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ANTIMUTAGENIC ACTIVITY OF *MOMORDICA COCHINCHINENSIS* EXTRACTS

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การศึกษานี้ทำการประเมินฤทธิ์ก่อกลายพันธุ์ ในสภาวะที่มีและไม่มีไนไตรทในการทำปฏิกิริยา รวมทั้งการปรับเปลี่ยนฤทธิ์ก่อกลายพันธุ์ต่ออะมิโนพัยรีนที่ทำปฏิกิริยากับไนไตรท ซึ่งเป็นสารก่อกลายพันธุ์มาตรฐาน ของสารสกัดด้วยน้ำจากเนื้อผลระยะอ่อน เนื้อผลระยะสุกปานกลาง เนื้อผลระยะสุกเต็มที่ และเยื่อหุ้มเมล็ด ของฟักข้าว (*Momordica cochinchinensis*) ต่อเชื้อ *Salmonella typhimurium* สายพันธุ์ TA98 และ TA100 เมื่อไม่มีระบบกระตุ้นเอนไซม์ โดยวิธีทดสอบเอมส์ ร่วมกับการดัดแปลงด้วยเทคนิค pre-incubation ผลการศึกษาพบว่าสารสกัดฟักข้าวทั้งหมด ทั้งในสภาวะที่ทำปฏิกิริยาและไม่ทำปฏิกิริยากับไนไตรท ไม่แสดงฤทธิ์ก่อกลายพันธุ์ต่อเชื้อ *S. typhimurium* ทั้งสองสายพันธุ์ สำหรับการศึกษาการปรับเปลี่ยนฤทธิ์ก่อกลายพันธุ์ พบว่าสารสกัดจากฟักข้าวทุกส่วน โดยเฉพาะส่วนเนื้อผลระยะอ่อน แสดงฤทธิ์ยับยั้งก่อกลายพันธุ์ของอะมิโนพัยรีนที่ทำปฏิกิริยากับไนไตรทต่อเชื้อ *S. typhimurium* สายพันธุ์ TA98 โดยฤทธิ์ในการยับยั้งจะเพิ่มขึ้นเป็นสัดส่วนแปรผันตามปริมาณสารสกัดที่เพิ่มขึ้น สำหรับผลการทดสอบต่อเชื้อ *S. typhimurium* สายพันธุ์ TA100 พบว่าสารสกัดจากเนื้อผลระยะอ่อน และระยะสุกปานกลาง ที่ความเข้มข้นสูงสุด สองความเข้มข้น (100 และ 200 ไมโครลิตร) ยับยั้งการก่อกลายพันธุ์ของอะมิโนพัยรีนที่ทำปฏิกิริยากับไนไตรทในระดับอ่อนถึงปานกลาง ในขณะที่ไม่พบฤทธิ์ยับยั้งการก่อกลายพันธุ์ดังกล่าวเมื่อใช้สารสกัดจากเนื้อผลระยะสุกเต็มที่ นอกจากนี้ยังพบว่าสารสกัดจากเยื่อหุ้มเมล็ดไม่มีผลปรับเปลี่ยนฤทธิ์ก่อกลายพันธุ์ตามความเข้มข้นที่ใช้ในการศึกษา แต่มีแนวโน้มในการเพิ่มฤทธิ์การก่อกลายพันธุ์ของอะมิโนพัยรีนที่ทำปฏิกิริยากับไนไตรท อย่างไรก็ตามสารสกัดจากเนื้อผลระยะอ่อนและระยะสุกปานกลางข้าวสามารถยับยั้งฤทธิ์การก่อกลายพันธุ์ของอะมิโนพัยรีนที่ทำปฏิกิริยากับไนไตรทได้

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NAPAD TRITEERADEJ: ANTIMUTAGENIC ACTIVITY OF *MOMORDICA COCHINCHINENSIS* EXTRACTS. ADVISOR: ASST. PROF. LINNA TONGYONK, D.Sc., CO-ADVISOR: TIPPAWAN SIRITIENTONG, Ph.D., 77 pp.

The water extracts from unripe pulp, half-ripe pulp, fully ripe pulp and aril of gac fruit (*Momordica cochinchinensis*) were investigated for their mutagenicity using the Ames test with modified pre-incubation method on *Salmonella typhimurium* strains TA98 and TA100 without enzymatic activation. The modification effect of these fruit extracts on the mutagenicity of standard mutagen; nitrite treated 1-aminopyrene (1-AP) was also evaluated. The results showed that none of them exhibited mutagenicity on both strains of *S. typhimurium* either with or without nitrite treatment. For the modification on mutagenicity assay, the extracts from various parts of gac fruit, especially the extracts from unripe pulp inhibited the mutagenicity of nitrite treated 1-AP with dose response manner on *S. typhimurium* strain TA98. According to the results from *S. typhimurium* strain TA100, two amounts (100 and 200 µl) of the extracts from unripe pulp and half-ripe pulp showed weak to moderate degree of inhibition while the extracts from fully ripe pulp showed negligible effect. The extracts from aril also showed negligible effect by the study concentrations but they tend to enhance the mutagenicity of nitrite treated 1-AP. However, the mutagenicity of nitrite treated 1-AP can be inhibited by the addition of the extracts from unripe pulp and half-ripe pulp.

Department:	Food and Pharmaceutical Chemistry	Student's Signature	.....
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LIST OF ABBREVEATIONS

1-AP	1-aminopyrene
℃	degree Celsius
DMSO	dimethyl sulfoxide
DPPH	diphenyl-picryhydrazyl
et al.	et alii (and others)
FRAP	ferric reducing antioxidant power
g	gram
HA	heterocyclic amines
HPLC	high performance liquid chromatography
His^-	histidine dependent
His^+	histidine independent
h	hour
H_2O_2	hydrogen peroxide
LPS	lipopolysacchalide
µg	microgram
µl	microliter
µm	micrometre
mg	milligram
ml	millilitre

mM	millimolar
MGA	minimal glucose agar
min	minute
M	molarity
MI	mutagenicity index
NOCs	*N*-nitroso compounds
N	normality
No.	number
$O^{\bullet-}$	superoxide anion radical
PAHs	polycyclic aromatic hydrocarbons
pH	potential of hydrogen ion
rpm	revolution per minute
RIPs	ribosome-inactivating proteins
S. typhimuriun	*Salmonella typhimurium*
SD	standard deviation
TBHP	tertiary butyl hydroperoxide
TFC	total flavonoid contents
TPC	total phenolic contents
UV-C	ultraviolet light in C Spetrum
VB	Vongel-Bonner medium E

CHAPTER I

INTRODUCTION

1.1 Background and rationale

Gac fruit (*Momordica cochinchinensis*) is widely used in different purposes such as food, colorant and medicine (Chuyen et al., 2015; Kha et al., 2013). In Thailand, gac fruit is consumed for a long period of time. Especially, gac pulp is prefered to eat as side dish vegetable and gac aril is preferred to eat as juice. Gac fruit contains several nutrients , as well as a wide range of bioactive compounds which have pro-vitamin A activity (Voung, Dueker and Murphy, 2002), antioxidant activity (Klungsupya et al., 2012; Kubola and Siriamornpun, 2011; Praychoen, Praychoen and Phongtongpasuk, 2013) and anticancer activity (Chuethong et al., 2007; Petchsak and Sripanidkulchai, 2015; Tien et al., 2005; Zheng et al., 2013). All these activities may be related to their bioactive compounds such as vitamin C, vitamin E, β-carotene, lycopene, phenolic acid, flavonoid and some identified proteins which found in different amounts in each part of gac fruit at different ripening stages (Kubola and Siriamporn, 2011). Almost these compounds are also known as antimutagens (Bhattacharya, 2011; Bronzetti, 1994).

The Ames test is a bacterial reverse mutation assay for the purpose of determining a wide range of mutagens as well as antimutagens (Calomme et al., 1996; Maron and Ames, 1983). Interestingly, a wide range of antimutagens have antioxidant

activity that protect cells from oxidative damage involving in the induction of mutagenesis, initial stage of carcinogenesis (Bhattacharya, 2011; Cooke et al., 2003; Ferguson, 1994). In the aspect of health benefit, the finding whether the edible parts (pulp and aril) of gac fruit from different growth stages, including unripe, half-ripe and fully ripe fruit have any mutagenic activity or antimutagenic activity against a mutagen is important for consumer health.

1.2 Objectives of the study

1.2.1 To investigate the mutagenicity of gac fruit extracts with or without nitrite treatment using pre-incubation method of the Ames test without enzymatic activation.

1.2.2 To investigate the modification effect of gac fruit extracts on the mutagenicity of nitrite treated 1-AP using pre-incubation method of the Ames test without enzymatic activation.

1.3 Benefits of the study

This study provides the information on the mutagenicity and antimutagenicity of the water extracts from various parts of gac fruit. Moreover, the results of this study may be a beneficial recommendation for consumers.

CHAPTER II

LITERATURE REVIEW

2.1 Mutagens and antimutagens in food

Mutation occurs when DNA is injured or changed in the DNA sequence due to alteration, removal or insertion of one or more bases, resulting in DNA damage. These processes are also associated with the initial stage of carcinogenesis. The physical factors or chemical agents that induce mutation are known as mutagens whereas antimutagens are various substances which reduce or abolish the effects of mutagens (Bronzetti, 1994; Cooke et al., 2003). Food is one of the most important factors that can strongly influence the occurrence of cancer (Anand et al., 2008). Food contains several mutagens which some naturally occur, namely aflatoxin B_1 (fungal toxin) contaminated in grain products and others that occur via cooking process such as heterocyclic amines (HAs) (Kizil, Oz and Besler, 2011), polycyclic aromatic hydrocarbons (PAHs) and *N*-nitroso compounds (NOCs) (Goldman and Shields, 2003). HAs are formed when amino acids, sugar and creatine react in the high temperature of cooking (Kizil, Oz and Besler, 2011). PAHs are formed during incomplete combustion of organic substances. In terms of food, fat and juice from meat, poultry or fish are grilled directly over the fire dripping onto the fire, causing flame. The flame containing PAHs adheres onto the surface of such food. PAHs are commonly found in broiled food, smoked

food and charred food (Hamidi et al., 2016). NOCs are formed by the reaction between nitrite and nitrosable mutagen precursors such as secondary or tertiary amines and amides (Mirvish, 1995). NOCs are mainly found in cured meat such as sausage, ham and bacon (Lijinsky, 1999). The consumption of such food mutagens is associated with carcinogenesis (Goldman and Shield, 2003; Sugimura, 2000).

On the other hand, food, especially fruits and vegetables also contains several antimutagens (Bhattacharya, 2011; Bronzetti, 1994). They can be classified as bio-antimutagens and desmutagens. Bio-antimutagen are substances which reduce the effect of mutagens on DNA damage by modification the cellular process, especially DNA repair process. Desmutagens are substances which act directly on mutagens before they attack DNA by several mechanisms such as chemical or enzymatic inactivation, mutagens adsorption, complex formation with mutagens, and free radical scavenging or antioxidant activity (Figure 1) (Table 1). Thus, fruits and vegetables rich in antimutagens may be involved in protection of DNA damage which initializes carcinogenesis (Bhattacharya, 2011; Bronzetti, 1994).

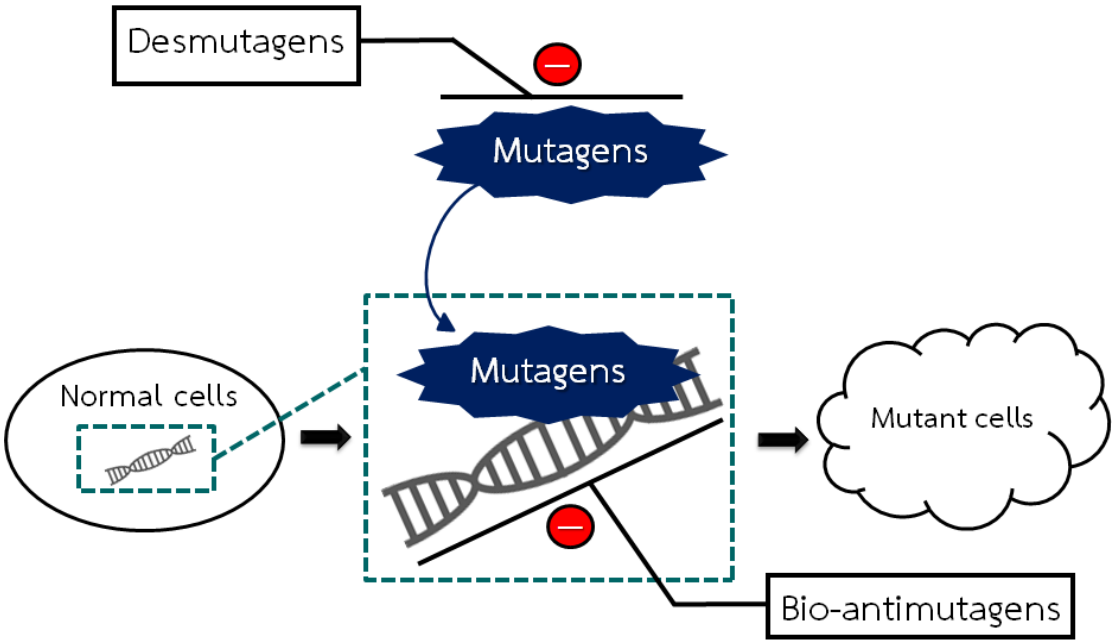

Figure 1. Mechanisms of bio-antimutagens and desmutagens

Table 1. Antimutagens in food and mechanisms (Bhattacharya, 2011; Ferguson, 1994)

Food sources	Bioactive compounds	Mechanisms
Yellow to red fruits and vegetables	Carotenoids	Antioxidant activity
Fruits and vegetables, mainly citrus fruits	Vitamin C	Antioxidant activity, nitrite scavenging
Plant oils; wheat germ, rice and cotton seed	Vitamin E	Antioxidant activity, nitrite scavenging
Green leafy vegetables	Chlorophyll, chlorophyllin	Antioxidant activity, complex formation with mutagens
Fruits and vegetables	Dietary fibers	Mutagens adsorption
Cruciferous vegetables	Isothiocyanate	Stimulation of phase II enzyme activity
Fruits and Vegetables; green tea, black tea, red wine	Phenolic acids, flavonoids	Antioxidant activity, stimulation of phase II enzyme activity
Vanilla pods	Vanillin	Stimulation of DNA repair process

2.2 Gac fruit

Gac (*Momordica cochinchinensis*) is classified in the Cucurbitaceae family which is cultivated throughout South China to Southeast Asia. The shape of fruit is oval or round shape, full of short spines which categorizes into three stages of maturity, including unripe, half-ripe and fully ripe fruit (Table 2) (Kubola and Siriamornpun, 2011). Its colour changes from green to yellow then red when the fruit becomes mature (Figure 2). Fully ripe gac fruit has bright red aril inside occupying 25% of the fruit weight (Figure 3). The oil content in gac aril is approximately 102 mg/g of fresh weight that is composed of several types of fatty acids, especially oleic acid and linoleic acid (Table 3) (Ishida et al., 2004; Voung et al., 2002). Besides essential fatty acids, gac aril also contains α-tocopherol with a mean concentration of 76 μg/g of fresh weight (Vuong et al., 2006).

