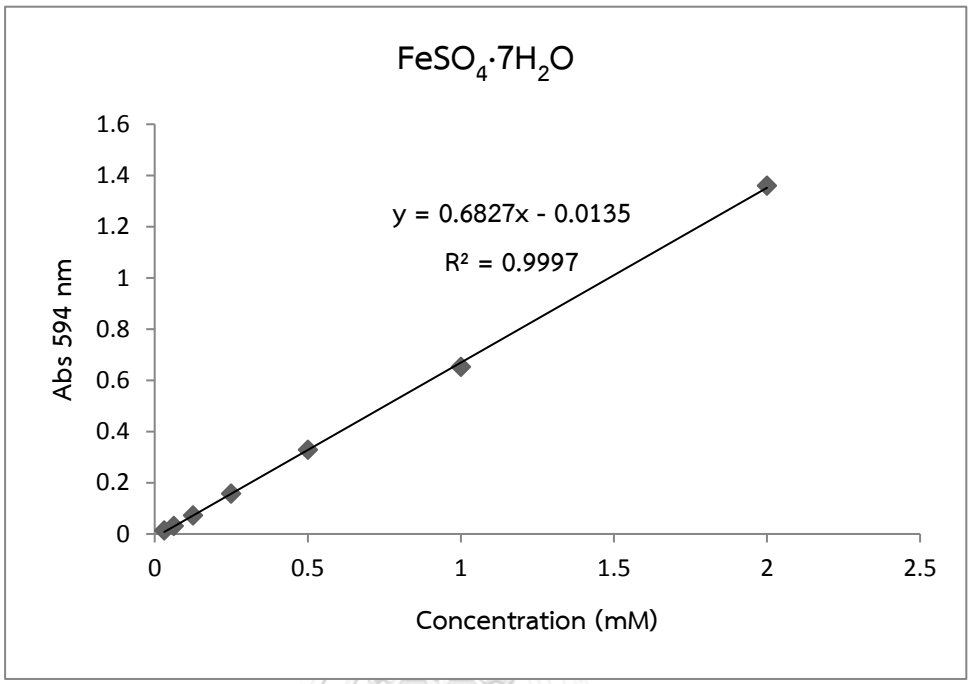
2. DPPH assay

Standard curve for Trolox



3. FRAP assay

Standard curve $FeSO_4 \cdot 7H_2O$



APPENDIX B

PREPARATION OF REAGENTS FOR THE AMES TEST

1. Preparation of stock solution, medium and reagents

1.1 Vogel-Bonner medium E stock salt solution (VB salts)

Ingredient	1000 ml
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	10 g
Citric acid monohydrate ($C_6H_8O_7.H_2O$)	100 g
Potassium phosphate dibasic anhydrous (K_2HPO_4)	500 g
Sodium ammonium hydrogen phosphate tetrahydrate ($NaHNH_4PO_4.4H_2O$)	175 g
Warm distilled ultra-pure water (45°C)	670 ml

Salt were added to warm distilled ultra-pure water (45°C) in order that indicated above and allowed each salt to dissolve completely before adding the next one. The solution were filtered into bottles with screw caps and then autoclaved at 121°C for 20 minutes.

1.2 40 % (w/v) Glucose solution

Ingredient	100 ml
D (+) – Glucose	40 g
Distilled ultra-pure water qs to	100 ml

Dissolve glucose in distilled ultra-pure water around 60 ml placed on a magnetic striring hot plate. After the solution dissolve completely, adjusted volume to 100 ml with distilled ultra-pure water. Then filter and transfer to a glass bottle with screw cap. Autoclaved at 121°C for 20 minutes.

1.3 Minimal glucose agar plate (MGA)

Ingredient	300 ml
Bacto agar or agar agar	4.5 g
VB salts (sterile)	6 ml
40% (w/v) glucose (sterile)	15 ml
Distilled ultra-pure water	279 ml

Bacto agar was added to distilled ultra-pure water in erlenmeyer flask and autoclave at 121°C for 20 minutes. Then added sterile VB salts and sterile 40% (w/v) glucose, swirled gently and poured 25-30 ml into sterile petri dish. Minimal glucose agar plates were incubated at 37°C for 48 hours before use.

