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Effect of bitter gourd extracts on the mutagenicity of 1-aminopyrene reacted with nitrite using ames test

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EFFECT OF BITTER GOURD EXTRACTS ON THE MUTAGENICITY OF 1-AMINOPYRENE REACTED WITH NITRITE USING AMES TEST

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A Thesis Submitted in Partial Fulfillment of the Requirements
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ผลของสารสกัดมะระต่อฤทธิ์ก่อกลายพันธุ์ของ 1-อะมิโนพัยรีนที่ทำปฏิกิริยากับไนไตรท์โดยใช้การทดสอบเอมส์

นายก่อเกียรติ์ ปานผาสุข

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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Thesis Title	EFFECT OF BITTER GOURD EXTRACTS ON THE MUTAGENICITY OF 1-AMINOPYRENE REACTED WITH NITRITE USING AMES TEST
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Field of Study	Food Chemistry and Medical Nutrition
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Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science in Pharmacy

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ก่อเกียรติ์ ปานผาสุข : ผลของสารสกัดมะระต่อฤทธิ์ก่อกลายพันธุ์ของ 1-อะมิโนพัยรีนที่ทำปฏิกิริยากับไนไตรท์โดยใช้การทดสอบเอมส์. (EFFECT OF BITTER GOURD EXTRACTS ON THE MUTAGENICITY OF 1-AMINOPYRENE REACTED WITH NITRITE USING AMES TEST) อ.ที่ปรึกษาหลัก : ผศ. ภญ. ดร.ลินนา ทองยงค์

การศึกษานี้เป็นการศึกษาฤทธิ์ก่อกลายพันธุ์ของสารสกัดจากมะระสดและมะระที่ผ่านการปรุง ได้แก่ สารสกัดมะระสด น้ำลวกมะระ สารสกัดจากมะระต้มที่ผ่านการลวก สารสกัดจากมะระผัด และสารสกัดจากมะระที่ขยำเกลือก่อนการผัด นอกจากนี้ยังศึกษาผลของสารสกัดมะระในการปรับเปลี่ยนฤทธิ์ของสารก่อกลายพันธุ์ที่เกิดจากปฏิกิริยาระหว่าง 1-อะมิโนพัยรีนกับโซเดียมไนไตรท์ และผลของการเติมสารสกัดมะระในการปรับเปลี่ยนปฏิกิริยาดังกล่าว โดยใช้วิธีเอมส์แบบไม่มีระบบเอนไซม์กระตุ้นสารพิษ ทดสอบโดยใช้เชื้อแบคทีเรีย *Salmonella typhimurium* สายพันธุ์ TA98 และ TA100 ผลการทดสอบฤทธิ์ก่อกลายพันธุ์ แสดงเป็นค่าเฉลี่ยของจำนวนโคโลนีกลายพันธุ์ของแบคทีเรียต่อจานเพาะเชื้อ และผลการปรับเปลี่ยนฤทธิ์ก่อกลายพันธุ์ แสดงเป็นร้อยละของการปรับเปลี่ยนฤทธิ์ก่อกลายพันธุ์ ผลการศึกษาพบว่าสารสกัดมะระทุกตัวอย่าง ไม่มีฤทธิ์ก่อกลายพันธุ์โดยตรงในสภาวะที่ทำ และไม่ทำปฏิกิริยากับโซเดียมไนไตรท์ต่อเชื้อแบคทีเรียทั้ง 2 สายพันธุ์ สำหรับการศึกษาผลของสารสกัดมะระในการปรับเปลี่ยนฤทธิ์ของสารก่อกลายพันธุ์ที่เกิดจากปฏิกิริยาระหว่าง 1-อะมิโนพัยรีนกับโซเดียมไนไตรท์ พบว่าสารสกัดมะระสดมีฤทธิ์ส่งเสริมการก่อกลายพันธุ์ของสารก่อกลายพันธุ์ต่อเชื้อ *S. typhimurium* สายพันธุ์ TA98 (ร้อยละของการปรับเปลี่ยนฤทธิ์ก่อกลายพันธุ์ตั้งแต่ 9.54 ถึง 147.62) แต่ไม่มีผลปรับเปลี่ยนเมื่อทดสอบด้วยเชื้อสายพันธุ์ TA100 (ร้อยละของการปรับเปลี่ยนฤทธิ์ก่อกลายพันธุ์น้อยกว่า 20) เมื่อนำสารสกัดมะระที่ผ่านการปรุงมาทดสอบพบว่า ค่าร้อยละของการส่งเสริมฤทธิ์การก่อกลายพันธุ์ของน้ำลวกมะระ (ต่อเชื้อ TA98 คือ 116.12 ถึง 338.06 และต่อเชื้อ TA100 คือ 74.93 ถึง 109.95) มีแนวโน้มที่สูงกว่าค่าที่ได้จากสารสกัดจากมะระต้มที่ผ่านการลวกต่อเชื้อทั้ง 2 สายพันธุ์ (ต่อเชื้อ TA98 คือร้อยละ 42.61 ถึง 140.85 และต่อเชื้อ TA100 คือร้อยละ 8.59 ถึง 77.29) นอกจากนี้ยังพบแนวโน้มที่คล้ายกันจากผลการศึกษาของมะระผัด กล่าวคือการขยำด้วยเกลือก่อนนำไปผัดมีผลทำให้ร้อยละของการส่งเสริมฤทธิ์การก่อกลายพันธุ์มีแนวโน้มที่ต่ำกว่าค่าที่ได้จากสารสกัดมะระผัดที่ไม่ผ่านการขยำด้วยเกลือก่อน ผลของการเติมสารสกัดมะระในช่วงตั้งต้นของการเกิดปฏิกิริยาระหว่าง 1-อะมิโนพัยรีนกับโซเดียมไนไตรท์ พบว่าสารสกัดทั้งหมดมีฤทธิ์ส่งเสริมการก่อกลายพันธุ์ต่อเชื้อแบคทีเรีย นอกจากนี้ค่าร้อยละของการส่งเสริมฤทธิ์การก่อกลายพันธุ์ของน้ำลวกมะระ (ต่อเชื้อ TA98 คือ 74.21 ถึง 348.69 และต่อเชื้อ TA100 คือ 13.10 ถึง 74.97) แสดงแนวโน้มที่สูงกว่าค่าร้อยละที่ได้จากสารสกัดจากมะระต้มที่ผ่านการลวกต่อเชื้อ *S. typhimurium* ทั้ง 2 สายพันธุ์ (ต่อเชื้อ TA98 คือ 37.19 ถึง 57.46 และต่อเชื้อ TA100 คือ 3.54 ถึง 34.66) และสารสกัดจากมะระที่ขยำเกลือก่อนการผัดแสดงแนวโน้มการส่งเสริมฤทธิ์การก่อกลายพันธุ์ที่ลดลงเมื่อเปรียบเทียบสารสกัดจากมะระผัดต่อเชื้อ *S. typhimurium* สายพันธุ์ TA98 อย่างไรก็ตามเมื่อทดสอบกับ *S. typhimurium* สายพันธุ์ TA100 พบว่าได้ผลที่แตกต่างไปกล่าวคือ ค่าร้อยละของการส่งเสริมฤทธิ์การก่อกลายพันธุ์ของสารสกัดจากมะระที่ขยำเกลือก่อนการผัด (19.06 ถึง 88.48) มีแนวโน้มที่สูงกว่าสารสกัดจากมะระผัด (2.90 ถึง 57.09) ผลการศึกษานี้แสดงให้เห็นว่ามะระดิบและมะระที่ผ่านการปรุงมีความปลอดภัยในการบริโภคเมื่อพิจารณาจากการเกิดฤทธิ์ก่อกลายพันธุ์ และวิธีการเตรียมมะระก่อนการปรุง เช่น การลวกก่อนต้ม หรือการขยำด้วยเกลือก่อนผัดเป็นวิธีที่ไม่เพียงแต่ช่วยลดความขมของมะระ แต่ยังอาจลดความเสี่ยงในการส่งเสริมการก่อกลายพันธุ์ของสารก่อกลายพันธุ์บางชนิดที่อาจปนเปื้อนในอาหาร

สาขาวิชา	อาหารเคมีและโภชนศาสตร์ทางการแพทย์	ลายมือชื่อนิสิต .....
ปีการศึกษา	2561	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5976101533 : MAJOR FOOD CHEMISTRY AND MEDICAL NUTRITION
KEYWORD: BITTER GOURD, COOKING METHOD, MUTAGENICITY, AMES TEST
Korkiat Panpasuk : EFFECT OF BITTER GOURD EXTRACTS ON THE MUTAGENICITY OF 1-AMINOPYRENE REACTED WITH NITRITE USING AMES TEST. Advisor: Asst. Prof. Linna Tongyonk, D.Sc.

This study was conducted to investigate the mutagenic activity of raw and cooked bitter gourd (*Momordica charantia*) including raw fruit extract (RW), blanching water (BW), hot water extract from blanched fruit (HB), stir-fried fruit extract (SF), and stir-fried fruit (pre-squeezed with salt) extract (SS). In addition, the effects of extracts in modifying the mutagenic activity of mutagen freshly prepared by the reaction between 1-aminopyrene and sodium nitrite and the effects when adding each extract at the beginning of the same reaction using Ames test on *Salmonella typhimurium* strains TA98 and TA100 without enzymatic activation were also determined. The data for mutagenic activity were expressed as a number of histidine revertant colonies/plate and expressed as percentage of modification for mutagenic modification effect. The results showed that all extracts had no direct mutagenic activity either with or without sodium nitrite treatment on both tester strains. The effect of bitter gourd extracts in modifying the mutagenic activity of newly formed mutagen showed that RW had the mutagenic enhancing effect on *S. typhimurium* strain TA98 (%enhancement = 9.54-147.62). However, there was no mutagenic enhancing effect on *S. typhimurium* strain TA100 (%enhancement <20%). For cooked bitter gourd extract, it was found that the percentage of mutagenic enhancement of BW (116.12%-338.06% and 74.93%-109.95% for TA98 and TA100, respectively) tended to be higher than that of HB on both stains (42.61%-140.85% and 8.59%-77.29% for TA98 and TA100, respectively). In addition, the similar trend was found from the results of the stir-fried bitter gourd. SS showed lower enhancing effect on the mutagenicity of newly formed mutagen than SF. The results of the effect of bitter gourd extracts added before starting the mutagen forming reaction showed that all extracts exhibited enhancing effects on *S. typhimurium* strains TA98 and TA100. In addition, the percentage of mutagenic enhancement of BW (74.21%-348.69% and 13.10%-74.97% for TA98 and TA100, respectively) showed a higher trend than that of HB on both strains of *S. typhimurium* (37.19%-57.46% and 3.54%-34.66% for TA98 and TA100, respectively). SS showed lower values of the percentage of mutagenic enhancement when comparing with SF on *S. typhimurium* strain TA98. However, when tested with *S. typhimurium* strain TA100, it was found that the results were different. These values obtained from SS (19.06%-88.48%) tend to be higher than that of SF (2.90%-57.09%). The present study provides the information that raw and cooked bitter gourd are safe for consumption in consideration of mutagenicity. In the case of cooking, the fruit should be pretreatment first. These pretreatment methods such as blanching before boiling or squeezing with salt before stir frying not only reduce the bitter taste of this fruit, but may also reduce the risk of promoting mutagenic effects of some mutagens that may be contaminated in food.