Gac fruit is widely used in different cultures. In Vietnam, gac aril is used as a natural colorant for cooking red glutinous rice which is served in special occasions such as wedding and New Year celebration. In China, gac seed is used as a traditional medicine, known as mubiezhi for treatment of skin disorders (Chuyen et al., 2015). In Thailand, this fruit is known as fak khao, commonly used as food. Generally, gac pulp is cooked curries or blanched to eat with chili sauce. Gac aril is preferred to make juices due to its bright red and appetizing colour (Praychoen et al., 2013).

Table 2. Description of gac fruit on three stages of maturity.

Stages of maturity	Description
Unripe gac fruit	Unripe gac fruit ages between 2 to 3 months after flowering. There is a green peel with short spines, white pulp and small transparent seeds.
Half-ripe gac fruit	Half-ripe gac fruit ages between 3 to 4 months after flowering. There is a yellow peel with short spines, light yellow pulp and white seeds.
Fully ripe gac fruit	Fully ripe gac fruit ages between 4 to 5 months after flowering. There is a red peel with short spines, yellow pulp, bright red aril and black seeds.

Figure 2. Three stages of maturity of gac fruit, including unripe gac fruit (green), half-ripe gac fruit (yellow) and fully ripe gac fruit (red)

Figure 3. Bright red aril covered the seeds and yellow pulp of fully ripe gac fruit

Table 3. Fatty acid composition and percentage of total fatty acids of gac aril (Vuong et al., 2002)

Fatty acid	Concentration (mg/g)	Percentage of total fatty acids (%)
Myristic acid	0.89	0.87
Palmitic acid	22.48	22.04
Palmitoleic acid	0.27	0.26
Stearic acid	7.20	7.06
Oleic acid	34.76	34.08
Vaccenic acid	1.15	1.13
Linoleic acid	32.06	31.43
α-linolenic acid	2.18	2.14
Eicosanoic acid	0.40	0.39
Gadoleic acid	0.15	0.15
Arachidonic acid	0.10	0.10
Behenic acid	0.19	0.19
Lignoceric acid	0.14	0.14
Total	101.98	100.0

2.3 Health benefits of gac fruit

Gac fruit contains several nutrients including carbohydrate, protein, fat and fibre (Table 4) (Nagarani, Abirami and Siddhuraju, 2014) as well as non-nutritive substances known as phytochemicals and contributed to health benefit. Interestingly, the aril part of gac fruit contain high contents of carotenoids higher than the other parts. Gac aril is used to promote healthy vision and treat eye disorder due to its high contents of β-carotene, the precursor of vitamin A. (Chuyen et al., 2015; Kubola and Siriamornpun, 2011). In Vietnam, gac aril is added in food like red glutinous rice known as xoi gac. Vuong et al. (2002) evaluated the effects of gac fruit mixed-rice consumption on the plasma β-carotene and retinol concentration in vitamin A deficient children for 30 days. The 185 children aged between 2.5 – 5.8 years were enrolled and classified into three groups: a fruit group, who consumed xoi gac (cooked rice with fully ripe gac fruit) that contained 3.5 mg β-carotene per serving; a powder group, who consumed rice mixed with 5 mg synthetic β-carotene powder; and control group, who consumed rice without fortification. After 30 days of supplementation, the mean plasma β-carotene concentrations in the fruit and powder groups were significantly higher than those in the control group. The mean plasma retinol concentrations in the fruit group were also significantly higher than those in the powder and control groups. Additionally, gac seed has been extensively used as a traditional Chinese medicine for treatment of skin disorders, including abscesses, bruises and sores. Ground gac seed mixed with alcohol

or vinegar is also used to treat haemorrhoid, mumps and swelling in Vietnam countryside (Chuyen et al., 2015; Kha et al., 2013).

Table 4. Nutrient composition of gac fruit

Composition	Edible portion of unripe gac fruit/100 g[a]	Aril/ 100 g[b]
Water	88.6 g	77 g
Carbohydrate	7.6 g	10.5 g
Protein	1.5 g	2.1 g
Fat	0.1 g	7.9 g
Fibre	1.1 g	1.8 g
Ash	-	0.7 g
Calcium	64.0 mg	56.0 mg
Phosphorus	89.0 mg	6.4 mg
Iron	0.34 mg	-
Vitamin C	0.04 mg	-
Vitamin E	-	76.3 µg

[a] Nagarani, Abirami and Siddhuraju, 2014

[b] Vuong et al., 2006

2.4 Phytochemicals and antioxidants of gac fruit

Several studies reported that gac fruit, especially in gac aril contained high amount of carotenoids mainly lycopene and β-carotene (Table 5). Aoki et al. (2002) evaluated the carotenoid contents in gac fruit using high performance liquid chromatography (HPLC) and found that gac aril contained both β-carotene and lycopene higher than gac pulp. Interestingly, lycopene and β-carotene contents in gac aril were also higher than other fruits and vegetables (Figures 4 and 5). Ishida et al. (2004) reported the higher contents of β-carotene and lycopene in gac aril whereas no lycopene and low β-carotene contents in gac pulp. Vuong et al. (2006) evaluated the carotenoids in gac fruit and also found α-tocopherol in gac aril.

Table 5. The comparison of β-carotene and lycopene contents in gac aril (μg/g of wet weight)

Reference	β-carotene	Lycopene	Methods
Aoki et al. (2002)	101	380	HPLC
Ishida et al. (2004)	718	2227	HPLC
Vuong et al. (2006)	83	408	HPLC

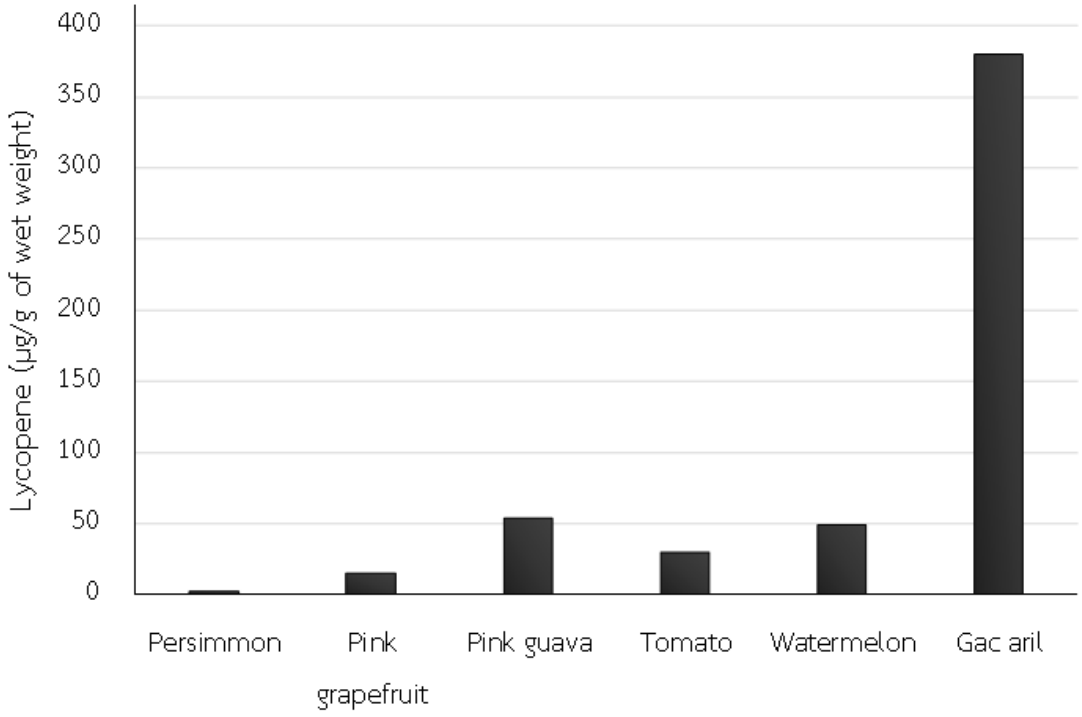

Figure 4. Lycopene contents of fruits and vegetable (Modified from Holden et al., 1999; Aoki et al., 2002)

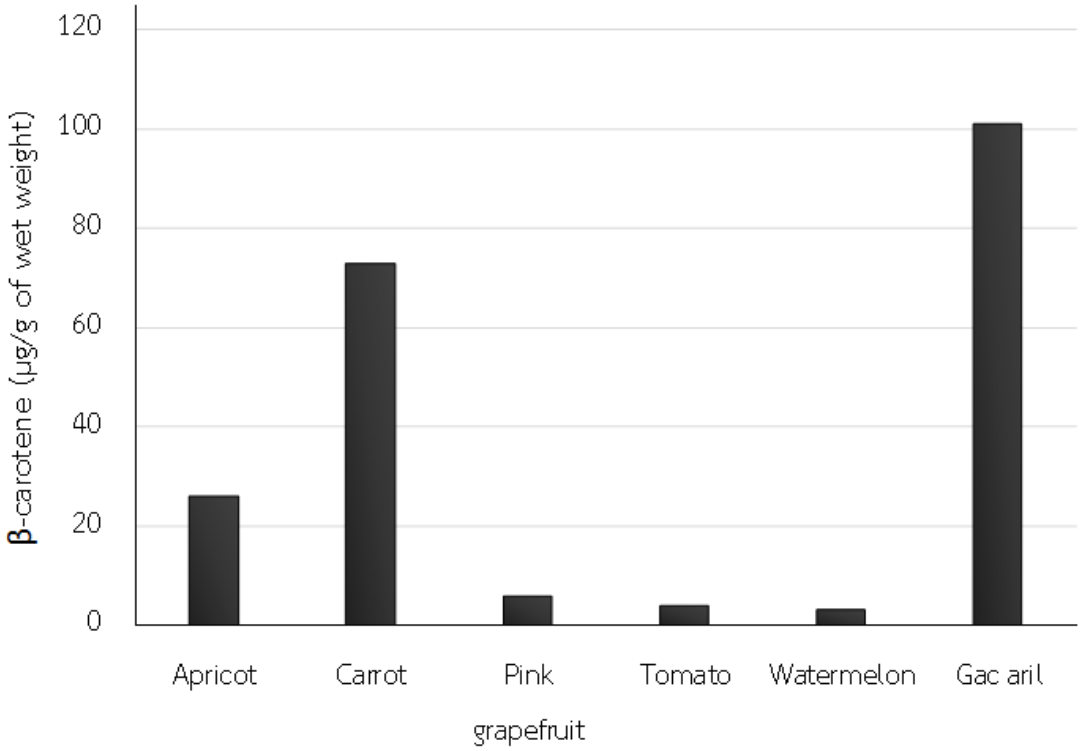

Figure 5. β-carotene contents of fruits and vegetables (Modified from Holden et al., 1999; Aoki et al., 2002)

In 2011, Kubola and Siriamornpun investigated the phytochemicals of different parts of gac fruit, including peel, pulp, aril and seed using HPLC and antioxidant activities using ferric reducing antioxidant power (FRAP) assay and diphenyl-picryhydrazyl (DPPH) radical scavenging assay. The results showed that its aril contained the highest levels of β-carotene, lycopene and total flavonoid contents (TFC) whereas unripe pulp contained the highest levels of total phenolic contents (TPC) and half-ripe peel contained the highest levels of lutein. For the antioxidant activities, aril showed the highest FRAP value, followed by peel, pulp and seed, while the DPPH radical scavenging activity decreased when the gac fruit was more ripeness. In 2012, Klungsupya et al. evaluated the effect of gac extracts on the hydrogen peroxide (H_2O_2) and ultraviolet light in C Spectrum (UV-C) induced oxidative DNA damage of by comet assay in TK6 human lymphoblast cells. The results showed that the water and ethanol extracts from peel, pulp and aril reduced the oxidative DNA damage induced by H_2O_2 and UV-C up to 30-60% and 20-30%, respectively. In 2013, Praychoen et al. evaluated the effect of thermal treatment on phytochemicals and antioxidant activities of gac juice at the concentration of 20% (v/v). The results showed that gac juice heated at 60°C for 2 minutes exhibited the highest FRAP value whereas gac juice heated at 80°C for 2 minutes exhibited the highest DPPH radical scavenging activities. Moreover, the highest amounts of phytochemicals such as TPC, TFC, β-carotene and lycopene were detected when gac juice was heated at 80°C for 2

minutes. However, the temperature higher than 80°C reduced the amounts of phytochemicals as well as antioxidant activities of gac juice.

2.5 Anticancer activity of gac fruit

Several studies demonstrated that the extracts of gac fruit, especially gac seed possessed anticancer activity. Tien et al. (2005) evaluated the anticancer activity of water extract of gac seed on Balb/c mice transplanted with the colon 26-20 adenocarcinoma and human hepatoma (HepG2) cells. The results showed that the water extract of gac seed inhibited the tumor growth and angiogenesis in the treated mice. The active component was confirmed as a water-soluble protein with molecular weight of 35 kDa which was different from β-carotene, lycopene and other compounds in gac fruit. Chuethong et al. (2007) found Cochinin B, ribosome-inactivating proteins (RIPs), with molecular weight of 28 kDa in gac seed. Cochinin B exhibited strong antitumor activity on the human cervical epithelial carcinoma (HeLa), human embryonic kidney (HEK293) and human lung cancer (NCI-H187) cells. Zheng et al. (2013) evaluated the anticancer activity of gac seed extract on the human breast cancer cells (ZR-75-30). They found that gac seed extract exhibited strong inhibitory effect on the proliferation and moderate inhibitory effect on the invasion of human breast cancer cells (ZR-75-30). In addition to gac aril, Petchsak and Sripanidkulchai (2015) evaluated the anticancer activity of aril extract on human breast cancer cells (MCF-7) and found that aril extract induced cells apoptosis via upregulation of

proapoptotic BCL2-associated X Protein (BAX) gene expression and activation of caspase 6, 8 and 9 activities.