1.4 Top agar

Ingredient	50 ml
Bacto agar or agar agar	0.3 g
Sodium chloride (NaCl)	0.25 g
0.5 mM Histidine/biotin solution	5 ml
Distilled ultra-pure water	50 ml

Bacto agar and sodium chloride were dissolved in distilled ultra-pure water and then autoclave at 121°C for 20 minutes. Added 0.5 mM Histidine/biotin sterile solution, mixed and warmed on water bath at 45° C before using.

1.5 0.1 M L-histidine

Ingredient	10 ml
L-histidine	0.1552 g
Distilled ultra-pure water qs to	10 ml

Dissolve histidine (MW = 155.2 g/mol) in distilled ultra-pure water. Then adjusted volume to 10 ml and autoclave at 121°C for 20 minutes.

1.5 1 mM L-histidine

Ingredient	100 ml
0.1 M L-histidine	1 ml
Distilled ultra-pure water	99 ml

0.1 M L-histidine was pipetted into volumetric flask and then adjusted the volume to 100 ml. The solution was autoclaved at 121°C for 20 minutes.

1.6 1 mM biotin

Ingredient	100 ml
Biotin	0.0244 g
Distilled ultra-pure water	100 ml

Dissolve biotin (MW = 244.3 g/mol) in distilled ultra-pure water. Then adjusted volume to 100 ml and autoclave at 121°C for 20 minutes.

1.7 0.5 mM histidine/biotin

Ingredient	200 ml
1 mM L-histidine	100 ml
1 mM biotin	100 ml

1 mM L-histidine and 1 mM biotin were mixed thoroughly and autoclave at 121°C for 20 minutes.

1.8 Oxoid nutrient

Ingredient	100 ml
Oxiod nutrient broth No.2	2.5 g
Distilled ultra-pure water	100 ml

Oxiod nutrient broth No.2 was dissolved in distilled ultra-pure water and adjusted volume to 100 ml. The solution was transferred into each flask covered with Cotton wool cork then autoclave at 121°C for 20 minutes.

1.9 0.9% Sodium chloride solutions

Ingredient	100 ml
Sodium chloride (NaCl)	0.9 g
Distilled ultra-pure water	100 ml

Sodium chloride was dissolved in distilled ultra-pure water and adjusted volume to 100 ml. Then autoclave at 121°C for 20 minutes.

1.10 M Sodium nitrite ($NaNO_2$)

Ingredient	10 ml
Sodium nitrite ($NaNO_2$)	1.38 g
Distilled ultra-pure water	10 ml

Sodium nitrite (MW = 68.99 g/mol) was dissolved in distilled ultra-pure water and adjusted volume to 10 ml. The solution was transferred into light protection glass bottle with screw cap then autoclave at 121°C for 20 minutes.

1.11 Ammonium sulfamate

Ingredient	10 ml
Ammonium sulfamate	2.2824 g
Distilled ultra-pure water	10 ml

Sodium nitrite (MW = 68.99 g/mol) was dissolved in distilled ultra-pure water and adjusted volume to 10 ml. The solution was transferred into glass bottle with screw cap and then autoclave at 121°C for 20 minutes.

1.12 Ampicillin solution (8 mg/ml)

Ingredient	10 ml
Ampicillin sodium	0.08 g
Distilled ultra-pure water	10 ml

Ampicillin sodium was dissolve in 10 ml distilled ultra-pure water and stored in sterile glass bottle with screw cap.

1.13 0.1% Crystal violet

Ingredient	10 ml
Crytal violet	0.01 g
Distilled ultra-pure water	10 ml

Crytal violet was dissolve in 10 ml distilled ultra-pure water and stored in sterile glass bottle with screw cap.

1.14 0.2 N Hydrochloric acid

Ingredient	100 ml
Conc. hydrochloric acid	1.67 ml
Sterile distilled ultra-pure water	100 ml

Aseptic technique

Conc. hydrochloric acid was pipetted into sterile volumetric flask 100 ml and then adjusted volume with sterile distilled ultra-pure water to 100 ml. Then stored in sterile glass bottle with ground glass plug.