Field of Study:	Food Chemistry and Medical Nutrition	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....

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LIST OF ABBREVIATIONS

1-AP	1-aminopyrene
°C	degree Celsius
BW	blanching water
et al.	et alia (and others)
g	gram
h	hour
HB	hot water extract from blanched fruit
µg	microgram
µl	microliter
mg	milligram
ml	millilitre
mM	millimolar
min	minute
M	molarity
MB	the effect of bitter gourd extracts in modifying the mutagenic activity of newly formed mutagen
MF	the effect of bitter gourd extracts added before starting the mutagen forming reaction
MI	mutagenicity index

N	normality
PAHs	polycyclic aromatic hydrocarbons
pH	potential of hydrogen ion
rpm	revolution per minute
RW	raw fruit extract
SD	standard deviation
SF	stir-fried fruit extract
SS	stir-fried fruit (pre-squeezed with salt) extract

CHAPTER I

INTRODUCTION

1.1 Background and rationale

Momordica charantia or bitter gourd is a plant in the family Cucurbitaceae which can be found in tropical countries such as India, China, Vietnam, Indonesia, and Thailand[1, 2]. The general characteristics of *Momordica charantia* are herbaceous plant with tendrils, simple alternate leaf and yellow unisexual solitary flower in leaf-axil[3, 4]. Bitter gourd fruits are green with ridges which will turn yellowish orange when ripe. The unripe fruits are often eaten as raw or cooked to varieties of menu such as chicken bitter gourd noodles, stuffed bitter gourd soup and stir fried bitter gourd with eggs[5-7]. Numerous biological substances can be found in bitter gourd, for example, cucurbitane type triterpenoids, cucurbitane type triterpene glycosides, phenolic compounds, flavonoids, and saponins[8, 9]. These substances made the bitter gourd having various biological activities such as antioxidant[2, 10], antiviral[11, 12], antidiabetic[13, 14], antiobesity[15, 16] and most importantly anticancer[17-19].

Cancer is a health problem that currently is one of the leading cause of death in the world. The underlying causes of cancer are living habits, external stimuli and ingested foods that may be contaminated with natural carcinogens and harmful food additives[20, 21]. In addition, consumption of processed meat that used nitrite and

nitrate for preservation causes the forming of *N*-nitroso compounds in the stomach, resulting in a high incidence of stomach cancer development[22, 23].

Mutagenicity testing is a process of identifying substances that may cause genetic alterations in somatic and/or germ cells. A quick and easy-to-use test is the Ames test that uses *Salmonella typhimurium* with S-9 mix for testing[24, 25]. The S-9 mix can also be replaced as nitrite salts, such as sodium nitrite and potassium nitrite in acidic conditions[26].

1.2 Objectives of the study

This study aimed to investigate the mutagenic effects of raw or cooked *Momordica charantia* extracts. In addition, the effects of raw or cooked *Momordica charantia* extracts in modifying the mutagenic activity of mutagen obtained by the reaction between 1-aminopyrene and sodium nitrite and in modifying the reaction between 1-aminopyrene and sodium nitrite using the Ames test in the absence of metabolic activation were also determined.

1.3 Benefits of the study

This study provided information on the mutagenic activity and mutagenicity modification effect on standard mutagen of raw or cooked *Momordica charantia* extracts, which may be useful as a guidance for selecting a suitable method of cooking bitter gourd.

CHAPTER II

LITERATURE REVIEW

2.1 Bitter gourd

Momordica charantia is a plant in the Cucurbitaceae family and can be found in tropical areas such as China, India, Vietnam and Indonesia[1, 2]. In 2016, Ministry of Agriculture and Cooperatives, Thailand[27] reported that the large number of bitter gourd cultivation areas are Pathumthani, Ratchaburi, Surat Thani, and Phichit provinces.

The botanical characteristics of bitter gourd is a climbing herb with tendrils. Leaves are simple alternate, palmate with 5-7 lobes, 5-8 cm wide and 10-12 cm long. Flowers are solitary in leaf-axil, unisexual to monoecious, yellow and rotate-campanulate. Fruit is bitter, oblong-fusiform-cylindrical, coarsely ridged and irregular warty, green when young and yellowish orange when ripe[3, 4] (Figure 1).

2.1.1 Edible plant parts and uses

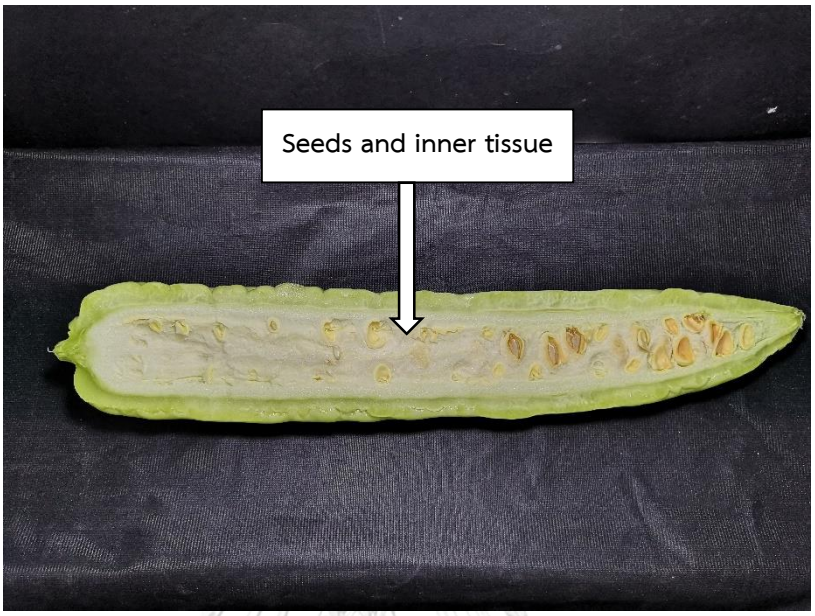
The young tender leafy shoots and green fruits of the bitter gourd are popular parts for consumption. The seed and inner tissue in the fruit will be discarded because it may cause nausea[4] (Figure 2). The bitter taste of bitter gourd could be reduced before cooking by boiling, soaking in saline or squeezing with salt[1, 9, 28].

Figure 1 The bitter gourd fruit

In China, bitter gourd is cooked by stir frying or boiling technique, and the dried sliced bitter gourds are consumed as herbal tea[4]. In Vietnam, the raw bitter gourd is eaten with dried meat floss and also cooked with eggs in soups[4]. In Japan, bitter gourd is cooked in various foods such as dumplings, omelets or soups. Moreover, bitter gourd is also produced as a fermented tea[4]. The bitter gourd dishes in the Philippines are cooked by roasting and eaten with salt and meat. The bitter gourd salad is also consumed with cooked or smoked fish[4]. In Indonesia, the bitter gourd is cooked with coconut milk[4].

In Thailand, the bitter gourd has been eaten raw and cooked such as chicken bitter gourd noodles, stuffed bitter melon soup, stir fried bitter gourd with eggs, bitter gourd salad with prawns, and red curry bitter gourd with catfish[4, 5, 7, 29].

A) With seeds and inner tissues



B) Without seeds and inner tissues

Figure 2 Longitudinal section of bitter gourd fruit

2.1.2 Nutrients and phytochemicals

The water is a main component in bitter gourd. It is also a source of nutrients such as carbohydrate, fiber, vitamin C, vitamin B, vitamin A, β-carotene, potassium, phosphorus, and calcium[30] (Tables 1 and 2).

Table 1 Nutrient composition of bitter gourd

Nutrient (Unit)	Value per 100 g
Water (g)	94.03
Energy (Kcal)	17
Protein (g)	1.0
Fat (g)	0.17
Ash (g)	1.10
Carbohydrate (g)	3.70
Fiber (g)	2.8

Modified from United States Department of Agriculture (USDA)[30]

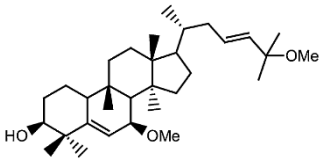
Table 2 Vitamins and minerals in bitter gourd

Vitamins and minerals	Value per 100 g
Vitamins	
Vitamin C (mg)	84.0
Thiamin (mg)	0.040
Riboflavin (mg)	0.040
Niacin (mg)	0.400
Pantothenic acid (mg)	0.212
Vitamin B 6 (mg)	0.043
Folate (total) (mg)	72
β-carotene (µg)	190
α-carotene (µg)	185
Vitamin A (IU)	471
Minerals	
Potassium (mg)	296
Phosphorus (mg)	31
Calcium (mg)	19
Magnesium (mg)	17
Sodium (mg)	5
Zinc (mg)	0.8
Iron (mg)	0.43
Manganese (mg)	0.089
Copper (mg)	0.034
Selenium (µg)	0.2

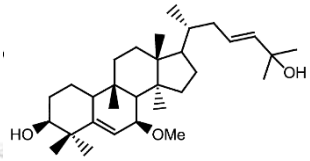
Modified from USDA[30]

Various kinds of phytochemicals were also found in bitter gourd such as pentacyclic triterpenes, cucurbitane-type triterpenoid, cucurbitane-type triterpene glycosides, cucurbitane-type triterpenoid saponins, phenolic acids, and flavonoids[4, 8, 9] (Figure 3).