2.6 The Ames test

The Ames test is a short-term bacterial reverse mutation assay for the purpose of determining several chemicals that lead to gene mutations on histidine dependent (His^-) *Salmonella* tester strains. The mutations of the *Salmonella* tester strains occur in different histidine operons such as the *hisC3076*, *hisD3052*, *hisG428* and *hisG46* that encode enzymes required for the histidine biosynthesis. According to the mutations, they can be classified by two mechanisms, including base-pair substitution mutation (single base change) and frameshift mutation (insertion or deletion of one or more bases). Moreover, the *Salmonella* tester strains contain other mutations which enhance their ability to detect mutagen. One mutation (rfa mutation) causes loss of the lipopolysaccharide (LPS) barrier in bacterial cell wall to improve the permeability of large chemical molecules. Another mutation (uvrB mutation) is a deletion of a gene coding for the DNA excision repair system to enhance bacterial sensitive to mutagens. The deletion also affects the biotin synthase gene that causes deficient in biotin synthesis. The *Salmonella* tester strains such as TA98, TA100 and TA102 contain the R-factor plasmid, pKM101 that enhances the mutagenesis by chemicals and ultraviolet radiation via an error-prone DNA repair system and encode resistance to ampicillin.

TA102 strain also contains the plasmid, pAQ1 that encode resistance to tetracycline (Table 6) (Ames, 1975; Maron and Ames, 1983; Mortelmans and Zeiger, 2000).

Table 6. The properties of the *Salmonella* tester strains (modified from Maron and Ames, 1983; Mortelmans and Zeiger, 2000)

Allele/ Strain	Plasmid	rfa mutaion	uvrB mutation	Reversion event
hisC3076/				
TA1537	No plasmid	Yes	Yes	Frameshift mutation
hisD3052/				
TA98	pKM101	Yes	Yes	Frameshift mutation
TA1538	No plasmid	Yes	Yes	Frameshift mutation
hisG428/				
TA102	pKM101, pAQ1	Yes	No	Base-pair substitution mutation
TA104	No plasmid	Yes	Yes	Base-pair substitution mutation
hisG46/				
TA100	pKM101	Yes	Yes	Base-pair substitution mutation
TA1535	No plasmid	Yes	Yes	Base-pair substitution mutation

2.7 The standard method of the Ames test (the plate incorporation method) and the modified pre-incubation method

The standard method of the Ames test (the plate incorporation method) is based on the use of histidine dependent (His^-) bacteria that revert to histidine independent (His^+) bacteria. When a mutagen is added, the number of His^+ revertants is higher than the number of spontaneous revertants (Figure 6). Briefly, the mixture of bacteria, mutagen and molten top agar (45°C) is directly overlaid on minimal glucose agar plate before incubated at 37°C for 48 hours to obtain His^+ revertants (Ames, 1975).

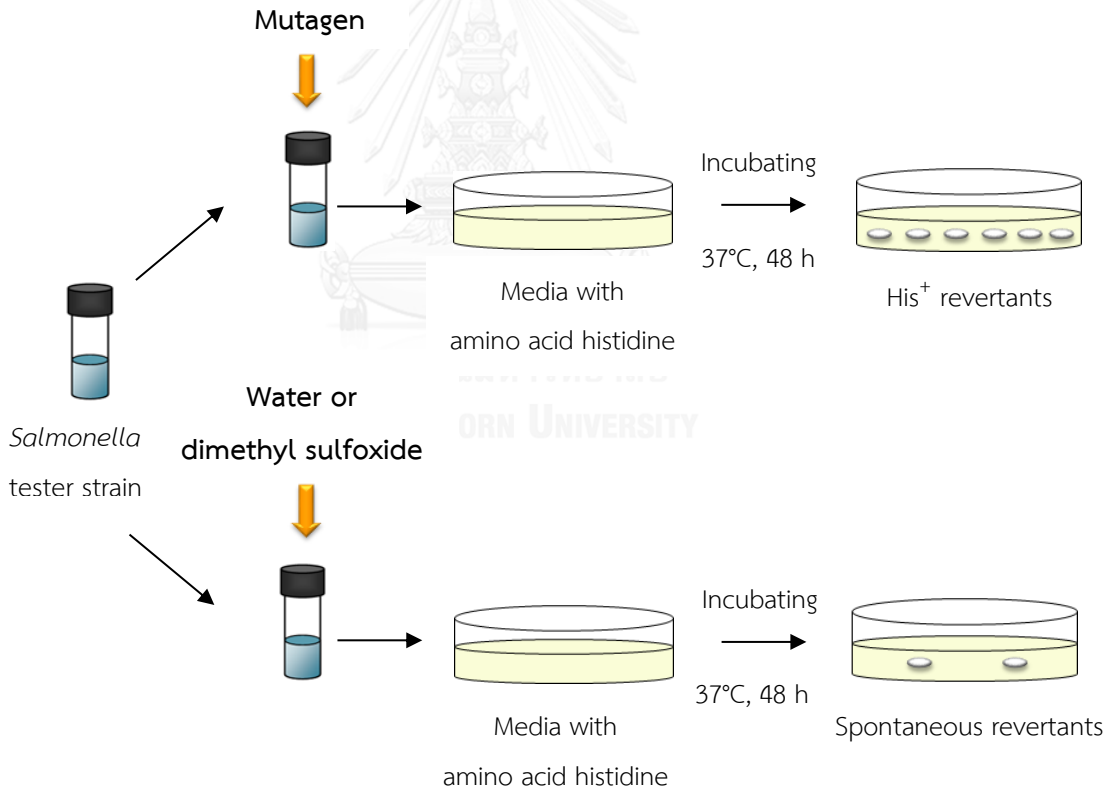

Figure 6. The standard method of the Ames test (the plate incorporation method)

Dimethylnitrosamine and diethylnitrosamine are mutagens which are poorly detected by the standard method of the Ames test (the plate incorporation method). It should be tested using a modification of the standard plate incorporation method. In 1975, Yahagi et al. modified the standard method of the Ames test to improve the sensitivity. In this modify method (the pre-incubation method of the Ames test), the mixture containing specific mutagen and bacteria is incubated at 37°C for 20 minutes then mix with molten top agar (45°C) and pour onto a minimal glucose agar plate. The mixture is then incubated at 37°C for 48 hours to obtain histidine revertants. The modified pre-incubation method was used to detect the mutagenic activity of 10 carcinogenic nitrosamines (Yahagi et al., 1977) and several carcinogenic alkaloids (Yamanaka et al., 1979). Furthermore, aflatoxin B_1, benzidine, benzo[*a*]pyrene and methylmethanesulfonate was determined for their mutagenic activity using both of the standard plate incorporation and modified pre-incubation method. The results showed that the sensitivity of modified pre-incubation method was equal or greater sensitivity than the standard plate incorporation method. This is due to the fact that the mixture of the modified pre-incubation method containing mutagen and bacteria is incubated at higher concentration of the mutagen than the standard plate incorporation method (Mortelmans and Zeiger, 2000). The modified pre-incubation method can be used when inconclusive results are obtained in the standard plate incorporation method. Furthermore, this method was recommended by de Serres and Shelby (1979) for detecting mutagenic activity of any chemicals.

2.8 Nitrite treated 1-AP as a mutagenic assay model

1-Nitropyrene is a predominant nitro-polycyclic aromatic hydrocarbons (nitro-PAHs) which can be generated by incomplete combustion of organic substances (Fu and Herreno-Saenz, 1999). Inhalation is one of the routes of human exposure to 1-nitropyrene which can be metabolised by intestinal microflora into 1-AP (Manning, Cerniglia and Federle, 1986). Kato et al. (1991) demonstrated that 1-AP treatment with nitrite in acidic condition (pH 3.0) at 37°C showed mutagenic activity on *S. typhimurium* strains TA98 and TA100 without enzymatic activation. This result agreed with Kangsadalampai et al. (1995) who reported that nitrite treated 1-AP in an acidic condition (pH 3.0) at 37°C was a direct-acting mutagen. Thus, the mutagenic activity of nitrite treated 1-AP in acidic condition has been established as a model for mutagenicity studies of some chemicals occurred during digestion in the stomach.

CHAPTER III

MATERIALS AND METHODS

3.1 Tester strains

S. typhimurium strains TA98 (to detect frameshift mutation) and TA100 (to detect base-pair substitution mutation) were provided by Assoc. Prof. Keaw Kangsadalampai (Institute of Nutrition, Mahidol University).

3.2 Chemicals

3.2.1 1-Aminopyrene (1-AP) (Sigma-Aldrich, St. Louis, USA)

3.2.2 Ammonium sulfamate ($NH_2SO_3NH_4$) (Sigma-Aldrich, St. Louis, USA)

3.2.3 Biotin (Sigma-Aldrich, St. Louis, USA)

3.2.4 Histidine (Sigma-Aldrich, St. Louis, USA)

3.2.5 Sodium nitrite ($NaNO_2$) (Sigma-Aldrich, St. Louis, USA)

3.2.6 Dipotassium hydrogen phosphate anhydrous (K_2HPO_4) (Fluka, Buchs, Switzerland)

3.2.7 Sodium ammonium hydrogen phosphate tetrahydrate ($NaNH_4HPO_4.4H_2O$) (Fluka, Buchs, Switzerland)

3.2.8 Disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$) (Fluka, Buchs, Switzerland)

3.2.9 Agar-Agar (Merck, Darmstadt, Germany)

3.2.10 Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) (Merck, Darmstadt, Germany)

3.2.11 Sodium chloride (NaCl) (Merck, Darmstadt, Germany)

3.2.12 Potassium chloride (Merck, Darmstadt, Germany)

3.2.13 Crystal violet (Merck, Darmstadt, Germany)

3.2.14 Citric acid monohydrate ($C_6H_8O_7.H_2O$) (Analar, Poole, England)

3.2.15 D(+)-Glucose (Analar, Poole, England)

3.2.16 Oxoid nutrient broth No.2 (Oxoid Ltd., Hampshire, England)

3.2.17 Hydrochloric acid (HCl) (Normapur, Prolabo, Belgium)

3.2.18 Sodium dihydrogen phosphate (NaH_2PO_4) (Sigma Chemical, St. Louis, USA)

3.2.19 Acetonitrile (J.T. Baker, Phillibsburg, USA)

3.2.20 Ampicillin sodium salt (General Drugs House, Bangkok, Thailand)

3.3 Instruments

3.3.1 Analytical balance (Sartorius Model BSA224S-CW, Germany)

3.3.2 Autoclave (Hirayama Model HG-80, Japan)

3.3.3 Blender (Otto BE-120, Thailand)

3.3.4 Biohazard laminar flow (BossTech Model HVB-120S, Thailand)

3.3.5 Colony counter (Funke Gerber Model ColonyStar 8505, Germany)

3.3.6 High performance centrifuge (Hermle Model Z323K, USA)

3.3.7 Incubator (Memmert Model INE 200-800, Germany)

3.3.8 Pipette tip 2-200 µl, 50-1000 µl (Brand, Germany)

3.3.9 Repeater pipette syringe 1.5 ml, 6 ml (Nichiyo Model 8100, Japan)

3.3.10 Shaker bath (Hotech Model 905, Taiwan)

3.3.11 Spectrophotometer (Thermo Scientific Model GENESYS 20, USA)

3.3.12 Syringe dispenser (Nichiyo Model 8100, Japan)

3.3.13 Syringe driven filter unit 0.22 μm (Millex-GV (PVDF), Ireland)

3.3.14 Test tube culture, 16x125 mm (Pyrex Model 9820-16X, New York, USA)

3.3.15 Transfer pipette (Thermo Scientific Model F2-20S, F2-100, F2-1000, USA)

3.3.16 UltraBasic Benchtop pH Meter (Denver Instrument Model UB-10, Germany)

3.3.17 Vortex-genie 2 (Scientific Industries Model G560E, USA)

3.4 Fruit samples

Three stages of gac fruit, including unripe, half-ripe and fully ripe fruit were purchased from a floating market in Samutprakan, Thailand during October to November, 2014.

3.5 Sample preparation

After cleaning with tap water, unripe and half-ripe gac fruit were peeled off, cut into halves to remove the seeds and then chopped into small pieces. Aril and pulp of fully ripe gac fruit were separately scooped out and the seeds were removed. Thus, the fruit samples divided into four parts, including unripe pulp, half-ripe pulp, fully ripe pulp and aril. Each part of gac fruit (100 g wet weight) was blended with 200 ml of distilled water in the ratio of 1:2 w/v and centrifuged at 4,500 rpm for 15 min. The supernatant of each fruit sample was collected in a tight protecting from light container

at -20°C until required. The fruit sample extract was thawed and sterilized by filtration through a sterile 0.2 micron filter paper before assay (Figure 7).

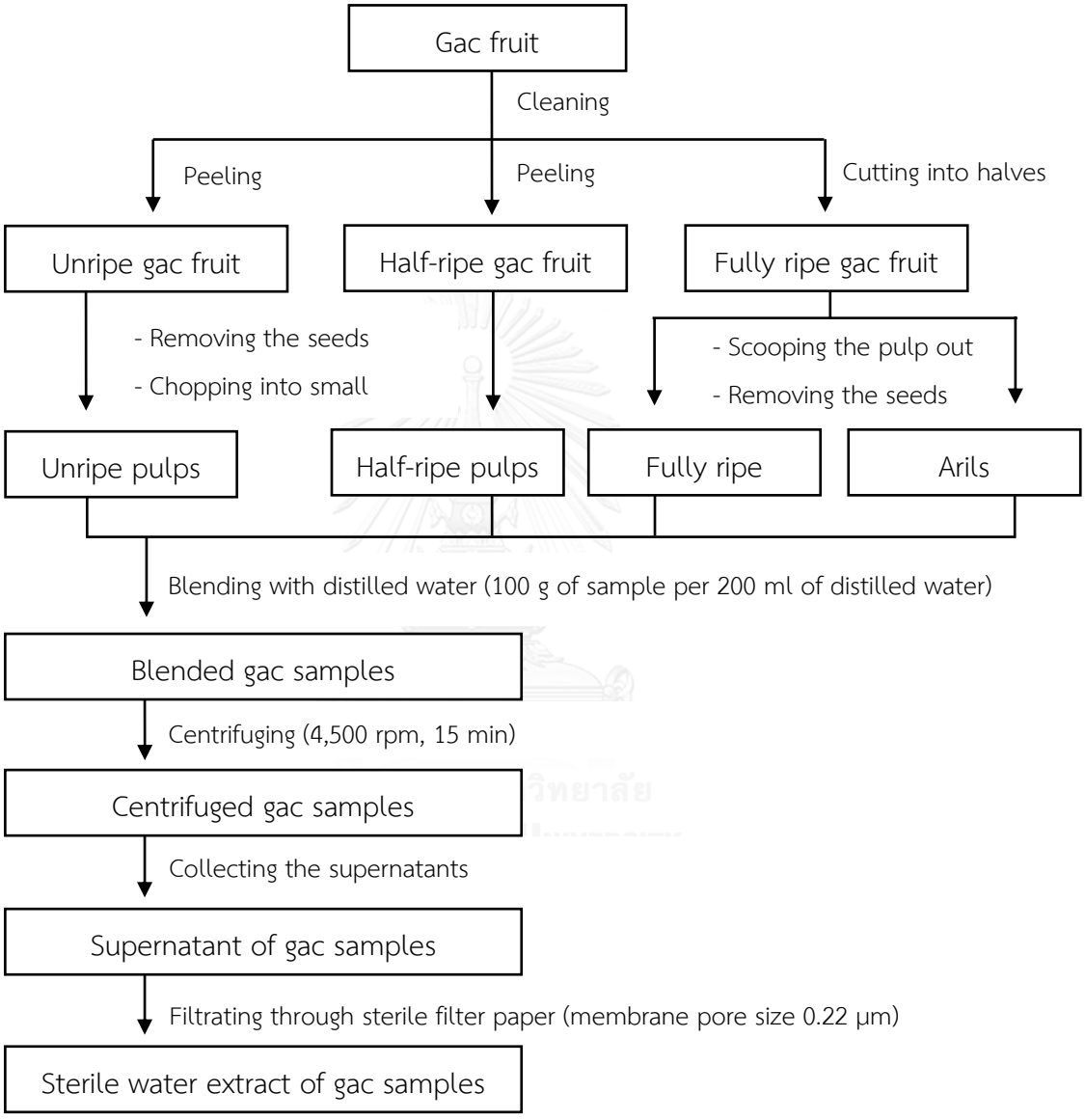

Figure 7. Sample preparation

3.6 Experimental design on the mutagenicity and antimutagenicity against nitrite treated 1-AP of gac fruit

Sterile water extract of gac samples from 3.5 were treated with nitrite in an acidic condition (pH 3.0-3.5) for 4 hours and investigated their mutagenic activity on *S. typhimurium* strains TA98 and TA100 without enzymatic activation using the Ames test with modified pre-incubation method. The mutagenicity of each sample extract was also determined without nitrite treatment. The modifying effect of each sample extract on the mutagenicity of nitrite treated 1-AP was then investigated on *S. typhimurium* strains TA98 and TA100 using pre-incubation method of the Ames test without enzymatic activation (Figure 8).