1.15 1-aminopyrene 0.075 mg/ml in acetonitrile

Ingredient	1 ml
1-aminopyrene (1-AP)	0.003 g
Acetonitrile	1 ml

Aseptic technique

0.003 g 1-AP in acetonitrile was dissolved in 1 ml acetonitrile. Pipette 0.1 ml of 1-AP 3 mg/ml in acetonitrile in sterile glass bottle with screw cap before adding 0.9 ml of acetonitrile and mixing thoroughly. 0.5 ml of This solution was pipetted into a new sterile glass bottle with screw cap and then added 1.5 ml of acetonitrile and mix thoroughly before keeping in freezer until use.

1.16 1 M Potassium chloride

Ingredient	100 ml
Potassium chloride (KCl)	7.456 g
Sterile distilled ultra-pure water qs to	100 ml

Potassium chloride was dissolve in distilled ultra-pure water and adjusted volume to 100 ml. Then autoclave at 121°C for 20 minutes.

1.17 0.5 M Sodium dihydrogen phosphate solution

Ingredient	100 ml
Sodium dihydrogen phosphate (NaH_2PO_4)	6 g
Sterile distilled ultra-pure water qs to	100 ml

NaH_2PO_4 was dissolve in distilled ultra-pure water and adjusted volume to 100 ml. Then autoclave at 121°C for 20 minutes.

1.18 0.5 M Disodium hydrogen phosphate dihydrate solution

Ingredient	100 ml
Disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$)	8.9 g
Sterile distilled ultra-pure water qs to	100 ml

$Na_2HPO_4.2H_2O$ was dissolve in distilled ultra-pure water and adjusted volume to 100 ml. Then autoclave at 121°C for 20 minutes.

1.19 0.5 M Sodium phosphate pH 7.4

Ingredient	100 ml
0.5 M Disodium hydrogen phosphate dehydrate	100 ml
0.5 M Sodium dihydrogen phosphate	add to pH 7.4

0.5 M Sodium dihydrogen phosphate (1.17) was added in 0.5 M Disodium hydrogen phosphate dehydrate (1.18) until to pH 7.4.

1.20 Sodium phosphate - potassium chloride buffer

Ingredient	330 ml
0.5 M Sodium phosphate pH 7.4	100 ml
1 M KCl	16.5 ml
Sterile distilled ultra-pure water	213.5 ml

Mix all ingredients together then autoclave at 121°C for 20 minutes.

2. Procedure for Re-isolation and Growing culture

Each tester strains (*S. typhimurium* strains TA98 and TA100) were incubated in 12 ml of Oxoid nutrient broth No.2 at 37°C for 16 hours in a shaking water bath. The overnight culture strains were re-isolated by streaking on a MGA plate which the surface were spread with the 100 µl of ampicillin solution (8 mg/ml), 300 µl of 0.1 M histidine and 100 µl of 1 mM biotin. Then these plates were incubated at 37°C for 48 hours. After incubation, the 4 single colonies of each strain were picked up and grown in oxoid nutrient broth No.2 37°C for 16 hours in a shaking water bath. Each tester strain was confirmed genotypes. Then 5 ml of each overnight culture was mixed with 450 µl of dimethyl sulfoxide (DMSO) in a sterile tube and distribute 200 µl of the mixed culture in a sterile micro-centrifuge tube before storing in a refrigerator at -80°C for mutagenic assay (Figure 19).