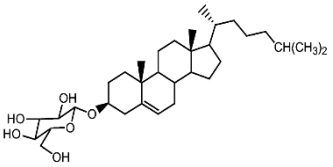
A) Cucurbitane-type triterpenoids



Karavilagenin A

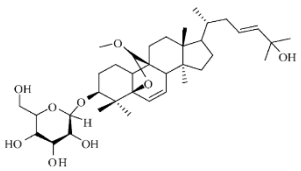


Karavilagenin B

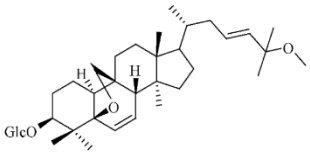


Charantin

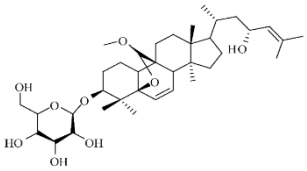
B) Cucurbitane-type triterpene glycosides



Goyaglycoside-b

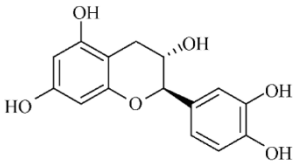


Momordicoside F1

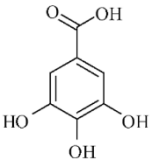


Momordicoside U

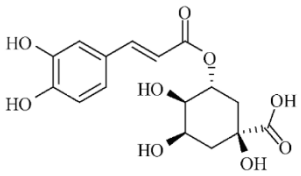
C) Phenolic acids and flavonoids



Catechin



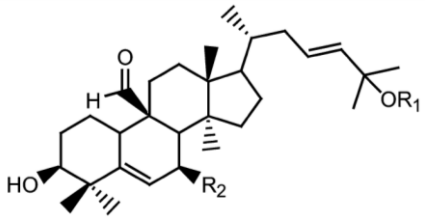
Gallic acid



Chlorogenic acid

Figure 3 Phytochemicals in bitter gourd

The bitter substances that are found in bitter gourd are momordicoside K and momordicoside L[9] (Figure 4).



Momordicoside K:

R_1 = Me, R_2 = β-D-glucopyranosyl

Momordicoside L:

R_1 = H, R_2 = β-D-glucopyranosyl

Figure 4 The bitter compounds in bitter gourd

2.1.3 Health benefit

The bitter gourd is a traditional medicinal herb. It is used as anthelmintic, antipyretic, anti-inflammatory, antimalarial, emetic, emmenagogue, and laxative[3, 8, 31]. Furthermore, the phytochemical substances found in the bitter gourd showed several biological activities such as antioxidant, antiviral, antidiabetic, antiobesity, and anticancer activities.

2.1.3.1 Antioxidant activity

The extracts of leaf, stem and fruit from *Momordica charantia* showed different levels of antioxidant activities from *in vitro* antioxidant assay. The leaf extract demonstrated the highest antioxidant activity with DPPH radical scavenging assay and ferric reducing antioxidant power assay. The fruit extract exhibited the highest antioxidant activity when tested with β-carotene-linoleate bleaching assay and total antioxidant capacity. The most abundant phenolic compounds were gallic acid followed by caffeic acid and catechins[32].

In 2010, Tripathi et al.[33] tested the hypoglycemic effect and antioxidant activity of *Momordica charantia* extract in alloxan-induced diabetic rats. They found that the extracts reduced blood sugar levels and enhanced the antioxidant activity of superoxide dismutase (SOD), catalase (CAT) and glutathione-s-transferase (GST) enzymes in the kidney, cardiac and liver tissues. Moreover, Horax et al.[34] reported that the compounds of catechin, gallic acid, gentisic acid, chlorogenic acid, and epicatechin were the major constituents of the phenolic compounds in the ethanol extracts of the bitter gourd. These compounds are natural antioxidants and can be used as an antioxidant in food products.

Subramaniam et al.[35] tested the antioxidant activity and phenolic content of bitter gourd with different cooking methods for different periods of time. The results showed that boiling, and microwave cooking increased antioxidant activity when cooked for 5 min, and these properties decreased when cooking time increased.

2.1.3.2 Antiviral activity

The bitter gourd exhibited antiviral activity in *in vitro* testing with multiple viruses, for instance, Epstein-Barr, herpes, and HIV virus. Proteins isolated from bitter gourd seed extracts inhibited replication of herpes simplex, type (HSV-1) and poliovirus 1[36]. MAP 30 (*Momordica* anti-HIV, 30 kDa protein) was a substance that inhibited the human immunodeficiency virus (HIV). It was isolated and purified from seeds and fruits of bitter gourd[11]. Moreover, MAP 30 was a type I ribosome

inactivating protein that showed anti-cancer activity and anti-HIV activity. It bound to ribosomal RNA and HIV-1 long-terminal repeat DNA[37].

MAP 30 inhibited HIV-1 infection in T lymphocytes and monocytes, including replication of the virus in infected cells; however, MAP30 was not toxic to non-infected cells due to inability to enter normal cells[38]. In addition, MAP30 was more effective in treating herpes simplex virus (HSV) and HIV than other ribosome inactivating protein[39].

2.1.3.3 Antidiabetic activity

The bitter gourd is used as a traditional medicine for the treatment of diabetes in Asia, Africa and South America. Indian traditional medicine (Ayurveda) recommends the use of *Momordica charantia* as a supplement to prevent diabetes and related complications[40]. The bitter gourd contains substances that have properties in the treatment of diabetes, for example, charantin, vicine, polypeptide-p including other biological components such as antioxidants[41].

The antidiabetic effect of *Momordica charantia* water extract was evaluated in KK-Ay mice with type 2 diabetes and hyperinsulinemia. After 3 weeks of treatment, the extract was able to decrease serum insulin levels of KK-Ay mice. However, the normal mice receiving this extract showed no change in blood sugar levels[42]. In addition, Virdi et al.[43] found that the aqueous extract powder of raw fruit at a dose of 20 mg/kg body weight reduced blood sugar levels by 48%. These effects were similar to the effect of glibenclamide.

The juice and the alcohol extract of *Momordica charantia* showed the decreasing effect on blood sugar levels in normal and diabetic rats. Both extracts did not affect the levels of urea, creatinine, AST (aspartate aminotransferase), ALT (alanine transaminase), and AP (alkaline phosphatase) in normal rats, but significantly reduced the levels of those parameters, triglyceride, and cholesterol in diabetic rats[44]. Moreover, Baldwa et al.[45] found that the bitter gourd extract could reduce blood sugar levels in diabetic patients in clinical trials and reported no allergic reactions to this extract.

2.1.3.4 Antiobesity activity

Chen et al.[46] found that diet supplemented with freeze dried bitter gourd juice reduced visceral fat in rats fed high fat (HF; 30%) diet. In addition, the juice of bitter gourd also had an antiobesity effect. It was found that the values of visceral fat mass, serum glucose, and insulin resistance index in supplemented rats were lower than those without supplementation[47].

In 2005, Chen et al.[16] reported that after feeding a high fat diet supplemented with freeze dried bitter gourd juice for 4 weeks, rats had a slight increase in weight and decrease in visceral fat and hepatic triacylglycerol. Moreover, the bitter gourd juice also inhibited lipogenesis and stimulated lipolysis in human adipocytes[48].

2.1.3.5 Anticancer activity

Several studies showed that the *Momordica charantia* extracts were effective in the inhibition of various cancers, including lymphoid leukemia, lymphoma, colorectal cancer, skin cancer, breast cancer, skin tumors, prostate cancer, squamous cell carcinoma of the tongue and larynx, human bladder cancer, and Hodgkin's disease[49]. The crude extract of *Momordica charantia* inhibited cyclase guanylate activity from various tissues and prevented the growth of concanavalin A-stimulated rat splenic lymphocytes[50]. The crude extract eliminated human leukemic lymphocytes in a dose dependent manner[51]. The crude extract which was resistant to heat exhibited cytostatic and cytotoxic effects and also showed antiviral activity[52]. In addition, the bitter gourd ethanol extract was found to inhibit growth and caused apoptosis in HL60 human leukemia cells[53].

Kwatra et al.[18] studied the effect of gourd extract on resistant doxorubicin (DOX) intestinal cancer cells (HT-29). It was found that the extract used together with DOX inhibited the number of cell growth in a dose dependent manner. The 25 μg/ml of the extracts were tested in HT-29, and then DOX was added after 24 h. It was found that the extracts increased the sensitivity of DOX response. The bitter gourd juice extract was found to reduce the number of living AsPC-1 pancreatic cancer cells resistant gemcitabine (GR AsPC-1) and increase the number of dead cells. The extract treated cells showed a significant increase in the necrotic cell death in the GR AsPC-1 cells[19].

Momorcharaside A, isolated from *Momordica charantia* seed, inhibited DNA and RNA syntheses in S 180 cancer cells[54]. α-momorcharin (α-MMC) was a ribosome inactivating protein. There was cytotoxicity in tumor cells. However, It showed that plasma was short half-life in clinical application and could be solved by PEGylation of α-MMC[55].

The bitter gourd extract which was tested against human breast cancer cells, MCF-7 and MDA-MB-231, and primary human mammary epithelial cells was found to reduced cell proliferation and promote apoptosis[56]. In addition, MAP30 was tested on MDA-MB-231 breast cancer cells. The results showed that MAP30 inhibited proliferation and HER2 gene expression[57]. Fan et al.[58] also demonstrated that MAP30 significantly inhibited the proliferation of human colorectal carcinomar LoVo cells. These inhibitions depended on the time and dose of MAP30.

2.2 Cooking methods

Foods are composed of protein, fat, carbohydrates, and water, as well as other compounds such as minerals, vitamins, colorants, and flavors[59]. With cooking, the physical and chemical properties of the food, such as color, smell, texture, and taste often change[60-62]. There are 2 types of cooking methods [59, 63, 64].

1. Moist-heat methods

The heat is transferred to the food by using moisture in the food, water or vapor. This cooking method is found in blanching, steaming and boiling.