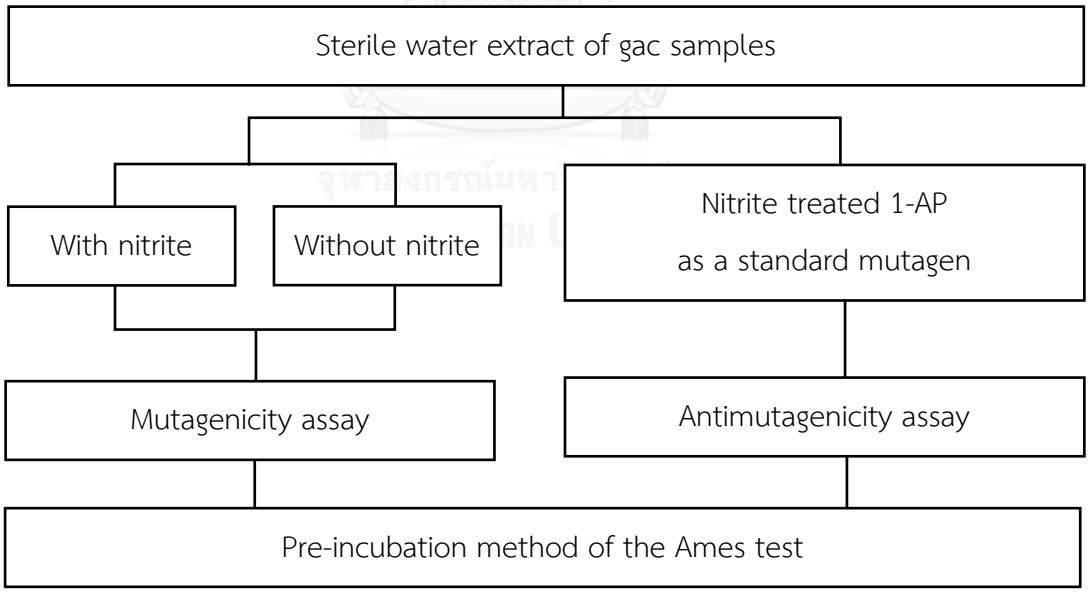

Figure 8. Experimental design on the mutagenicity and antimutagenicity effects against nitrite treated 1-AP of gac fruit

3.7 Nitrite treatment procedure

For mutagenicity assay, each fruit sample extract was treated with nitrite in an acidic condition. Briefly, an aliquot of each part of fruit sample extract (50, 100, 200 µl) was adjusted pH to 3.0-3.5 by 0.2 N hydrochloric acid and then mixed with 250 µl of 2 M sodium nitrite in a sterile tube with plastic cap to obtain the final volume of 1,000 µl. The mixture was incubated in a shaking water bath at 37°C for 4 h before dipped into an ice bath for 1 min to stop the reaction. The mixture was then added with 250 µl of 2 M ammonium sulfamate to decompose the residual nitrite and dipped into an ice bath for 10 minutes before mutagenicity assay (Figure 9).

3.8 Standard mutagen model

Each volume of 1-AP (0.075 mg/ml in acetonitrile; 10 µl and 20 µl for *S. typhimurium* strain TA98 and TA100, respectively) was treated with nitrite in an acidic condition according to the procedure described in 3.7. The nitrite treated product obtained from this reaction was used as a standard mutagen or positive control of this study.

3.9 Mutagenicity assay

The mutagenicity of water extract of gac fruit was determined using the plate incorporation procedure of Ames (1975) with modified by Yahagi et al. (1975) The pre-incubation of sample without enzymatic activation was tested on *S. typhimurium* strains TA98 and TA100.

In the pre-incubation assay, 100 µl of the reaction mixture obtained from 3.7 was mixed with 500 µl of 0.5 M phosphate buffer (pH 7.4) and 100 µl of each overnight culture in a sterile tube with plastic cap before being shaken in a water bath at 37°C. After 20 min of pre-incubation, 2 ml of molten top agar (45°C) containing histidine and biotin (see appendix) was added. The mixture was carefully poured onto a minimal glucose agar (MGA) plate after thoroughly mixed. The number of His^+ revertants per plate was counted after turned plate upside down in an incubator at 37°C for 48 hours (Figure 10).

For determining the mutagenicity of each sample extract without nitrite treatment, the method was done as previously described in 3.7 but distilled water was placed instead of sodium nitrite and ammonium sulfamate.

3.10 Antimutagenicity assay

After 4 h of incubation, 100 µl of the reaction mixture of nitrite treated 1-AP obtained from 3.8 was mixed with each sample extract (50, 100 or 200 µl) and made up to the volume of 300 µl with distilled water. The modifying effect of each sample extract on the mutagenicity of nitrite treated 1-AP was evaluated according to the mutagenicity assay as described above (Figure 11).

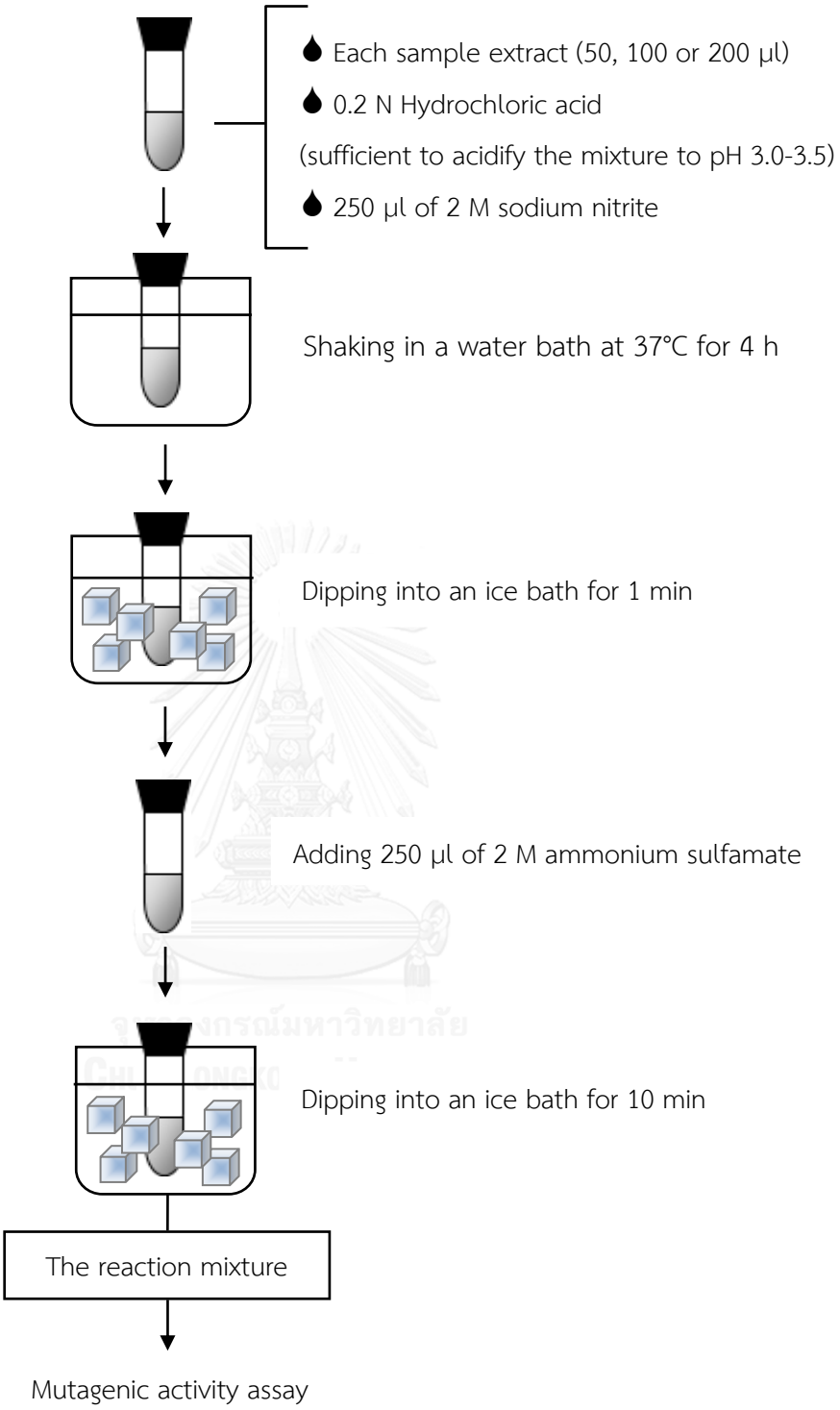

Figure 9. Nitrite treatment procedure

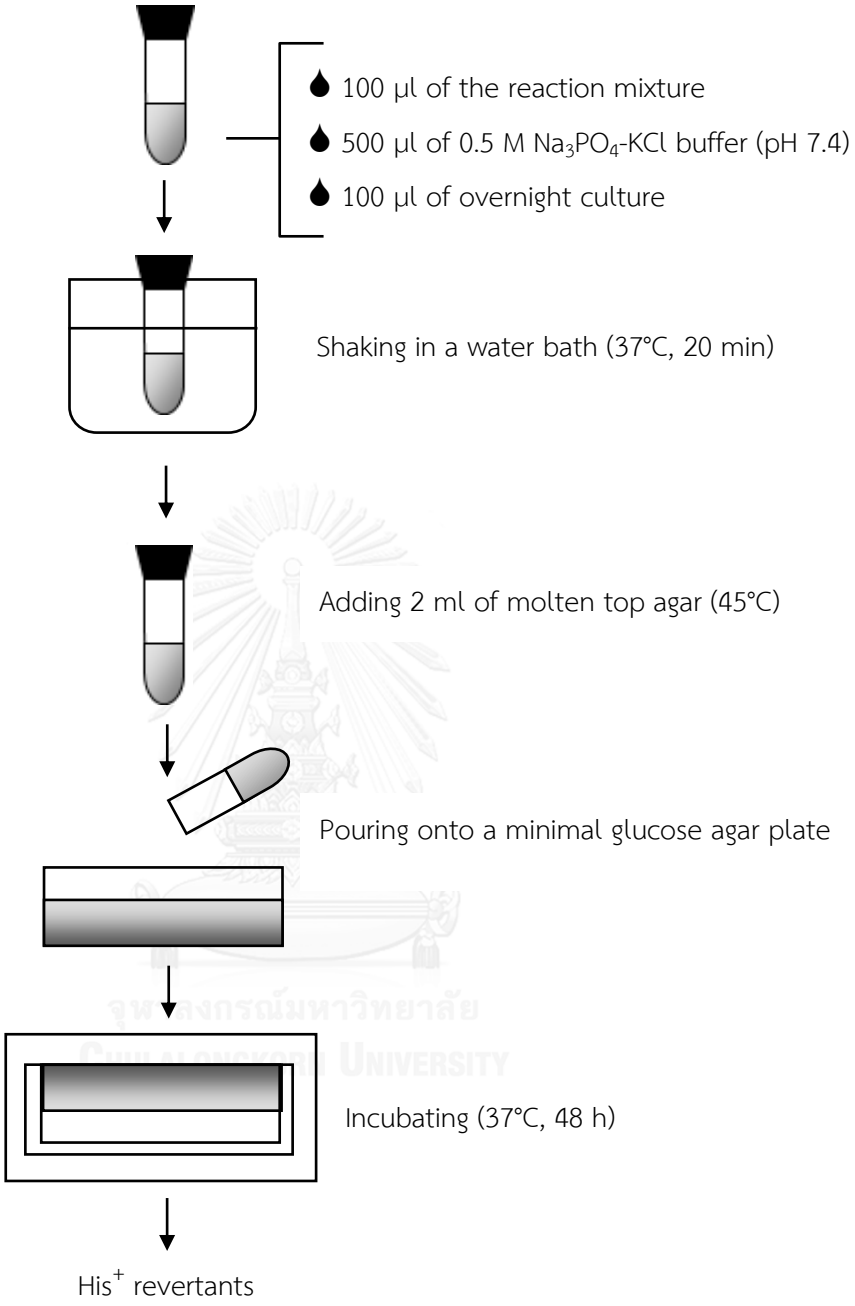

Figure 10. The experimental procedures to investigate the mutagenicity using pre-incubation method of the Ames test