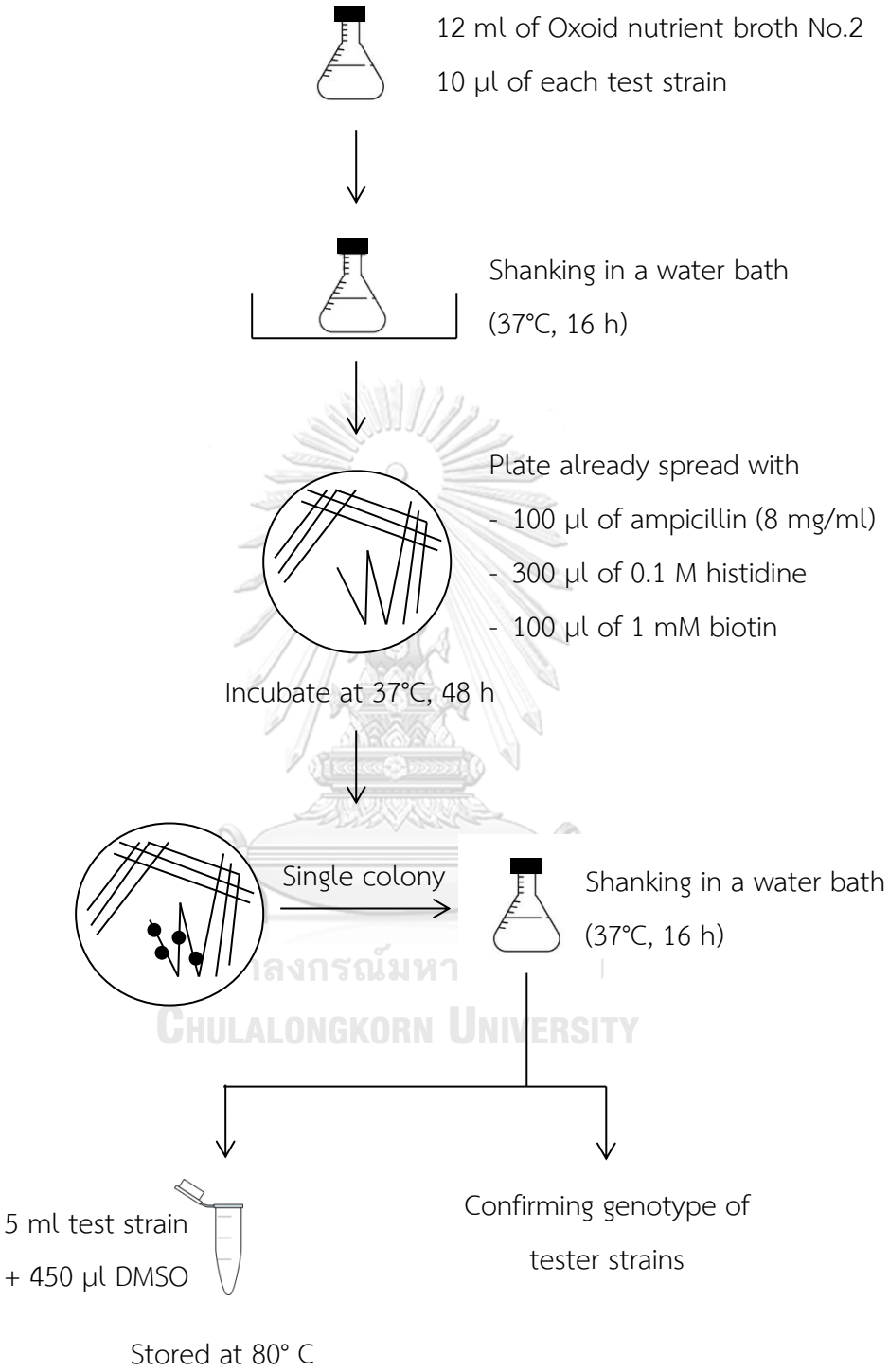


Figure 13 Re-isolation of bacteria

3. Confirming genotype of tester strains

3.1 Histidine requirement

Procedure: The growth of bacteria on minimal glucose agar (MGA) plates enriched with histidine and biotin used to confirm Histidine requirement of *S. typhimurium*. Four MGA plates were used to confirm for each strain. Each MGA plate was spread on the surface with 100 µl of 1 mM biotin, 300 µl of 0.1 M histidine and 100 µl of 1 mM biotin plus 300 µl of 0.1 M histidine onto the MGA plate b, c and d, respectively. Each strain was steaked across on the same plate and then incubated at 37°C for 24 hours. The growing of tester strains on plate d (containing biotin plus histidine) exhibit the results of histidine requirement (Figure 20).