2. Dry-heat methods

Cooking with heat, free from moisture, may use air, metal surface of the cooking utensils, radiation or oils (fats) to transfer heat to the food. There are two categories to cook with dry heat method: using and not using fat. The dry heat cooking method with fat finds in sauté, pan fry, stir fry, or deep fry. The grilling, broiling, baking, or roasting are the dry heat cooking method without fat.

The details of the cooking method are shown in Table 3.

Table 3 Cooking method

Cooking method	Definition
Blanching	Cooking food partially and very quickly in boiling water, hot oil or hot fat
Steaming	Cooking food with direct contact with steam
Boiling	Cooking food in the bubbling water rapidly, with the maximum possible temperature of boiling water being a boiling point of 100 °C at sea level
Grilling	Cooking food on grid that are open over heat sources such as charcoal, electric stoves, or gas stoves
Broiling	Cooking food with the radiant heat from the top
Roasting	Cooking food by using dry heating, which uses equipment such as ovens
Sauté	Cooking food using a small amount of oil or fat in a shallow pan with relatively high heat
Pan frying	Cooking food using a moderate amount of oil or fat in a pan with moderate heat
Stir frying	Cooking food in a frying pan with a small amount of fat, setting the fire and turning it over until cooked
Deep frying	Cooking food that is submerged in enough hot oil or fat

Modified from McGee[59] and Gisslen[63]

2.3 Mutagens and antimutagens in food

Consumption of foods contaminated with toxic substances or mutagen may increase the risk of cancer and may be harmful to human health[65]. Contamination of toxic substances can occur at all stages of food production process. For example, raw materials may be contaminated with pesticides used in agriculture[66] or mycotoxins generated from fungi[67].

In addition to the contaminants in the food mentioned above, there are also contamination during cooking, for instance, acrylamide occurs when carbohydrate-rich foods are cooked at high temperatures such as baking or frying. It is derived from heat induced the reactions between the asparagine amino acid and the carbonyl group of reducing sugar. Heterocyclic amines are formed by the Maillard reaction between creatine or creatinine, amino acids and sugars. It is found in meat or fish that is cooked with temperatures above 200 °C. Polycyclic aromatic hydrocarbons (PAHs) are produced by grilling or broiling meat, fish or other food directly on the flame, and then the fat droplets fall on the heat source to produce flame with PAHs. The resulting PAHs are adhered to the surface of the food. Moreover, when the heat temperature is higher, the amount of PAHs that are produced will increase[68].

Foods that are contaminated with mutagen are responsible for the damage of DNA. Most mutagens cause mutations at the DNA level by the formation of carcinogens DNA-adducts. This is due to the formation of covalent bonds of carcinogens or some of the carcinogens to the nucleotides, resulting in genetic

alterations[69]. Foods are also a source of antimutagens, such as vitamin C, vitamin E, flavonoids, phenolic compounds and carotenoids, most commonly found in fruits and vegetables[70]. These antimutagens can be used to prevent transformation of non-mutagenic compounds into mutagen or to inhibit the interaction between mutagen and DNA. Antimutagens can be divided into desmutagens which are substances that inhibit the mutagen by chemical or enzymatic processes before they react with DNA and bioantimutagens are substances that inhibits the mutation of the damaged gene from mutagens in the repair and replication process of DNA[70-72]:

The mechanisms of action of antimutagens that can prevent mutations are shown in the Table 4.

Table 4 Mechanisms of action of antimutagens

Mechanism	Compounds/chemicals
1. Extracellular mechanisms	
1.1 Inhibition of mutagen uptake	Dietary fibres, probiotics
1.2 Inhibition of endogenous formation	Vitamin C, Vitamin E, phenolic compounds
1.3 Deactivation and/or complexation	Dietary fibres, chlorophyllin
1.4 Favoring absorption of protective agents	Vitamin D3 and analogues
2. Cellular mechanisms	
2.1 Competition or blocking	
- Scavenging of reactive oxygen species	Vitamins C, vitamin E, polyphenols, anthocyanins, diterpenes
- Protection of DNA nucleophilic sites	Ellagic acid, polyamines, retinoids
2.2 Stimulation of trapping and detoxification in non-target cells	*N*-Acetyl cysteine
2.3 Modification of transmembrane transport	Short chain fatty acids, acylglycosylsterols, dietary calcium
2.4 Modulation of xenobiotic metabolising enzymes	Isothiocyanates, monocyclic monoterpenoids, retinoids, flavonoids, wheat bran, polyphenols, diterpene esters
2.5 Enhancement of cell apoptosis	Retinoids, flavonoids, butyric acid

Modified from De Flora S[73]

2.4 Nitrite and gastric cancer

In 2018, the World Health Organization[21] reported a 9.6 million deaths from cancer and found that gastric cancer, also called stomach cancer, was one of the top five causes of death. Gastric cancer is also a major contributor to the disability-adjusted life-years (DALYs) burden of cancer in males, accounting for 20% of the total DALYs worldwide, following liver and lung cancer[74]. The burden of gastric cancer remains very high in Asia, Latin American and Central and Eastern European countries. The risks of gastric cancer are life style, genetics, medical conditions, *Helicobacter pylori* infection, and diet[75]. Consumption of some food including pickled, smoked, salted, and processed foods may increase the risk of gastric cancer[76].

Processed foods that use nitrate and nitrite in food preservation, have the potential for the formation of *N*-nitroso compounds such as nitrosamines and nitrosamides. This process occurs when the body absorbs nitrates in the stomach and then stores at the salivary glands in the mouth. Oral bacteria convert nitrates into nitrites. When nitrite enters the stomach, the nitrosation reaction occurs under the acidic condition to convert amines or amides to *N*-nitroso compounds. Thus, long-term exposure of these chemicals may lead to gastric cancer[22, 23, 77-80].

Numerous epidemiological studies have investigated the food intake and gastric cancer risk. Ward et al.[81] conducted a case-control study in patients aged 20 years and older in Mexico. The risk of gastric cancer increased with frequent

consumption of processed meat (OR = 3.2; 95% CI = 1.5-6.6). The prospective cohort study in men and women aged 55-65 years was performed by van den Brandt et al.[82]. Eating processed meat such as bacon was positively associated with a risk of gastric cancer (RR = 1.33; 95% CI= 1.03-1.71). Rao et al.[83]carried out a case-control study for adults aged 30-75 years in India. Those who ate processed fish at least once a week had a 4-fold greater risk of gastric cancer than those who did not eat or who ate at least once in 2 weeks (OR = 4.6; 95% CI = 3.1- 6.8). Mayne et al.[84] reported a case-control study in men and women aged between 30 and 79. The high nitrite intake was associated with a risk of noncardia gastric cancer (OR = 1.64; 95% CI = 1.30-2.07). In 2011, Loh et al.[85] conducted a prospective study in men and women aged 40-79 years. Eating food contaminated with *N*-nitroso compounds was associated with gastrointestinal cancer in men (HR = 1.24; 95% CI = 1.07-1.44)

2.5 The Ames test

Mutation test is the first step in detecting whether substances such as pesticides, supplements, or medical substances are mutagenic. The most widely used mutagenicity test is the Ames test which is conducted in *Salmonella typhimurium*. This method is a short-term mutation test to detect various substances that can damage the DNA and lead to the mutation of the genes[86, 87].

The strains of the *Salmonella typhimurium* used in the experiments have mutated genes that control the production of histidine amino acids. Therefore, the bacteria cannot grow in non-histidine amino acids medium and is called Histidine-

dependent (His^-) strain. When the *Salmonella typhimurium* (His^-) strain is tested with mutagens, the bacteria will be mutated, and the histidine amino acids will be made up. This mutation is called reverse mutation. Each strain of *Salmonella typhimurium* (His^-) has different histidine operon mutations, such as *hisD6610, hisD3052, hisG46, hisG428 and hisC3076*[25, 86, 88]. In addition to these properties, the *Salmonella typhimurium* bacteria also possesses other properties, including *rfa* mutation, R-factor, and *uvrB* mutation, that allow the bacteria be sensitive to chemical mutagenicity tests[25, 88]. The *rfa* mutation causes the bacteria to lose of lipopolysaccharide on the cell wall. As a result, the large molecule mutants, such as benzo[a]pyrene can penetrate the cell wall. The R-factor is the addition of pKM101 plasmid into the bacteria cell. The repairing system was abnormal and ineffective. The sensitivity of bacteria to mutagens is increased. The *uvrB* mutation is the deletion of the DNA repair system, causing the bacteria to lose the ability to repair abnormal DNA and to increase the sensitivity of the bacterial response to the mutants. In addition, *uvrB* mutation results in the loss of biotin-producing genes. Thus, the bacteria require biotin for growth.

The characteristics of *Salmonella typhimurium* tester strains are shown in Table 5.

Table 5 The characteristics of Salmonella typhimurium tester strains

Bacterial strain	Histidine mutation	Type of mutation	*rfa* mutation	R-factor	*uvrB* mutation
TA97	*hisD6610*	Frameshift	✓	✓	✓
TA98	*hisD3052*	Frameshift	✓	✓	✓
TA100	*hisG46*	Base pair substitution	✓	✓	✓
TA102	*hisG428*	Base pair substitution	✓	✓	✗
TA104	*hisG428*	Base pair substitution	✓	✗	✓
TA1535	*hisG46*	Base pair substitution	✓	✗	✓
TA1537	*hisC3076*	Frameshift	✓	✗	✓

Modified from Maron et al.[88] and Kuma et al.[87]

2.5.1 The plate incorporation and pre-incubation method

The plate incorporation test is applied with the mutagen and His^- bacteria combined with top agar (45 °C). The mixture are poured onto agar plate then incubated at 37 °C for 48 h. The histidine independent (His^+) colonies are counted. The positive and negative control are included in each test[89]. (Figure 5)

Some mutagens, such as dimethylnitrosamine, pyrrolizidine alkaloids and diethylnitrosamine are poorly detected when using plate incorporation method. Yahagi et al.[90] introduced a modified method to make the test more sensitive. The method is called pre-incubation method. In the procedure of this method, the mixture of mutagen and His^- bacteria is incubated at 37 °C for 20 min. Then the top agar (45 °C) is added, and the mixture is poured onto agar plate. The plate is incubated at 37 °C for 48 h. The His^+ colonies are counted.