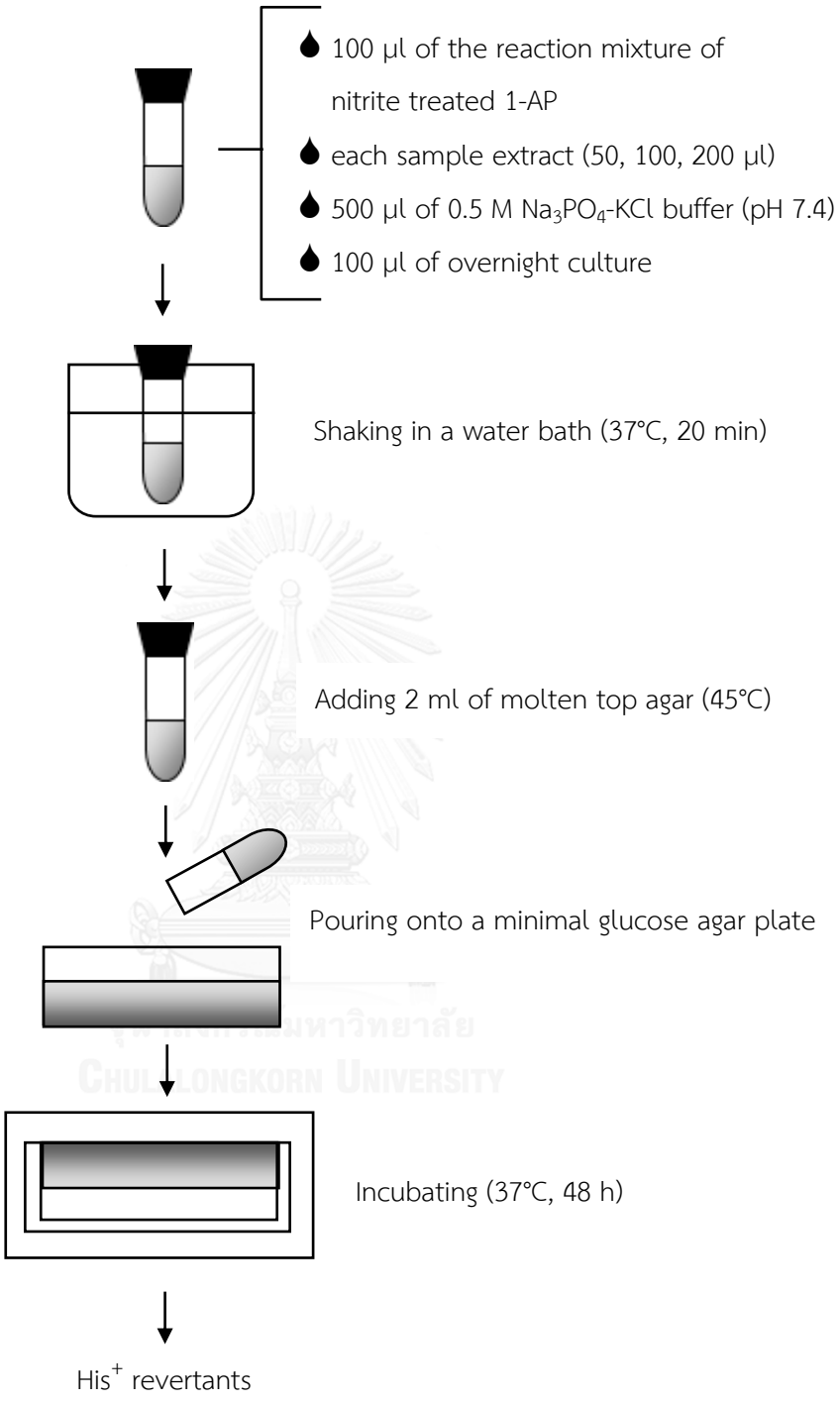

Figure 11. The experimental procedures to investigate the modifying effect of gac fruit on the mutagenicity of nitrite treated 1-AP using pre-incubation method of the Ames test

3.11 Data evaluation

The mutagenicity of each sample was presented as the number of histidine revertants per plate. The results were reported as means with standard deviation of three plates from two experiments. To compare the degree of mutagenicity, the results were also expressed as mutagenicity index (MI) which was calculated from the average number of His^+ revertants per plate of sample divides by the average number of spontaneous revertants. Samples which expressed their mutagenicity higher than two times of spontaneous revertants with a dose-response relationship which were considered to be mutagenic (de Serres and Shelby, 1979).

The degree of inhibition (+) or enhancement (-) of mutagenicity was classified into four levels as negligible (± <20 %), weak (± 20-<40 %), moderate (± 40-60 %) and strong (± >60 %) by the following formula (Calomme et al., 1996).

% Modification = [(A-B)/(A-C)] x 100

A = The average number of His^+ revertants per plate of nitrite treated 1-AP

B = The average number of His^+ revertants per plate of the extracts with nitrite treated 1-AP

C = The average number of spontaneous revertants

CHAPTER IV

RESULTS

4.1 The mutagenicity of the water extract of gac fruit

The mutagenicity of the water extract from various parts of gac fruit, including unripe pulp, half-ripe pulp, fully ripe pulp and aril was evaluated on *S. typhimurium* strains TA98 and TA100 without enzymatic activation in the Ames test. According to the results from this study, the number of His^+ revertants of each fruit sample extract did not meet the criteria for determining the mutagenicity and none of MI was higher than two indicating no direct mutagenicity on both strains of *S. typhimurium* of all extracts (Tables 7 and 8). After nitrite treatment, they also exhibited no mutagenicity on both strains of *S. typhimurium* (Tables 9 and 10).

Table 7. The mutagenicity of the water extract from various parts of gac fruit without nitrite treatment on *S. typhimurium* strain TA98

The water extract from each part of gac fruit	Amount (µl/plate)	No. of His^+ revertants/plate[a]	MI[b]
Unripe pulp	0[c]	29 ± 6	1.00
	4	31 ± 4	1.06
	8	30 ± 10	1.02
	16	35 ± 8	1.20
Half-ripe pulp	0[c]	37 ± 3	1.00
	4	31 ± 8	0.86
	8	29 ± 3	0.79
	16	32 ± 6	0.89
Fully ripe pulp	0[c]	22 ± 5	1.00
	4	18 ± 4	0.82
	8	17 ± 4	0.78
	16	19 ± 4	0.85
Aril	0[c]	20 ± 5	1.00
	4	17 ± 6	0.84
	8	19 ± 6	0.92
	16	17 ± 6	0.83

[a] The data were expressed as means ± standard deviation of six plates from two experiments

[b] Mutagenicity index (MI) was calculated from the average number of His^+ revertants per plate of the extracts divided by the average number of spontaneous revertants

[c] No extract was added which represented a negative control (spontaneous)

Table 8. The mutagenicity of the water extract from various parts of gac fruit without nitrite treatment on *S. typhimurium* strain TA100

The water extract from each part of gac fruit	Amount (µl/plate)	No. of His^+ revertants/plate[a]	MI[b]
Unripe pulp	0[c]	112 ± 11	1.00
	4	115 ± 12	1.03
	8	83 ± 17	0.74
	16	101 ± 8	0.90
Half-ripe pulp	0[c]	112 ± 8	1.00
	4	115 ± 12	1.03
	8	122 ± 15	1.09
	16	113 ± 15	1.00
Fully ripe pulp	0[c]	106 ± 14	1.00
	4	104 ± 10	0.97
	8	106 ± 9	0.99
	16	120 ± 11	1.13
Aril	0[c]	117 ± 4	1.00
	4	118 ± 6	1.01
	8	114 ± 7	0.98
	16	105 ± 11	0.90

[a] The data were expressed as means ± standard deviation of six plates from two experiments

[b] Mutagenicity index (MI) was calculated from the average number of His^+ revertants per plate of the extracts divided by the average number of spontaneous revertants

[c] No extract was added which represented a negative control (spontaneous)

Table 9. The mutagenicity of the water extract from various parts of gac fruit with nitrite treatment on *S. typhimurium* strain TA98

The water extract from each part of gac fruit	Amount (µl/plate)	No. of His^+ revertants/plate[a]	MI[b]
Unripe pulp	0[c]	29 ± 6	1.00
	4	42 ± 9	1.46
	8	43 ± 6	1.50
	16	46 ± 9	1.61
Half-ripe pulp	0[c]	37 ± 3	1.00
	4	31 ± 5	0.84
	8	30 ± 7	0.81
	16	31 ± 11	0.85
Fully ripe pulp	0[c]	22 ± 5	1.00
	4	21 ± 6	0.97
	8	22 ± 3	1.01
	16	17 ± 5	0.77
Aril	0[c]	20 ± 5	1.00
	4	25 ± 6	1.22
	8	38 ± 4	1.89
	16	39 ± 15	1.91

[a] The data were expressed as means ± standard deviation of six plates from two experiments

[b] Mutagenicity index (MI) was calculated from the average number of His^+ revertants per plate of the extracts divided by the average number of spontaneous revertants

[c] No extract was added which represented a negative control (spontaneous)

Table 10. The mutagenicity of the water extract from various parts of gac fruit with nitrite treatment on *S. typhimurium* strain TA100

The water extract from each part of gac fruit	Amount (µl/plate)	No. of His^+ revertants/plate[a]	MI[b]
Unripe pulp	0[c]	112 ± 11	1.00
	4	109 ± 9	0.97
	8	110 ± 9	0.98
	16	107 ± 13	0.96
Half-ripe pulp	0[c]	112 ± 8	1.00
	4	116 ± 22	1.03
	8	127 ± 15	1.13
	16	123 ± 19	1.10
Fully ripe pulp	0[c]	106 ± 14	1.00
	4	112 ± 14	1.05
	8	114 ± 17	1.07
	16	109 ± 9	1.03
Aril	0[c]	117 ± 4	1.00
	4	155 ± 10	1.32
	8	156 ± 7	1.33
	16	134 ± 12	1.15

[a] The data were expressed as means ± standard deviation of six plates from two experiments

[b] Mutagenicity index (MI) was calculated from the average number of His^+ revertants per plate of the extracts divided by the average number of spontaneous revertants

[c] No extract was added which represented a negative control (spontaneous)

4.2 The antimutagenicity of the water extract of gac fruit

The antimutagenicity against nitrite treated 1-AP of the water extract from various parts of gac fruit (unripe pulp, half-ripe pulp, fully ripe pulp and aril) were evaluated on *S. typhimurium* strains TA98 and TA100 without enzymatic activation in the Ames test. According to the results obtained from *S. typhimurium* strain TA98 (Table 11), the extracts from unripe pulp tended to reduce the mutagenic effect of nitrite treated 1-AP better than the other parts. At the highest amount (200 µl) of each fruit extract, the highest antimutagenicity against nitrite treated 1-AP was detected in the extract from unripe pulp (60% inhibition) followed by the extract from half-ripe pulp (49% inhibition), the extract from aril (42% inhibition) and the extract from fully ripe pulp (23% inhibition), respectively. Overall, all extracts exhibited dose-dependent inhibitory effects on mutagenicity of nitrite treated 1-AP (Figure 12).

According to the results from *S. typhimurium* strain TA100 (Table 12), weak to moderate degrees of inhibition were observed in the two higher amounts of the extracts from unripe pulp (39% and 44% inhibition for 100 and 200 µl, respectively) and the extract from half-ripe pulp (28% and 46% inhibition for 100 and 200 µl, respectively). While the two higher amount of the extract from fully ripe pulp exhibited negligible effects (±<20% modification) except at 50 µl per plate showed weak mutagenic effect (-22% enhancement). The extracts from aril exhibited negligible

effects (±<20% modification) although they tended to enhance the mutagenicity of nitrite treated 1-AP by the study concentrations (Figure 13).

Table 11. The modifying effects of the water extract from various parts of gac fruit on the mutagenicity of nitrite treated 1-AP on *S. typhimurium* strain TA98

The water extract from each part of gac fruit	Amount (µl/plate)	No. of His+ revertants/plate[a]	% Modification[b]
Unripe pulp	0[c]	29 ± 6	-
	0[d]	1699 ± 51	-
	50	1442 ± 79	+ 15
	100	853 ± 26	+ 51
	200	701 ± 41	+ 60
Half-ripe pulp	0[c]	17 ± 4	-
	0[d]	1337 ± 99	-
	50	1140 ± 49	+ 15
	100	892 ± 45	+ 34
	200	694 ± 55	+ 49
Fully ripe pulp	0[c]	22 ± 5	-
	0[d]	1741 ± 69	-
	50	1580 ± 90	+ 9
	100	1484 ± 80	+ 15
	200	1339 ± 65	+ 23
Aril	0[c]	35 ± 3	-
	0[d]	1123 ± 90	-
	50	1008 ± 59	+ 11
	100	780 ± 78	+ 31
	200	665 ± 118	+ 42

[a] The data were expressed as means ± standard deviation of six plates from two experiments

[b] + or - indicates that the extracts inhibited or enhanced the mutagenicity of the model, respectively

[c] No extract and no standard mutagen were added which represented a negative control (spontaneous)

[d] No extract was added to the standard mutagen represented a positive control

Table 12. The modifying effects of the water extract from various parts of gac fruit on the mutagenicity of nitrite treated 1-AP on *S. typhimurium* strain TA100

The water extract from each part of gac fruit	Amount (µl/plate)	No. of histidine revertants/plate[a]	% Modification[b]
Unripe pulp	0[c]	74 ± 6	-
	0[d]	841 ± 157	-
	50	788 ± 116	+ 7
	100	543 ± 115	+ 39
	200	503 ± 90	+ 44
Half-ripe pulp	0[c]	112 ± 8	-
	0[d]	604 ± 30	-
	50	568 ± 26	+ 7
	100	469 ± 69	+ 28
	200	378 ± 29	+ 46
Fully ripe pulp	0[c]	106 ± 14	-
	0[d]	921 ± 146	-
	50	1098 ± 176	- 22
	100	842 ± 131	+ 10
	200	771 ± 126	+ 18
Aril	0[c]	104 ± 4	-
	0[d]	1069 ± 103	-
	50	1063 ± 75	+ 1
	100	1088 ± 90	- 2
	200	1185 ± 61	- 12

[a] The data were expressed as means ± standard deviation of six plates from two experiments

[b] + or - indicates that the extracts inhibited or enhanced the mutagenicity of the model, respectively

[c] No extract and no standard mutagen were added which represented a negative control (spontaneous)