Plate A : no histidine and biotin

Plate B : 0.1 ml of mM biotin

Plate C : 0.3 ml of 0.1 M Histidine

Plate D : 0.3 ml of 0.1 M histidine + 0.1 ml of 1 mM biotin

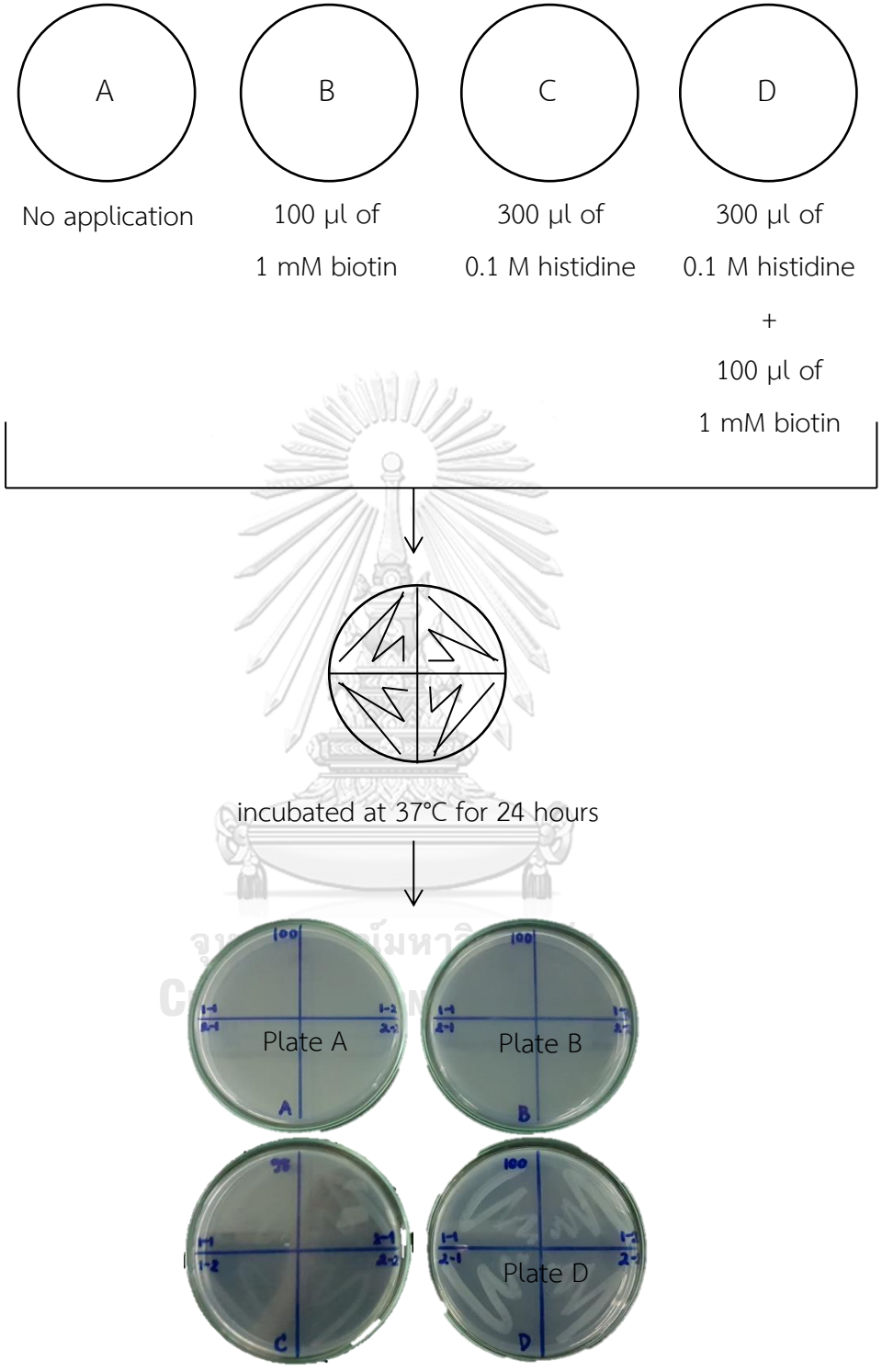


Figure 14 Histidine requirement

1.1 R-factor

Procedure: *S. typhimurium* strain should be tested for the presence of the resistance transfer factor (R-factor) due to the R factor plasmid (pKM101) makes the strains more responsive to a variety of mutagens. 300 µl each strain was added with 100 µl of 0.1 M histidine in a sterile tube with screw cap. Then added 2 ml of molten top agar (45°C) that contain 0.5 mM histidine and biotin. The solution was poured onto a MGA plate. R-factor and rfa mutation were examined in the same plate by dividing the plate into two equal parts. For R-factor, filter disc paper containing ampicillin solution (8 mg/ml) was applied on the surface of a MGA plate. Then the plates were incubated at 37°C for 24 hours. The absence of clear zone around the disc indicate resistance to ampicillin (Figure 21).