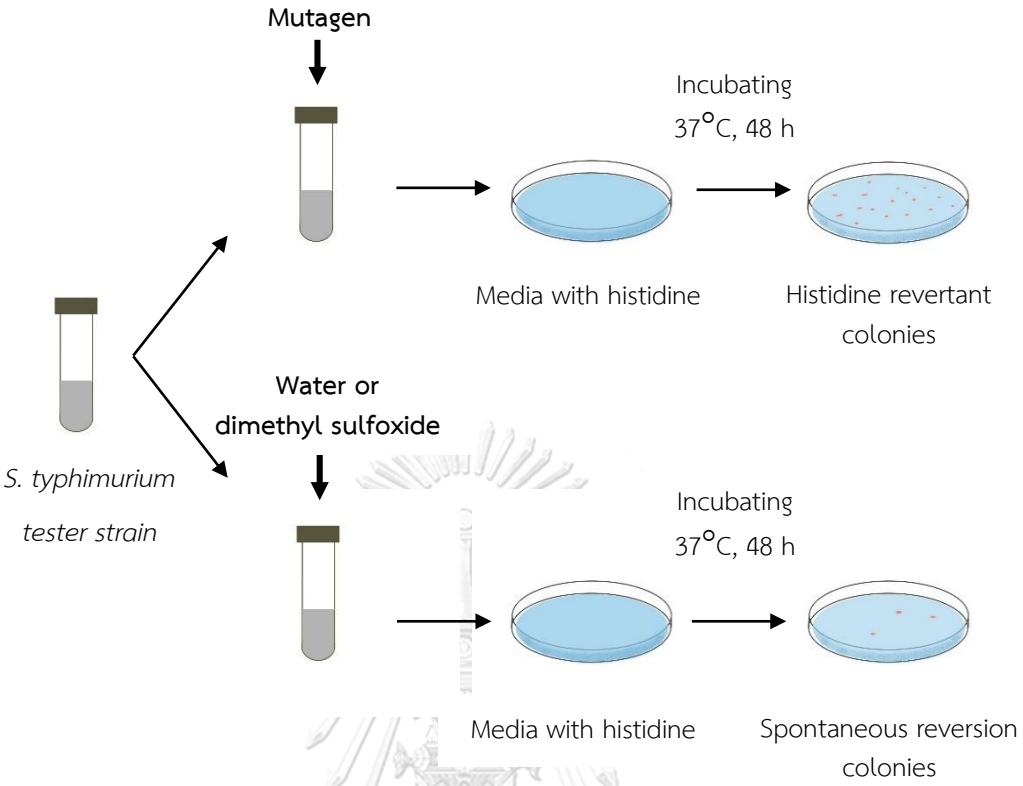


Figure 5 The plate incorporation method of the Ames test

From the previous studies, the studies of the mutagenic effects of 10 nitrosamines[91] and several carcinogenic alkaloids[92] were carried out using pre-incubation method. Matsushima et al.[93] also compared the sensitivity of the plate incorporation and pre-incubation method with aflatoxin B1, benzidine, benzo[a]pyrene, methylnitrosourea, and methylmethanesulfonate. The results demonstrated that the sensitivity of the pre-incubation method was equal or greater than that of plate incorporation method. Therefore, pre-incubation method can be used routinely or used when the results of the plate incorporation method are not clear. De Serres et al.[94] had recommended this method to detect mutagenic effects of any substance.

2.5.2 1-Aminopyrene reacted with nitrite mutagenicity model

1-nitropyrene is a nitro-polycyclic aromatic hydrocarbons (nitro-PAH) that occurs in incomplete combustion found from diesel exhaust, exhaust gas, and some of the food products[95-97]. The inhalation is one of the channels where 1-nitropyrene is able to enter the body[98]. 1-nitropyrene is metabolized by bacteria, to form 1-aminopyrene in the digestive tract[99]. 1-Aminopyrene is a substance that does not cause mutation. When it reacted with sodium nitrite at pH 3.0 at 37 °C, the mutagenic activity of *Salmonella typhimurium* strains TA98 and TA100 was increased[100]. In addition, Kangsadalampai et al.[101] reported that 1-aminopyrene reacted with sodium nitrite in acidic condition was considered to be direct-acting mutagen. Thus, the mutagenicity of the reaction between 1-aminopyrene and sodium nitrite in acidic condition is considered as model in the study of mutagenesis of certain chemicals that occur during gastric digestion.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

1-Aminopyrene (Sigma-Aldrich, St. Louis, USA) and sodium nitrite (Sigma-Aldrich, St. Louis, USA) were used to produce a standard direct mutagen of the Ames test. Ammonium sulfamate, biotin and histidine were purchased from Sigma-Aldrich (St. Louis, USA). Agar-agar was acquired from Merk (Darmstadt, Germany). Oxoid nutrient broth No.2 was a product of Oxoid Ltd. (Hampshire, England). Other chemicals were of analytical grade.

3.1.2 Bacterial strains

The strains of the *Salmonella typhimurium* (His^-) used in this study were TA98 and TA100, which were able to detect frameshift mutation and base-pair substitution, respectively. Both strains were provided by Assoc. Prof. Dr. Kaew Kangsadalampai. The overnight culture of each strain inoculated from frozen stock culture in Oxoid nutrient broth No.2 at 37 °C for 16 h was used for mutagenicity assay within 24 h.

3.1.3 Nutrient agars

Preparation of minimal glucose agar plate

Minimal agar containing 1.5% agar-agar was autoclaved (Model HG-80, Hirayama, Japan) and then mixed with 2% sterile glucose and Vogel-Bonner medium E stock salt solution (VB salts) (Appendix). Approximately 30 ml of molten agar was poured onto a sterile Petri dish. It was left to solidify and stored at 37 °C in the incubator (Model INE 500, Memmert, Germany).

Preparation of top agar

Top agar containing 0.6% agar-agar and 0.5% sodium chloride was autoclaved. A sterile solution 10 ml of 0.5 mM histidine/biotin was added to each 100 ml of the top agar, and it was maintained in a water bath at 45 °C (Model HS1, CTL, Thailand).

3.1.4 Sample

Fresh fruits of *Momordica charantia* were purchased from local markets in Nakhon Pathom, Thailand during January to August, 2018.

3.2 Experimental design

The mutagenic activities and the mutagenic modifying activity of *Momordica charantia* extracts on sodium nitrite - treated 1-aminopyrene were determined. Experimental design is shown in Figure 6.

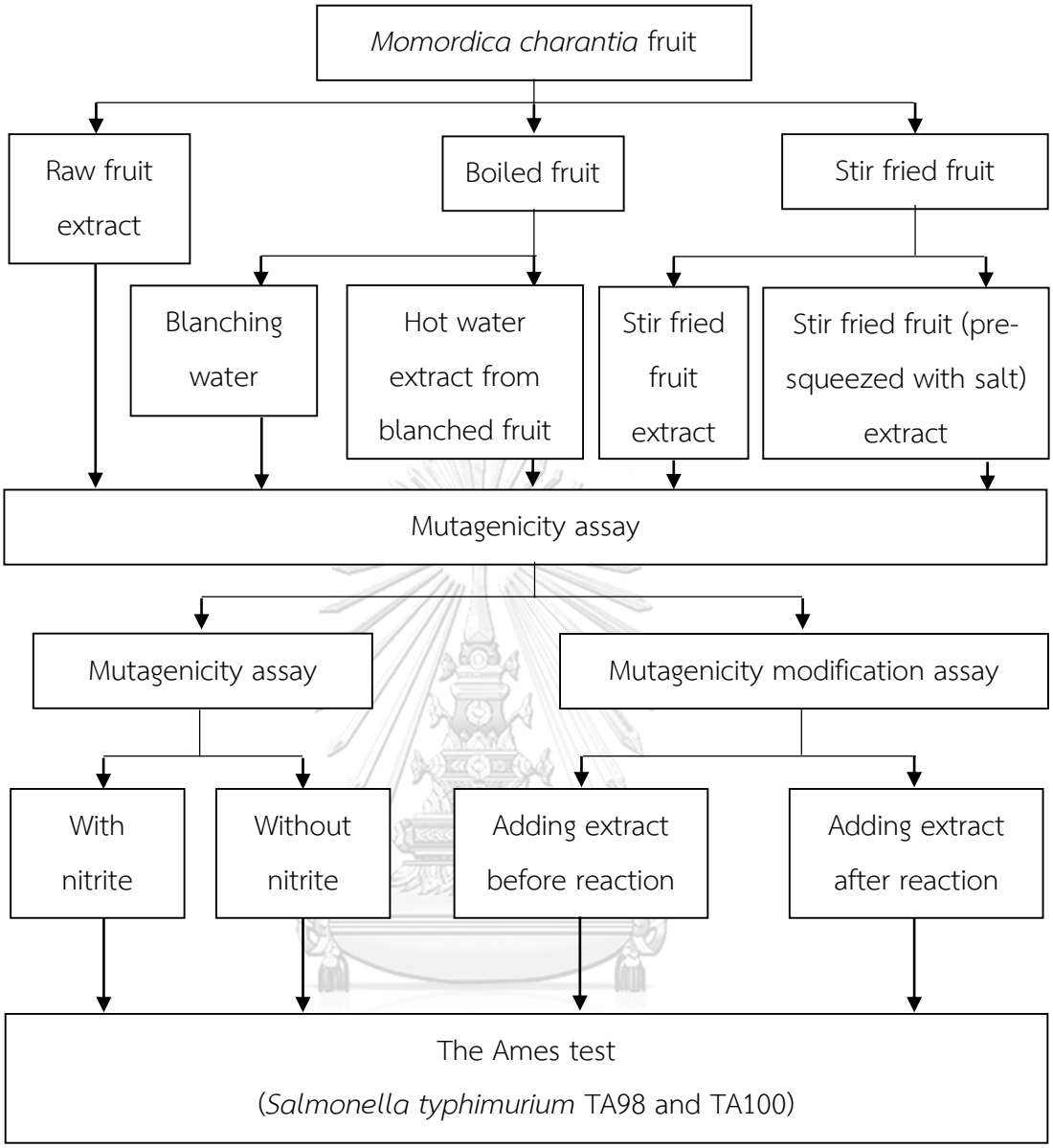


Figure 6 Experimental design

The raw or cooked bitter gourd extract was prepared by boiling or stir frying. The mutagenic activities of these extracts were assayed, either reacted or not reacted with sodium nitrite, using the Ames test in the absence of metabolic activation. Each extract was added to the 1-aminopyrene-sodium nitrite model, before and after the reaction process, and assayed using the Ames test in the absence of metabolic activation. These processes were conducted to find out whether the extracts from raw or cooked bitter gourd had any modification effect on the reaction between 1-aminopyrene and sodium nitrite, and also on the mutagenic product obtained from this reaction.