[d] No extract was added to the standard mutagen represented a positive control

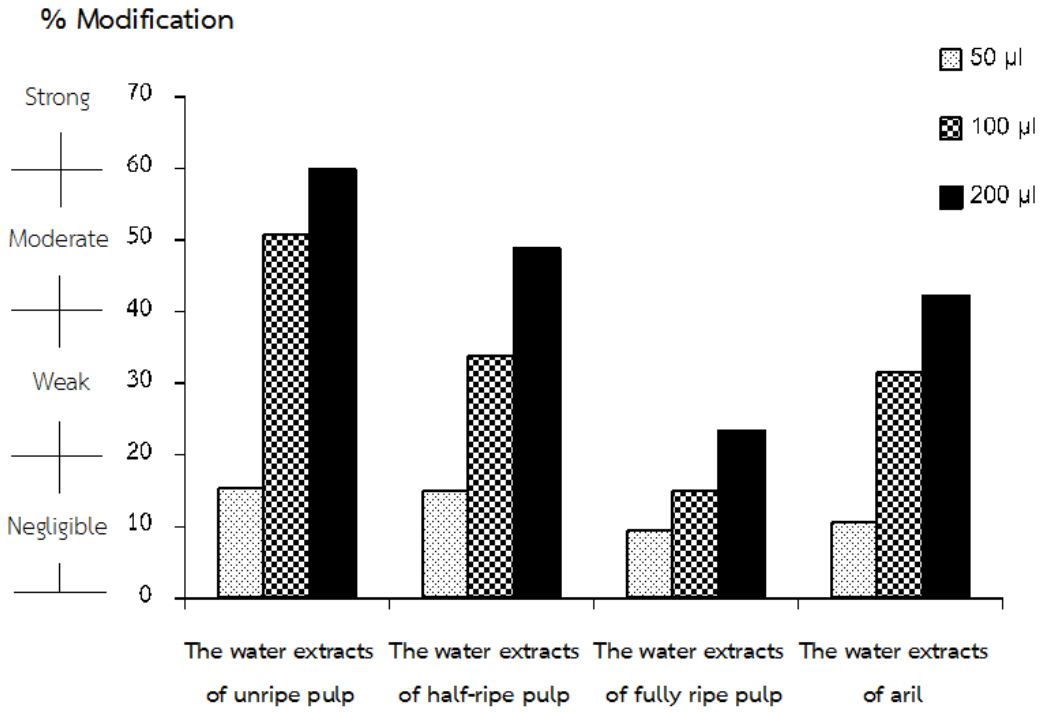

Figure 12. Percentage of modification of the water extract from various parts of gac fruit on the mutagenicity of nitrite treated 1-AP on *S. typhimurium* strain TA98

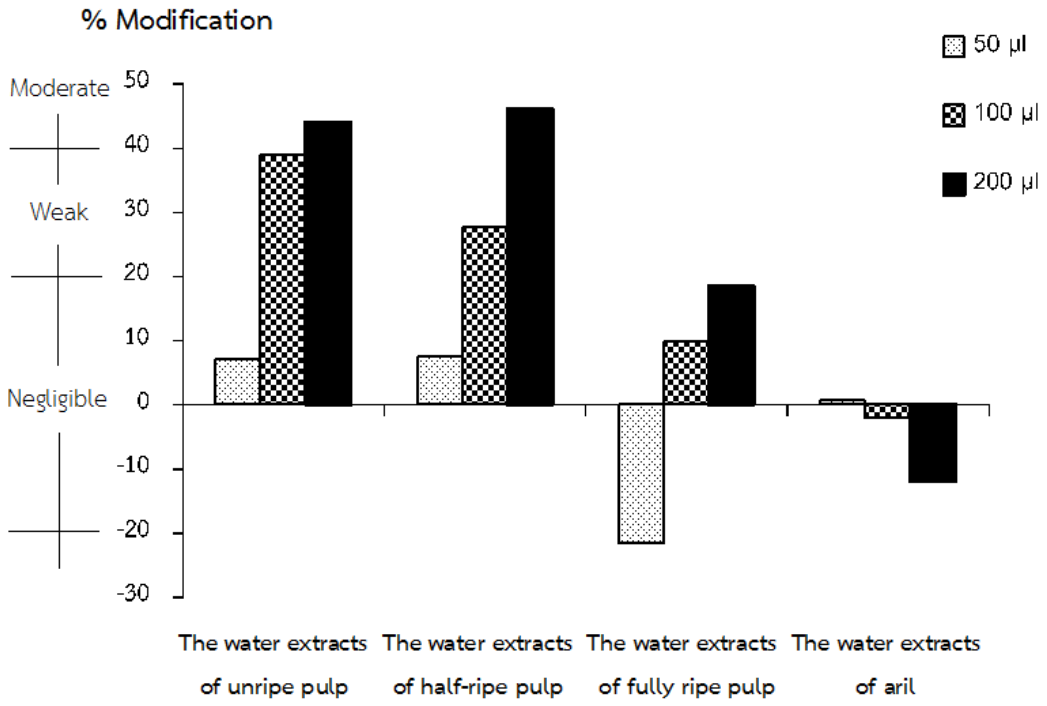

Figure 13. Percentage of modification of the water extract from various parts of gac fruit on the mutagenicity of nitrite treated 1-AP on *S. typhimurium* strain TA100

CHAPTER V

DISCUSSION

Recently, gac fruit is attracted more interest to consume due to its nutritive values (Nagarani, Abirami and Siddhuraju, 2014) and beneficial phytochemicals, especially in the aril part of the fruit. Different kinds of phytochemicals were found in different parts of gac fruit (Kubola and Siriamornpan, 2011). Therefore, in this study, the mutagenicity of the water extract from edible parts of gac fruit was revealed. In addition, the modifying effect of these gac extracts on the mutagenicity of nitrite treated 1-AP was also evaluated.

In mutagenicity assay, the water extract from various parts of gac fruit, including unripe pulp, half-ripe pulp, fully ripe pulp and aril showed no mutagenicity. After treatment with nitrite, they also showed no mutagenicity by the study concentrations which implied that some water soluble antioxidants of gac fruit may be involved in the inhibition of formation of *N*-nitroso compounds. Many investigators reported that the direct-acting mutagen like *N*-nitroso compounds causing gastric cancer in human were the by products from the reaction of nitrite and the nitrosable mutagen precursors containing in many food products (Choi et al., 1986; Mirvish, 1975; Montesano and Bartsch, 1976). After treatment with nitrite, a wide range of food such as pickled vegetable, sun-dried seafood, soy and fish sauces (Wakabayashi et al., 1985), broiled meat, poultry and fish (Yano et al., 1988), spices such as chili, laurel, nutmeg

and pepper (Namiki et al., 1984) and some edible fried-insects (Tongyonk et al., 2003) showed mutagenicity in the Ames test. Moreover, several food additives such as sorbic acid (Namiki et al., 1981), an artificial sweetener, namely aspartame (Shephard et al., 1993), synthetic colourant, Ponceau 4R and natural colourants from 5 plants, including butterfly pea, roselle, pandanus leaf, caramelized coconut sugar and safflower (Kangsadalampai and Butryee, 1995) also showed mutagenic effects according to the Ames assay. It suggested that gac fruit was rather safe for consumption due to gac fruit did not produce the direct mutagen when reacted with nitrite.

In this study, the protective effects of polar fraction of water extract from edible parts of gac fruit against the mutagenicity induced by nitrite treated 1-AP was revealed. 1-AP treated with nitrite was used as a standard mutagen because it was a direct-acting mutagen which required no enzymatic activation (Kangsadalampai and Butryee, 1995). Kato et al. (1991) reported that one of the product after nitrite treatment 1-AP was 1-nitropyrene. It is the predominant nitro-polycyclic aromatic hydrocarbons emitted in diesel exhaust, exhaust of kerosene heaters and petroleum gas burners. It also released during barbecuing meat products as a results of incomplete combustion or pyrolysis of fatty meat (Edenharder, Petersdorff and Rauscher, 1993; Handa et al., 1983; Rosenkranz and Mermelstein, 1983; Tokiwa, Nakagawa and Horikawa, 1985).

In antimutagenicity assay, all gac extracts had antimutagenic activity on *S. typhimurium* strain TA98 referred to the influence on frameshift-mediated mutagens. The extract from unripe gac pulp showed the highest antimutagenicity on *S.*

typhimurium strain TA98 (60% inhibition). Interestingly, the higher antimutagenicity of the extract of gac fruit was obtained from unripe stage and the effect tended to reduce by gac maturity on both strains of *S. typhimurium*. On the other hand, fully ripe pulp and aril showed no antimutagenicity which may be a results from water extraction method obtaining only polar compounds. Lipid soluble compounds in fully ripe pulp and aril were not irrelevant in this study. According to Kubola and Siriamornpun (2011) reported that the total phenolic contents in gac fruit of unripe stage were higher than other stages which decreased during the fruit ripening process (unripe > half-ripe > fully ripe). The phenolic antioxidants which were the main components in gac pulp may be involved in the antimutagenic effect. Previous studies reported that phenolic compounds had antimutagenic activity against many mutagens such as *N*-nitroso compounds, smoke-induced mutagens and oxidative mutagen, namely H_2O_2 and tertiary butyl hydroperoxide (TBHP) (Bhattacharya, 2011; Ferguson, 1994).

In the extract from aril, the enhancing trend of mutagenicity of nitrite treated 1-AP on *S. typhimurium* TA100 may be attributed to pro-oxidants at certain concentrations. Kubola and Siriamornpun (2011) reported that gac aril exhibited the highest luteolin content and its amount was four-fold higher than unripe gac pulp while not detected in half-ripe pulp and fully ripe pulp. Flavonoids like luteolin has the pro-oxidative catechol structural element (hydroxyl group at positions 3 and 4 of the B ring) which exhibit mutagenicity without enzymatic activation. The auto-oxidation

of luteolin to luteolin *ortho*-semiquinone anion radical and luteolin *ortho*-quinone generate superoxide anion radical ($O^{\bullet -}$). Moreover, quinones and their isomeric quinone methides form from auto-oxidation of catechol-type flavonoids are electrophiles that can react with DNA (Rietjens et al., 2002). Some natural antioxidants may exert toxic pro-oxidant activities by auto-oxidation and/or enzymatic activation. As results of previous reports, several fruits and vegetables containing natural antioxidants exhibited mutagenic activity on *Salmonella* tester strains using the Ames test. Six samples (grapes, onions, peaches, raisins, raspberries and strawberries) from these fruits and vegetables showed strong mutagenic activity (Stoltz et al., 1984). Citrus fruit juices which contained many flavonoid compounds exhibited mutagenic activity after enzymatic hydrolysis using the Ames test (Mazaki, Ishii and Uyeta, 1982). Moreover, the water extracts of red grapes enhanced the mutagenic activity of H_2O_2 with dose dependent manner testing with the Ames assay (Stagos et al., 2006).

Nitro-PAHs, the product of nitrosation of 1-AP required bacterial bio-transformation to exert their mutagenicity on bacterial DNA. The mechanism of inhibition of genotoxicity by gac extract may be due to its inhibitory effect on metabolic enzyme, nitroreductase and *O*-transferase. The nitro compounds reduce the nitro group to a hydroxylamine intermediate and esterified the OH function by *O*-acetyltransferase to reactive arylnitrenium ion which can bind to DNA (Kappers et al., 2000). Thus, the reduction of His^+ revertants of both strains of *S. typhimurium* suggested that gac fruit, especially from unripe gac fruit contained some water soluble

bioactive compounds, major contribution to the phenolic acids which inhibited the mutagenic activity of nitrite treated 1-AP

CHAPTER VI

CONCLUSION

This study indicated that the water extracts from edible parts of gac fruit, including unripe pulp, half-ripe pulp, fully ripe pulp and aril had no mutagenic activity either with or without nitrite treatment testing with the Ames assay. Although there was not mutagenic activity after being treated with nitrite, avoiding consumption of nitrite containing food products should be considered regarding to the etiology of gastric cancer. According to the antimutagenic effects, the presence of antimutagenic activity of these fruit extracts, especially from unripe gac pulp could be useful for further study.

However, the modifying effect of gac fruit on mutagenicity of nitrite treated 1-AP obtained from polar fraction (water extract). Thus, further investigation is still required the isolation of the bioactive compounds from gac fruit by organic solvent extracts.

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APPENDIX

APPENDIX

1. Preparation of stock solution, medium and reagents

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

Ingredient	1,000 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 water (45°C)	670 ml

Add salts in the order indicated above which allow each salt to dissolve completely before adding the next one. Filter and autoclave the solution at 121°C for 20 minutes.

1.2 40 % (w/v) Glucose solution

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

Dissolve D(+)-glucose in appropriated volume of distilled water before adjusting to 100 ml. After complete dissolution, transfer 15 ml of this solution into a glass bottle with screw cap then autoclave at 121°C for 20 minutes.

1.3 Minimal glucose agar (MGA) plate

Ingredient	1,000 ml
Agar agar	15 g
Sterile VB salts	20 g
Sterile 40% (w/v) glucose	50 g
Distilled water	930 ml

Add agar to distilled water in a screw cap Erlenmeyer flask and autoclave at 121°C for 20 minutes. After the solution cooling down, add sterile VB salts and sterile 40% (w/v) glucose, mix gently and pour 30 ml of the solution onto a sterile petri dish then incubate at 37°C for 48 hours before use.

1.4 Top agar

Ingredient	110 ml
Agar agar	0.6 g
Sodium chloride (NaCl)	0.5 g
0.5 mM Histidine/biotin solution	10 ml
Distilled water	100 ml

Dissolve agar and sodium chloride in distilled water then autoclave at 121°C for 20 minutes. Add 10 ml of a sterile solution of 0.5 mM histidine/biotin and mix thoroughly by swirling.

1.5 0.1 M Histidine stock solution

Ingredient	10 ml
Histidine	0.1552 g
Distilled water qs to	10 ml

Dissolve histidine (MW = 155.2 g/mol) in appropriate volume of distilled water before adjusting to 10 ml then autoclave at 121°C for 20 minutes.

1.6 1 mM Histidine stock solution

Ingredient	100 ml
0.1 M Histidine	1 ml
Distilled water qs to	100 ml

Pipette 1 ml of 0.1 M histidine into volumetric flask before adjusting the volume to 100 ml then autoclave at 121°C for 20 minutes.

1.7 1 mM Biotin stock solution

Ingredient	100 ml
Biotin	0.0244 g
Distilled water qs to	100 ml

Dissolve biotin (MW = 244.31 g/mol) in appropriated volume of distilled water before adjusting the volume to 100 ml then autoclave at 121°C for 20 minutes.

1.8 0.5 mM Histidine/biotin solution

Ingredient	200 ml
1 mM Histidine	100 ml

1 mM Biotin	100 ml

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

1.9 Oxoid nutrient broth No.2

Ingredient	100 ml
Oxoid nutrient broth No. 2	2.5 g
Distilled water	100 ml

Dissolve Oxoid nutrient broth No. 2 in distilled water before filling 12 ml of this solution into each narrow-mouth Erlenmeyer flask covered with cotton ball cork then autoclave at 121°C for 20 minutes.

1.10 0.9% Sodium chloride solution

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

Dissolve sodium chloride in distilled water then autoclave at 121°C for 20 minutes.

1.11 2 M Sodium nitrite

Ingredient	10 ml
Sodium nitrite ($NaNO_2$)	1.38 g
Distilled water qs to	10 ml

Dissolve sodium nitrite (MW = 68.99 g/mol) in appropriate volume of distilled water before adjusting the volume to 10 ml then autoclave at 121°C for 20 minutes.