1.2 rfa mutation

Procedure: The rfa mutation is mutated in gene of the bacteria cell wall and results in loss of the lipopolysaccharide barrier for increasing permeability of chemicals into cell. The rfa mutation is indicated by sensitivity to crystal violet. The procedure performed as described in R-factor procedure but 0.1% crystal violet is placed instead of ampicillin solution (8 mg/ml). The appearance of clear zone around the disc indicate the presence of the rfa mutation (Figure 21).

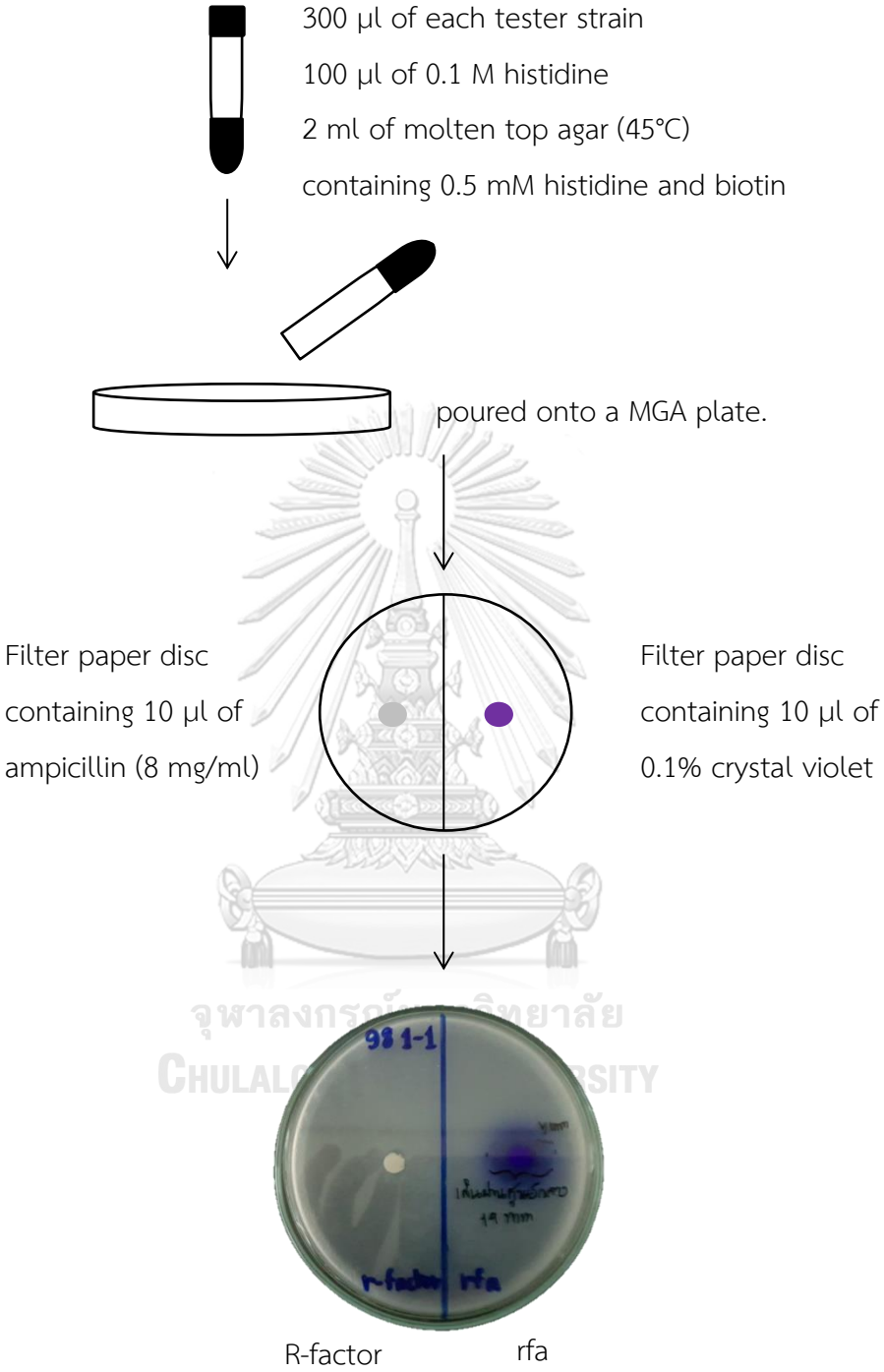


Figure 15 rfa and R-factor mutation

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