3.3 Sample preparations

Each sample preparation was done according to the home cooking method[5-7]. The bitter gourd fruit was cleaned with tap water and dried with paper towel. The cleaned fruit was cut in half to remove the seeds and the inner tissues and then chopped into small pieces. Several cooking methods were performed as followed.

3.3.1 Raw (RW)

The fruit sample (50 g) was blended (Model T-25, Ultra-turrax, Germany) with distilled ultrapure water 50 ml.

3.3.2 Boiling

The fruit sample (50 g) was blanched (Model HS1, CTL, Thailand) with 50 ml of distilled ultrapure water at 95 °C for 5 min. After that, the blanching water and

fruit were separated. The water sample was collected for use in the next step *(blanching water (BW))*. The blanched fruit was boiled again with 50 ml of distilled ultrapure water at 95 °C for 5 min, and it was blended together *(hot water extract from blanched fruit (HB))*.

3.3.3 Stir frying

Stir fried fruit (SF)

The fruit sample (50 g) was heated at 140 °C in a beaker placed in oil bath (Model FRI-1265, Fritlel, Belgium) for 5 min, and then it was blended with 50 ml of distilled ultrapure water. This heat treatment process is a simulation of stir fry cooking methods[102].

Stir fried fruit (pre-squeezed with salt) (SS)

The fruit sample (50 g) was squeezed with 8.16 %w/w table salt[5] and rinsed 3 times with distilled ultrapure water. After that, the sample was heated at 140 °C in a beaker placed in oil bath for 5 min, and then it was blended together with 50 ml of distilled ultrapure water.

Each fraction of sample (RW, BW, HB, SF or SS) was filtered separately through filter paper (Grade 1, Whatman, Germany) and centrifuged at 4000 rpm for 30 min (Model Rotofix 32, Hettich, Germany). The supernatant was filtered, and the filtrate was collected in the light protected container at 4 – 8 °C. Each extract was filtered

using a sterile 0.20 micron membrane filter (Type 25007, Sartorius AG, Germany) before use.

3.4 1-aminopyrene-sodium nitrite model

1-amoniopyrene treated with sodium nitrite in an acidic condition was used as a standard and positive mutagenic agent. Briefly, 1-aminopyrene 10 µl and 40 µl (0.0375 mg/ml) were used for testing on *Salmonella typhimurium* strains TA98 and TA100, respectively. 1-aminopyrene was added in a tube with a plastic cap and the pH was adjusted to 3.0 – 3.5 with 0.2 N hydrochloric acid. After mixing with 250 µl of 2 M sodium nitrite, the reaction tube was shaken in a water bath at 37 °C for 4 h and placed in an ice bath for 1 min to stop the reaction. The mixture was added with 250 µl of 2 M ammonium sulfamate in order to decompose the residue nitrite, and immersed in an ice bath for 10 min. The mutagenic activities of reaction product were tested using the Ames test with the modified pre-incubation method.

Pre-incubation method

The pre-incubation method recommended by Yahagi et al.[90] was used throughout this study. The reaction product (100 µl) was mixed with 500 µl of 0.5 M phosphate buffer (pH 7.4) in a tube with a plastic cap and added with 100 ml of overnight culture before being shaken in a water bath at 37 °C. After 20 min, 2 ml of molten top agar (45 °C) was added. The mixture was carefully poured onto a minimal glucose agar plate, and the plate was incubated at 37 °C for 48 h (Figure 7).

The number of histidine revertant colonies per plate was counted (Model ColonyStar 8505, Funke Gerber, Germany).

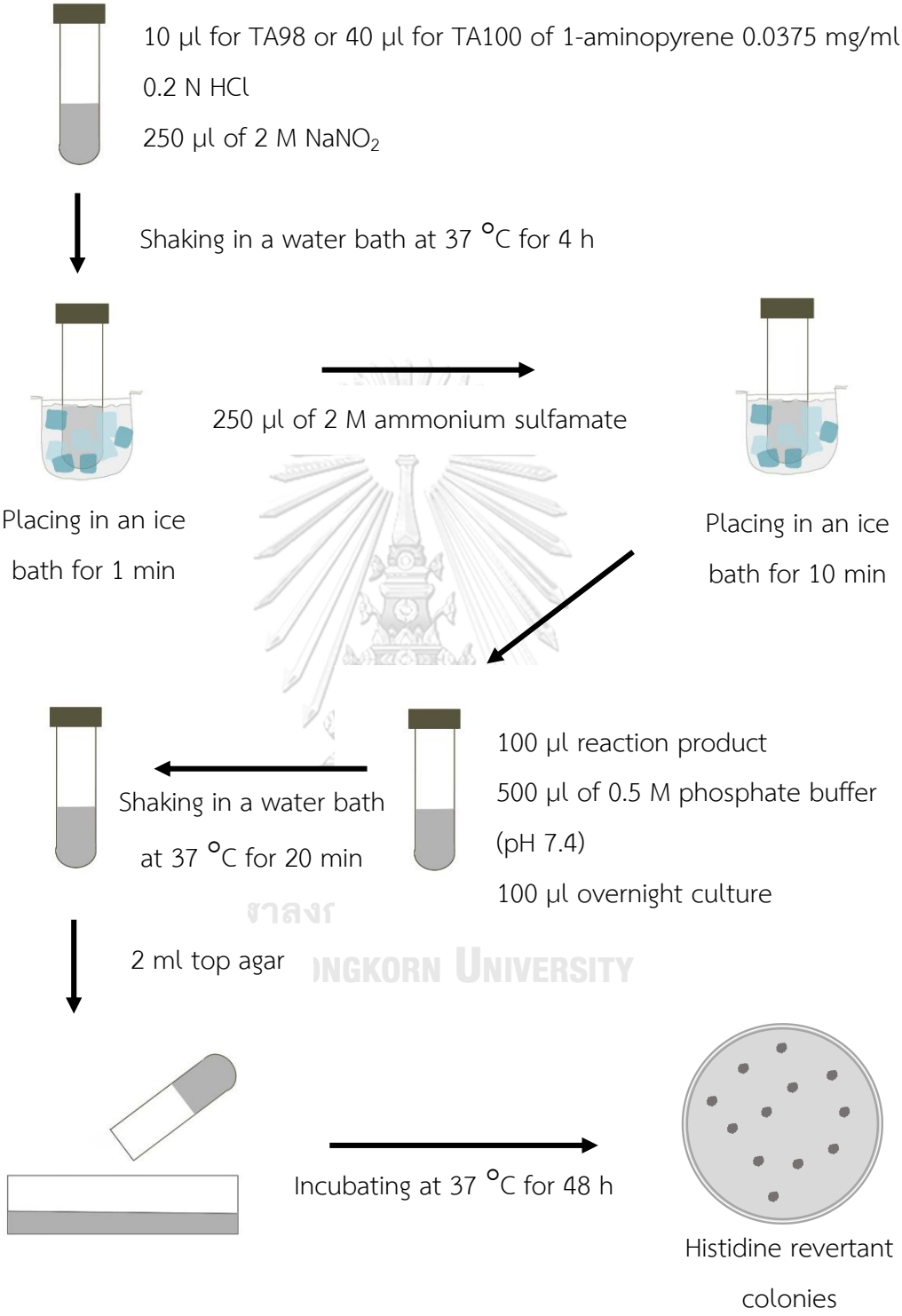


Figure 7 Experimental procedure for evaluating the mutagenic activities of 1-aminopyrene reacted with sodium nitrite using the Ames test (pre-incubation method)

3.5 Mutagenic activities of *Momordica charantia* extracts

3.5.1 With sodium nitrite treatment

The procedure was done as described in 3.4. The aliquot of each extract from 3.3 (25, 50 or 100 µl) was added instead of 1-aminopyrene (Figure 8).

3.5.2 Without sodium nitrite treatment

The procedure was done as described in 3.4. 1-aminopyrene was replaced by an aliquot of each extract from 3.3 (25, 50 or 100 µl), and the distilled ultrapure water was added instead of 2 M sodium nitrite and 2 M ammonium sulfamate (Figure 8).