1.12 2 M Ammonium sulfamate

Ingredient	10 ml
Ammonium sulfamate ($NH_2SO_3NH_4$)	2.2824 g
Distilled water qs to	10 ml

Dissolve ammonium sulfamate (MW = 114.12 g/mol) in appropriate volume of distilled water before adjusting the volume to 10 ml then autoclave at 121°C for 20 minutes.

1.13 1 M Potassium chloride

Ingredient	100 ml
Potassium chloride (KCl)	7.456 g
Distilled water qs to	100 ml

Dissolve potassium chloride in appropriate volume of distilled water before adjusting the volume to 100 ml then autoclave at 121°C for 20 minutes.

1.14 0.5 M Sodium dihydrogen phosphate solution

Ingredient	100 ml
Sodium dihydrogen phosphate (NaH_2PO_4)	6 g
Distilled water qs to	100 ml

Dissolve sodium dihydrogen phosphate (MW = 120 g/mol) in appropriate volume of distilled water then adjusting the volume to 100 ml.

1.15 0.5 M Disodium hydrogen phosphate dihydrate solution

Ingredient	100 ml
Disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$)	8.9 g
Distilled water qs to	100 ml

Dissolve disodium hydrogen phosphate dihydrate (MW = 177.99) in appropriate volume of distilled water then adjust to 100 ml.

1.16 0.5 M Sodium phosphate pH 7.4

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

Add 0.5 M sodium dihydrogen phosphate solution (from 1.14) in 0.5 M disodium hydrogen phosphate dihydrate solution (from 1.15) until to pH 7.4 then autoclave at 121°C for 20 minutes.

1.17 Sodium phosphate - potassium chloride buffer

Ingredient	330 ml
1 M Potassium chloride (KCl)	16.5 ml
0.5 M Sodium phosphate pH 7.4	100 ml
Distilled water qs to	213.5 ml

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

1.18 Ampicillin solution (8 mg/ml)

Ingredient	10 ml
Ampicillin sodium	0.08 g
Sterile distilled water qs to	10 ml

Aseptic technique

Dissolve ampicillin sodium in appropriate volume of sterile distilled water before adjusting the volume to 10 ml then store in sterile glass bottle with cap.

1.19 0.1% Crystal violet

Ingredient	10 ml
Crystal violet	0.01 g
Sterile distilled water qs to	10 ml

Aseptic technique

Dissolve crystal violet in appropriate volume of sterile distilled water before adjust to 10 ml then store in sterile glass bottle with cap.

1.20 0.2 N Hydrochloric acid

Ingredient	100 ml
Conc. hydrochloric acid	1.67 ml
Sterile distilled water qs to	100 ml

Aseptic technique

Pipette 1.67 ml of conc. hydrochloric acid in a sterile volumetric flask with appropriate volume of sterile distilled water before adjusting the volume to 100 ml then store in sterile amber glass bottle with ground glass plug.

1.21 1-Aminopyrene 3 mg/ml in acetonitrile

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

Aseptic technique

Dissolve 1-aminopyrene in acetonitrile (3 mg/ml) then store in sterile amber glass bottle with screw cap in freezer.

1.22 1-Aminopyrene 0.075 mg/ml in acetonitrile

Ingredient	2 ml
1-Aminopyrene solution 3 mg/ml	0.1 ml
Acetonitrile	2.4 ml

Aseptic technique

Pipette 0.1 ml of 1-aminopyrene 3 mg/ml in acetonitrile in a sterile amber glass bottle with screw cap before adding 0.9 ml of acetonitrile and mixing thoroughly. Pipette 0.5 ml of this solution in a new sterile amber glass bottle with screw cap and then add 1.5 ml of acetonitrile and mix thoroughly before keeping in freezer until use.

2. Re-isolation of bacteria (Figure 15)

Pipette 10 µl of each tester strains (*S. typhimurium* strains TA98 and TA100) in a sterile Erlenmeyer flask with 12 ml of Oxoid nutrient broth No.2 and incubate overnight at 37°C for 16 hours in a shaking water bath. After 16 hours of incubation, each overnight culture is streaked on a MGA plate which already 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 onto the surface evenly then turn plates upside down and place them in an incubator at 37°C for 48 hours. After 48 hours of incubation, four single colonies of each tester strain (Figure 16) are picked up and separately placed them in each sterile Erlenmeyer flask with 12 ml of Oxoid nutrient broth No.2 then incubate overnight at 37°C for 16 hours in a shaking water bath. After 16 hours of incubation, 7 ml of each overnight culture is identified and 5 ml of each overnight culture is mixed with 450 µl of dimethyl sulfoxide (DMSO) in a sterile tube then distribute 200 µl of the mixed culture in a sterile micro-centrifuge tube before storing in a refrigerator at -80°C for mutagenic assay.

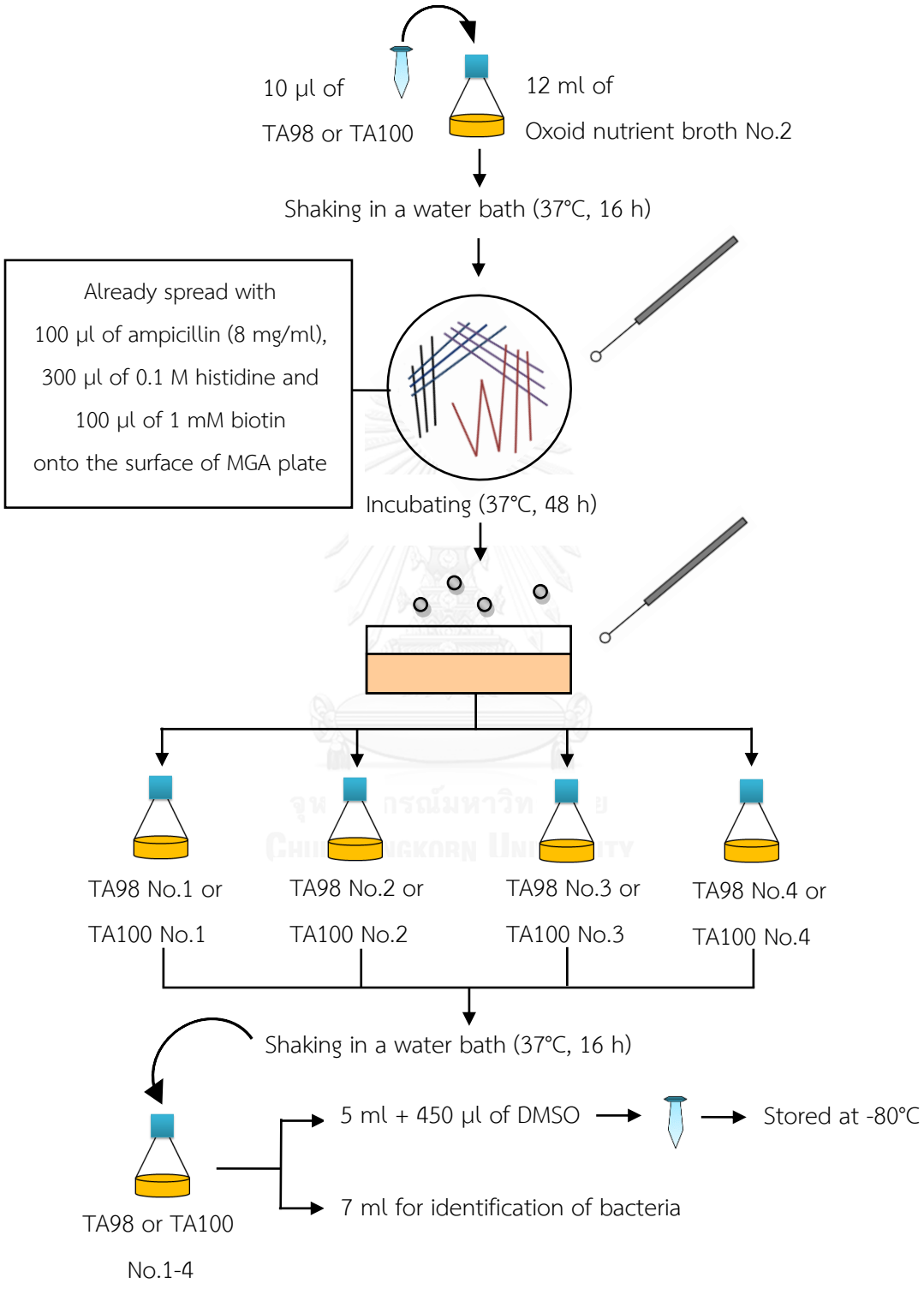
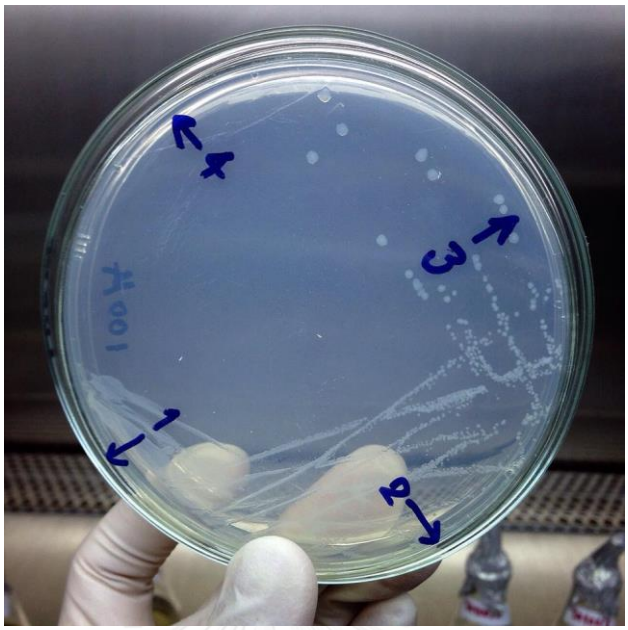
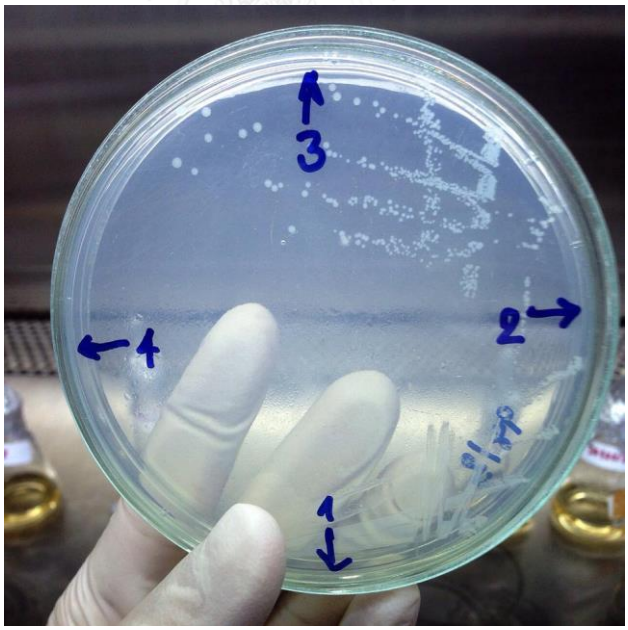


Figure 15. Re-isolation of bacteria



A



B

Figure 16. Re-isolation for a single colony. A: *S. typhimurium* strain TA98; B: *S. typhimurium* strain TA100

3. Identification of bacteria

3.1 Histidine requirement (Figure 17)

S. typhimurium is mutated in gene necessary for histidine synthesis. It requires amino acid histidine for growth but can not synthesize. Four MGA plates, including plate a, b, c and d are required for each tester strain (*S. typhimurium* strains TA98 and TA100). Briefly, spread the 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 while plate a is no application. Four strains (No.1, No.2, No.3 and No.4) of *S. typhimurium* strain TA98 or TA100 are tested on the same plate and single streaked across these plates then incubate at 37°C for 24 hours. The growing of tester strains on plate d (containing biotin plus histidine) exhibit the results of histidine requirement.

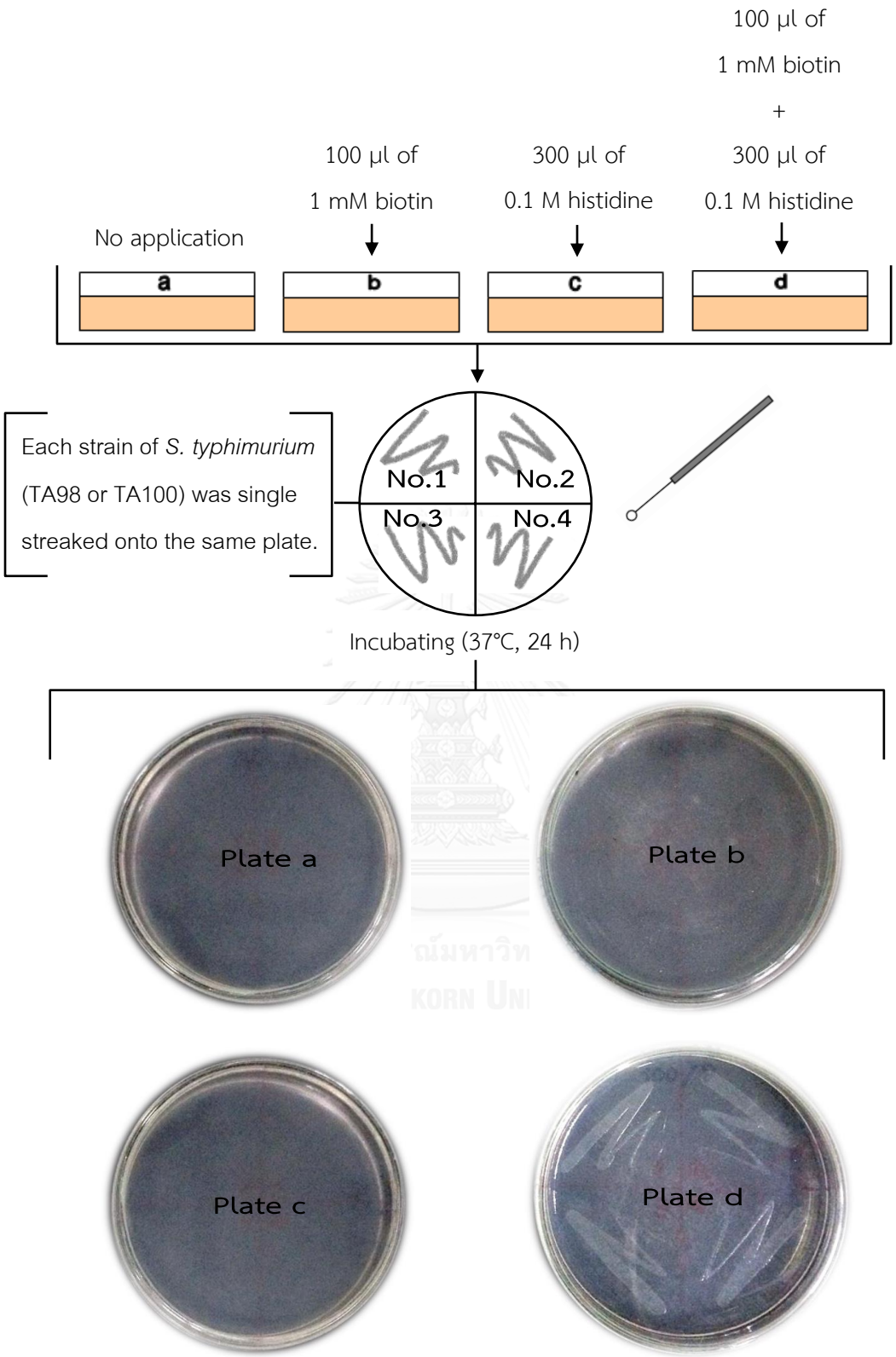


Figure 17. Histidine requirement

3.2 R-factor (Figure 18)

S. typhimurium is tested for the presence of the resistance transfer factor (R-factor) which is a plasmid that encoded resistance to ampicillin. Briefly, 300 µl of each tester strain (*S. typhimurium* strains TA98 and TA100) is mixed with 100 µl of 0.1 M histidine in a sterile tube with cap then added 2 ml of molten top agar (45°C) containing 0.5 mM histidine and biotin (add 10 ml to 100 ml of top agar), mixed well and poured onto a MGA plate. Divide a minimal glucose agar plate into two equal parts in order to perform R-factor and rfa mutation (see the next section) in the same plate. For R-factor, filter paper disc containing 10 µl of ampicillin solution (8 mg/ml) is placed onto a MGA plate by sterile forceps then incubated at 37°C for 24 hours. The absence of clear zone around the disc indicate that the strain retained the plasmid.