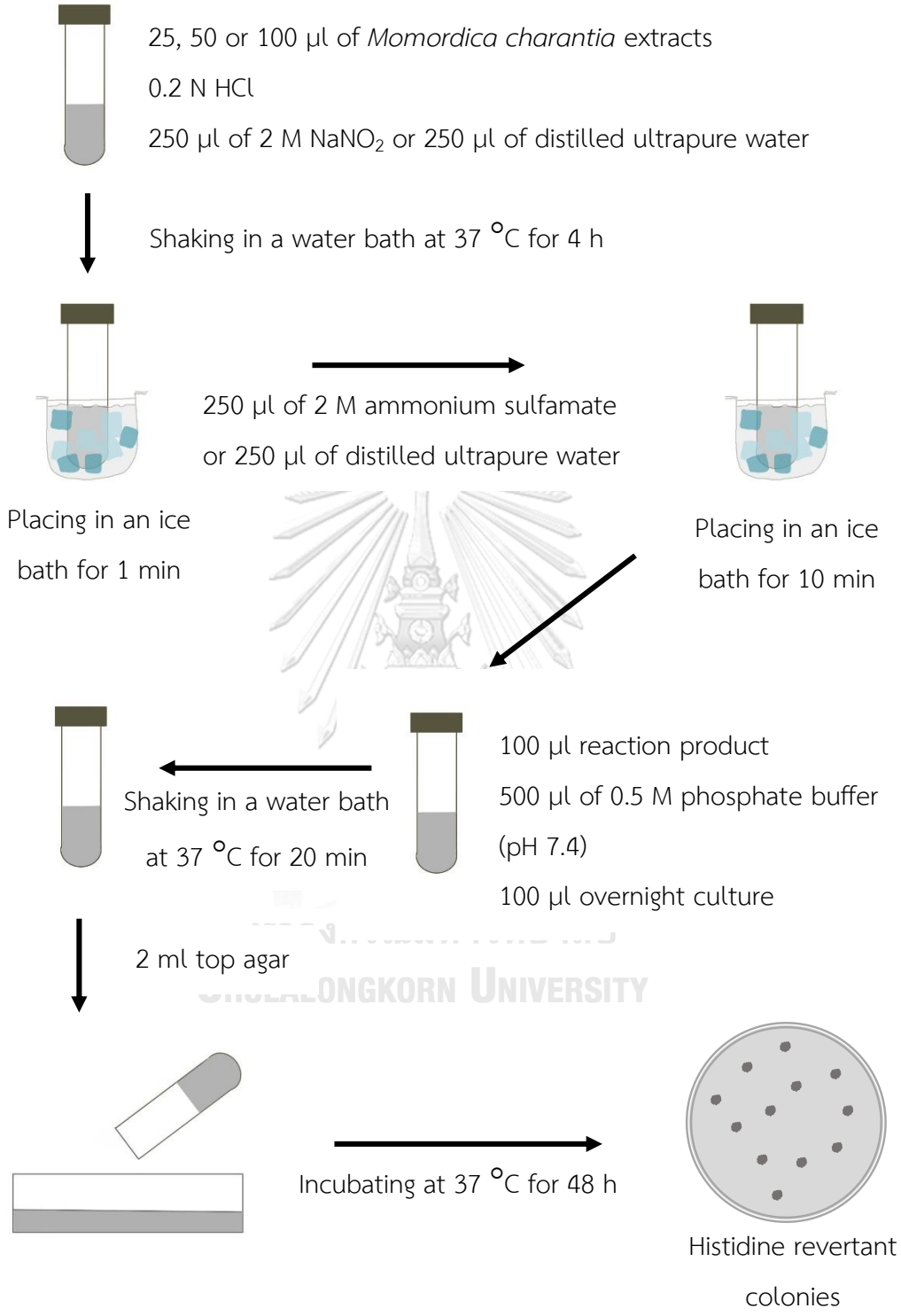


Figure 8 Experimental procedure for evaluating the mutagenic activities of *Momordica charantia* extracts with and without sodium nitrite using the Ames test (pre-incubation method)

3.6 Mutagenicity modification test

In this study, 1-aminopyrene reacted with sodium nitrite in an acidic condition was used as a standard mutagen. The mutagenicity modification effect of the extracts was performed using the Ames test in the absence of metabolic activation. There was divided into two conditions.

3.6.1 Adding the extract before starting the mutagen forming reaction (duration of presence = 4 h)

The procedure was done as described in 3.4. The aliquot of each extract from 3.3 (25, 50 or 100 µl) was added to a tube containing 1-aminopyrene and 0.2 N hydrochloric acid before mixing with 2 M sodium nitrite (Figure 9).

3.6.2 Adding the extract after completion of mutagen forming reaction (duration of presence = 0 h)

The procedure was done as described in 3.4. A tube containing the 4 h reaction mixture was mixed with the aliquot of each extract from 3.3 (25, 50 or 100 µl) before the pre-incubation process (Figure 10).

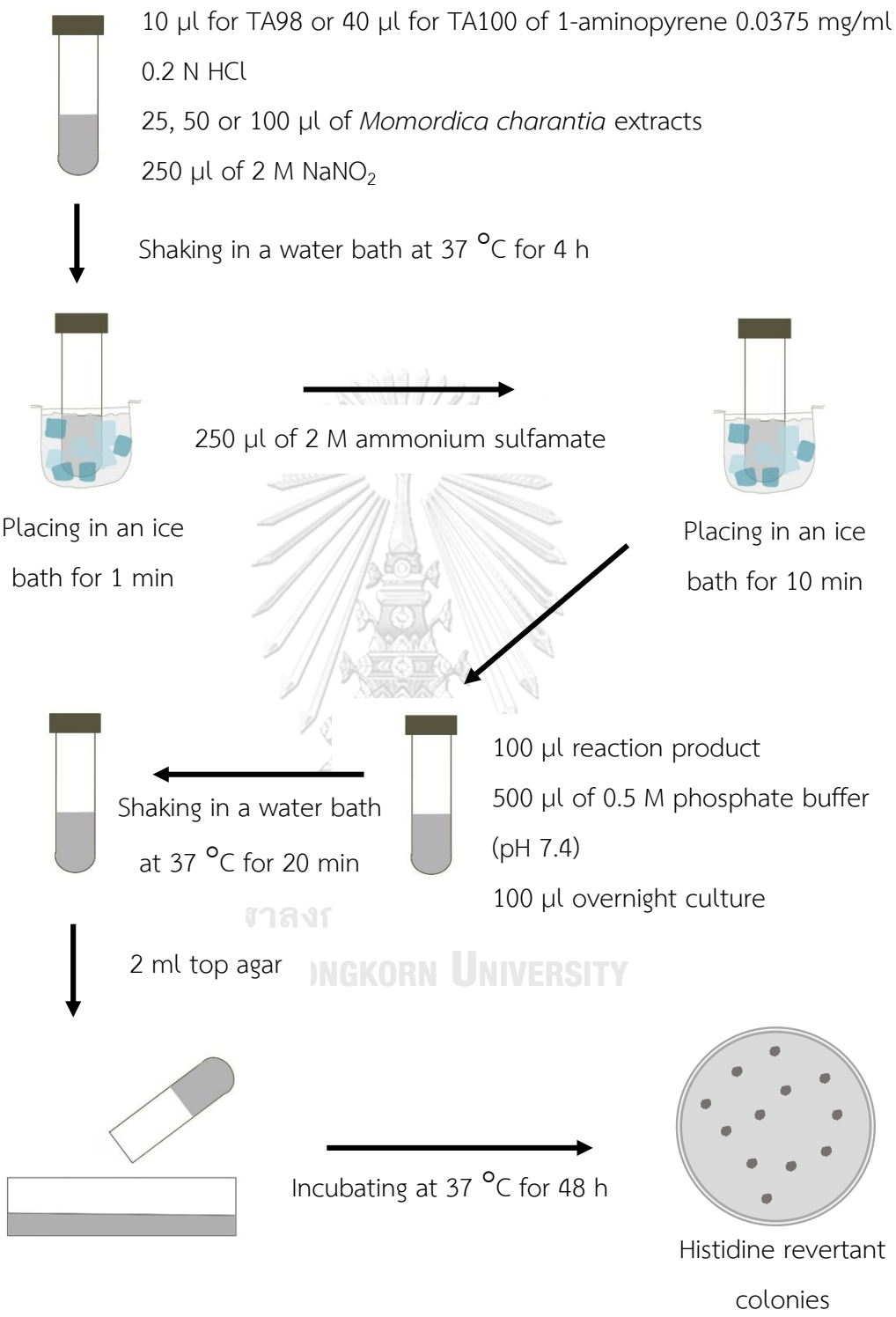


Figure 9 Experimental procedure for evaluating the mutagenic effect of *Momordica charantia* extracts before starting the mutagen forming reaction (duration of presence = 4 h) using the Ames test (pre-incubation method)

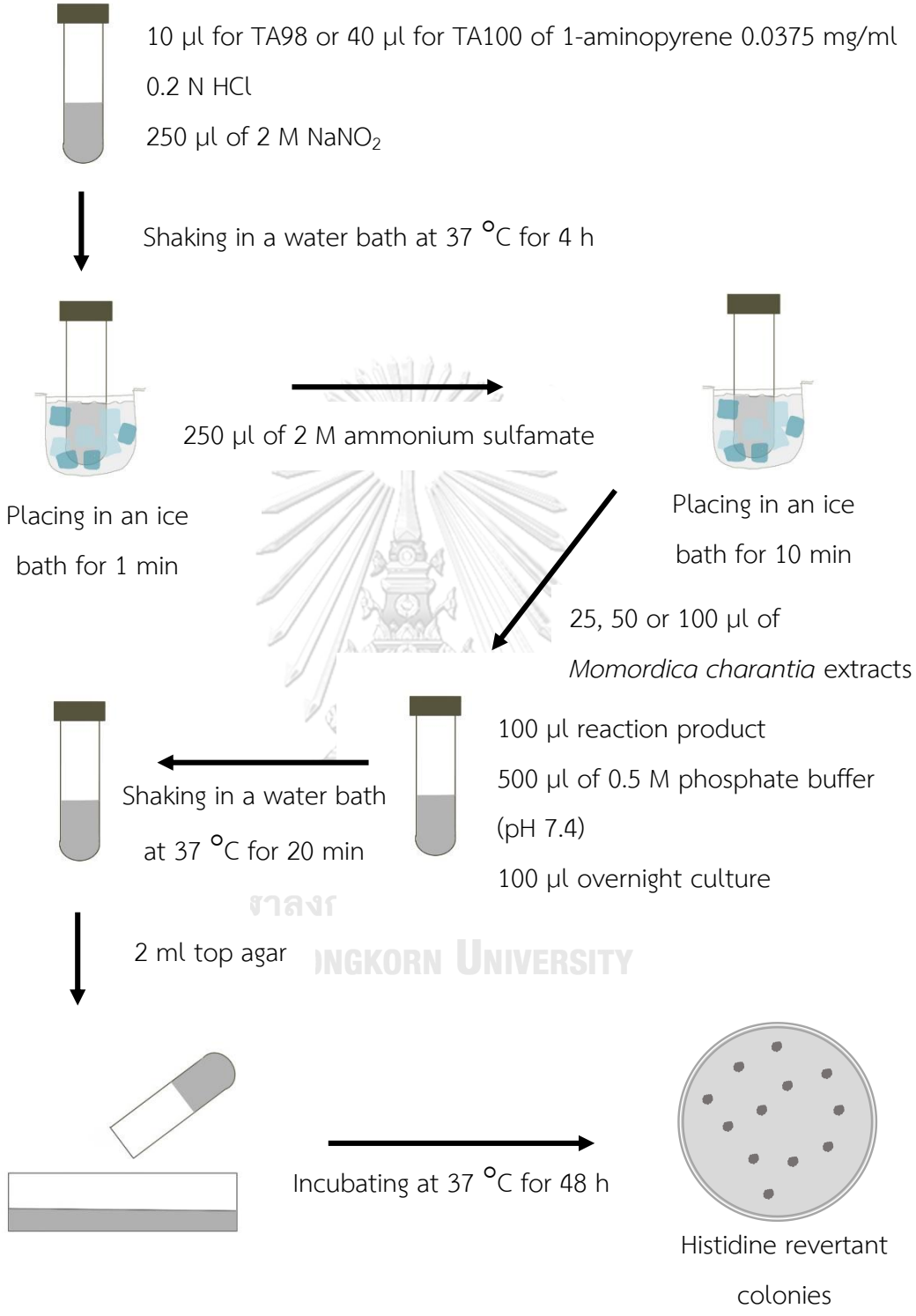


Figure 10 Experimental procedure for evaluating the mutagenic effect of *Momordica charantia* extracts after completion of mutagen forming reaction (duration of presence = 0 h) using the Ames test (pre-incubation method)