3.3 rfa mutation (Figure 18)

S. typhimurium is mutated in genes involved in lipopolysaccharide synthesis that makes the cell wall more permeable to large molecules. The rfa mutation is performed as described in R-factor except 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.

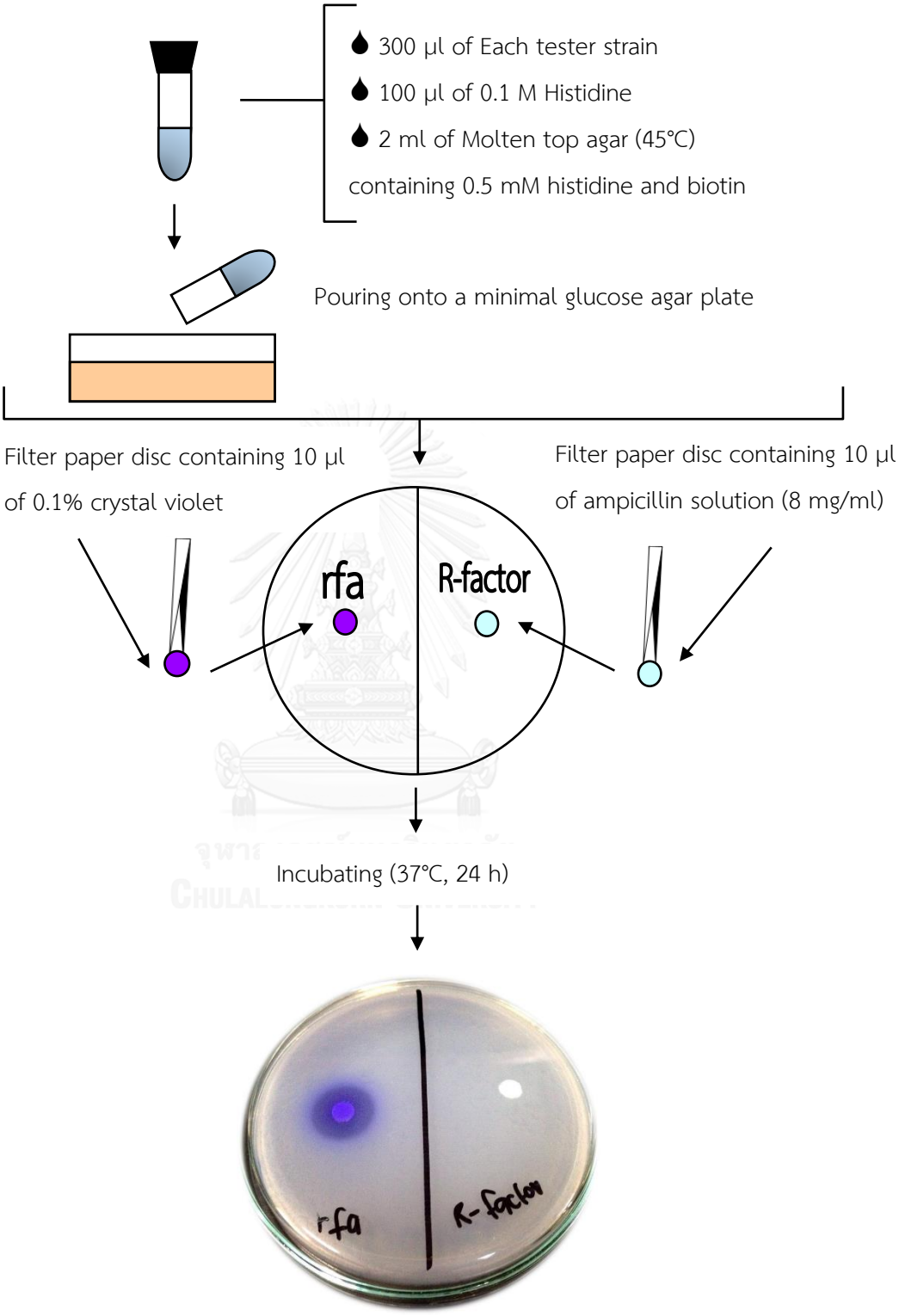


Figure 18. rfa and R-factor mutation

4. Spontaneous reversion or negative control

Spontaneous reversion of *S. typhimurium* is performed routinely in mutagenic assay. A range of the number of spontaneous revertants per plate without enzyme activation is about 20-50 and 75-200 on *S. typhimurium* strains TA98 and TA100, respectively (Mortelmans and Zeiger, 2000). However, the number of spontaneous revertants per plate is variability from one experiment to another and from one plate to another, and it is advisable to include at least 3 negative control plates for each strain in the mutagenicity assay (Maron and Ames, 1983). Briefly, 200 µl of distilled water, 500 µl of 0.5 M sodium phosphate-potassium chloride buffer (pH 7.4) and 100 µl of each overnight culture are mixed in sterile tube with cap. The mixture was incubated in a shaking water bath at 37°C for 20 minutes then added 2 ml of molten top agar (45°C) containing 0.5 mM histidine and biotin (add 10 ml to 100 ml of top agar). The mixture is carefully poured onto a MGA plate after thoroughly mixed. The number of spontaneous revertants per plate is counted after turned plate upside down in an incubator at 37°C for 48 hours (Figure 19).

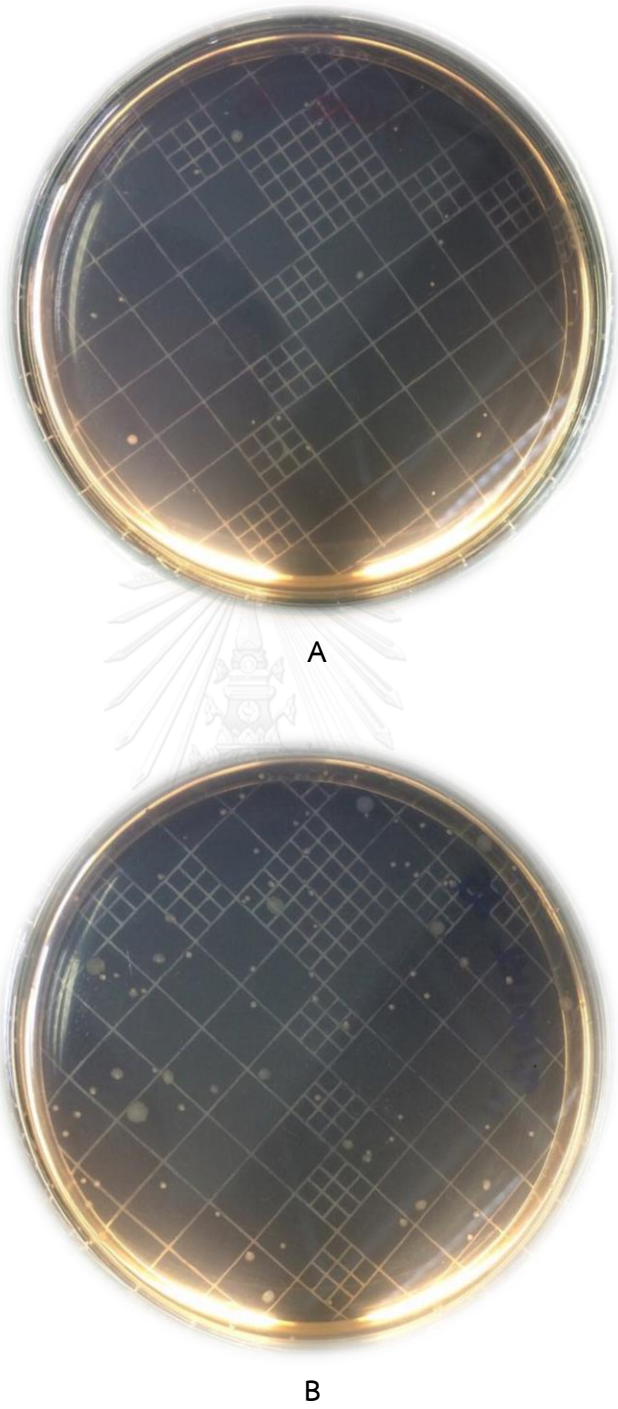


Figure 19. Spontaneous revertants after 48 hours of incubation. A: *S. typhimurium* strain TA98; B: *S. typhimurium* strain TA100

5. The response to standard mutagen or positive control

The response to standard mutagen of *S. typhimurium* is performed routinely in mutagenicity assay to confirm the reversion property and specificity of tester strains. In this study, nitrite treated 1-AP in an acidic condition is used as a standard mutagen because it is a direct-acting mutagen in the Ames test (Kangsadalampai et al., 1995). The procedure is performed as described in spontaneous reversion except 100 µl of the reaction mixture of nitrite treated 1-AP (1-AP 0.075 mg/ml in acetonitrile; 10 µl and 20 µl per plate for *S. typhimurium* strain TA98 and TA100, respectively) is placed instead of distilled water. The number of His^+ revertants per plate is counted after turned plate upside down in an incubator at 37°C for 48 hours (Figure 20).

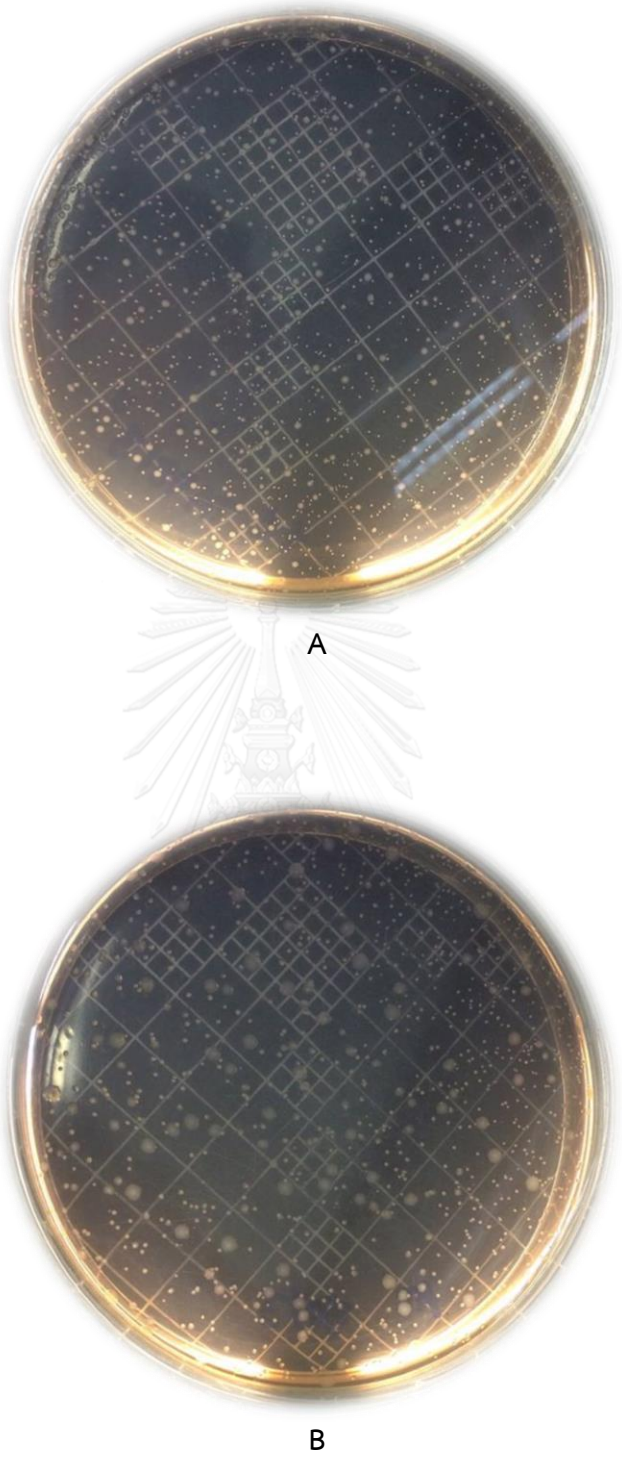


Figure 20. His^+ revertants of 1-AP 0.075 mg/ml. A: *S. typhimurium* strain TA98 (1-AP 10 µl/plate); B: *S. typhimurium* strain TA100 (1-AP 20 µl/plate)

VITA

Miss Napad Triteeradej was born on January 27, 1986 in Nakhon Ratchasima, Thailand. She received her Bachelor of Science in Pharmacy in 2010 from the Faculty of Pharmaceutical Science, Huachiew Chalermprakiet University, Thailand. After graduation, she worked as a pharmacist at Bangpakok 9 International Hospital. Her responsibilities were dispensing medicines and patients counselling. In 2013, she worked as a pharmacist at Pure drug store and a part-time pharmacist at Health Up drug store. Her responsibilities were dispensing medicines, patients counselling, educating for food supplements and skin care products for pharmacy assistants and warehouse management.