3.7 Data analysis and interpretation

The mutagenic activities of each sample was presented as a number of histidine revertant colonies/plate. The results were expressed as mean ± standard deviation (SD) of six plates from two different determinations. The samples were considered to be mutagenic if at least two different concentrations showed the number of histidine revertant colonies greater than that of spontaneous reversion with a dose-response relationship. In addition, at least one concentration exhibited the number of histidine revertant colonies higher than two times of the value obtained from spontaneous reversion[94].

To compare the degree of mutagenicity, the results were also presented as mutagenicity index (MI) which was calculated as following[103]:

$$MI = \text{average (N)} / \text{average (S)}$$

N = the number of histidine revertant colonies/plate of sample

S = the number of histidine revertant colonies/plate of spontaneous reversion

The percentage of modification was calculated as following:

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

A = the number of histidine revertant colonies/plate of sodium nitrite treated 1-AP

B = the number of histidine revertant colonies/plate of the extract with sodium nitrite treated 1-AP

C = the number of histidine revertant colonies/plate of spontaneous reversion

The degree of inhibition (+) or enhancement (-) of mutagenic activity from percentage of modification was divided into 4 classes as follows: ± < 20%: negligible; ± 20 – 40%: weak; ± 40 – 60%: moderate; ± > 60%: strong[104].

CHAPTER IV

RESULTS

4.1 Mutagenic activities of *Momordica charantia* extracts

Mutagenic activities of *Momordica charantia* extracts in the forms of raw fruit extract (RW), blanching water (BW), hot water extract from blanched fruit (HB), stir fried fruit extract (SF), and stir fried fruit (pre-squeezed with salt) extract (SS) were tested. The mutagenic effects of all extracts with and without sodium nitrite treatment on *Salmonella typhimurium* strains TA98 and TA100 are shown in Tables 6 and 7, respectively. Results indicated that the histidine revertant colonies induced by *Momordica charantia* extracts after reacting with and without sodium nitrite did not meet the mutagenicity criteria. None of the extracts showed a higher mutagenic index (MI) value than two, indicating that all the extracts had no mutagenic effect.

Table 6 The mutagenic activities of extracts from *Momordica charantia* with or without sodium nitrite treatment on *Salmonella typhimurium* strain TA98

Cooking method	Sample (µl/plate)	No. of Histidine revertant colonies/plate[a] (MI[b])	
		Without sodium nitrite treatment	With sodium nitrite treatment
RW[d]	0[c]	14 ± 3	14 ± 3
	0	14 ± 3 (1.00)	15 ± 2 (1.07)
	25	13 ± 2 (0.93)	16 ± 2 (1.14)
	50	10 ± 1 (0.71)	16 ± 3 (1.14)
	100	12 ± 3 (0.86)	16 ± 1 (1.14)
BW[e]	0[c]	20 ± 2	20 ± 2
	0	16 ± 3 (0.80)	24 ± 5 (1.20)
	25	14 ± 3 (0.70)	20 ± 3 (1.00)
	50	14 ± 3 (0.70)	21 ± 5 (1.05)
	100	17 ± 3 (0.85)	22 ± 4 (1.10)
HB[f]	0[c]	19 ± 4	19 ± 4
	0	14 ± 3 (0.74)	22 ± 4 (1.15)
	25	16 ± 3 (0.84)	23 ± 5 (1.21)
	50	19 ± 2 (1.00)	26 ± 2 (1.36)
	100	15 ± 2 (0.79)	21 ± 4 (1.11)

Table 6 The mutagenic activities of extracts from *Momordica charantia* with or without sodium nitrite treatment on *Salmonella typhimurium* strain TA98 (Cont.)

Cooking method	Sample (µl/plate)	No. of Histidine revertant colonies/plate[a] (MI[b])	
		Without sodium nitrite treatment	With sodium nitrite treatment
SS[g]	0[c]	12 ± 3	12 ± 3
	0	13 ± 2 (1.08)	14 ± 3 (1.17)
	25	13 ± 3 (1.08)	16 ± 3 (1.33)
	50	12 ± 2 (1.00)	15 ± 3 (1.25)
	100	12 ± 3 (1.00)	14 ± 3 (1.17)
SF[h]	0[c]	14 ± 1	14 ± 1
	0	13 ± 3 (0.93)	12 ± 2 (0.86)
	25	12 ± 2 (0.86)	16 ± 2 (1.14)
	50	14 ± 2 (1.00)	15 ± 4 (1.07)
	100	13 ± 2 (0.93)	14 ± 3 (1.00)

[a]Data were expressed as the mean ± standard deviation (SD) of six plates from two different determinations.

[b]Mutagenicity index (MI) was calculated from the average number of histidine revertant colonies/plate of each extract divided by that of spontaneous reversion.

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

[d]RW is raw fruit extract.

[e]BW is blanching water.

[f]HB is hot water extract from blanched fruit.

[g]SS is stir fried fruit (pre-squeezed with salt) extract.

[h]SF is stir fried fruit extract.

Table 7 The mutagenic activities of extracts from *Momordica charantia* with or without sodium nitrite treatment on *Salmonella typhimurium* strain TA100

Cooking method	Sample (µl/plate)	No. of Histidine revertant colonies/plate[a] (MI[b])	
		Without sodium nitrite treatment	With sodium nitrite treatment
RW[d]	0[c]	142 ± 13	142 ± 13
	0	140 ± 14 (0.98)	137 ± 26 (0.96)
	25	141 ± 13 (0.99)	137 ± 14 (0.96)
	50	149 ± 12 (1.05)	126 ± 11 (0.89)
	100	128 ± 16 (0.90)	141 ± 10 (0.99)
BW[e]	0[c]	119 ± 19	119 ± 19
	0	118 ± 22 (0.99)	109 ± 11 (0.92)
	25	114 ± 12 (0.96)	133 ± 7 (1.12)
	50	126 ± 12 (1.06)	119 ± 8 (1.00)
	100	135 ± 20 (1.13)	127 ± 20 (1.07)
HB[f]	0[c]	152 ± 18	152 ± 18
	0	149 ± 14 (0.98)	135 ± 11 (0.89)
	25	136 ± 26 (0.89)	134 ± 13 (0.88)
	50	145 ± 11 (0.95)	152 ± 28 (1.00)
	100	143 ± 9 (0.94)	148 ± 16 (0.97)

Table 7 The mutagenic activities of extracts from *Momordica charantia* with or without sodium nitrite treatment on *Salmonella typhimurium* strain TA100 (Cont.)

Cooking method	Sample (µl/plate)	No. of Histidine revertant colonies/plate[a] (MI[b])	
		Without sodium nitrite treatment	With sodium nitrite treatment
SS[g]	0[c]	125 ± 9	125 ± 9
	0	130 ± 20 (1.04)	118 ± 12 (0.94)
	25	119 ± 13 (0.95)	115 ± 12 (0.92)
	50	115 ± 13 (0.92)	125 ± 22 (1.00)
	100	122 ± 19 (0.98)	124 ± 10 (0.99)
SF[h]	0[c]	153 ± 26	153 ± 26
	0	139 ± 14 (0.90)	141 ± 25 (0.92)
	25	146 ± 18 (0.95)	146 ± 18 (0.95)
	50	147 ± 21 (0.96)	161 ± 21 (1.05)
	100	171 ± 17 (1.12)	152 ± 19 (0.99)

[a]Data were expressed as the mean ± standard deviation (SD) of six plates from two different determinations.

[b]Mutagenicity index (MI) was calculated from the average number of histidine revertant colonies/plate of each extract divided by that of spontaneous reversion.

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

[d]RW is raw fruit extract.

[e]BW is blanching water.

[f]HB is hot water extract from blanched fruit.

[g]SS is stir fried fruit (pre-squeezed with salt) extract.

[h]SF is stir fried fruit extract.

4.2 Mutagenic modification effects of *Momordica charantia* extracts

Effects of the extracts from *Momordica charantia* on the mutagenic activity of mutagen obtained from the reaction between 1-aminopyrene and nitrite (duration of presence = 0h) (MB) assayed using *Salmonella typhimurium* strains TA98 and TA100 are shown in Table 8. The RW, BW, HB, SF, and SS extracts exhibited mutagenicity enhancing effects with a dose-response relationship compared to the standard mutagen. Percentages of mutagenicity enhancement of RW on *Salmonella typhimurium* strain TA98 were 147.62% and 144.77% for 50 and 100 µl/plate, respectively, whereas there was no effect on *Salmonella typhimurium* strain TA100 (< 20% enhancement). For BW, the range of percentages of mutagenicity enhancement on *Salmonella typhimurium* strains TA98 and TA100 were 116.12%-338.06% and 74.93%-109.95%, respectively, whereas HB showed lower enhancement on both bacterial strains (42.61%-140.85% and 8.59%-77.29% for TA98 and TA100, respectively).

SS showed a weak to strong (23.38%-235.97%) degree of mutagenicity enhancement on *Salmonella typhimurium* strain TA98 and a negligible to strong (18.89%-93.38%) enhancing effect on *Salmonella typhimurium* strain TA100. The ranges of percentage enhancement of SF on *Salmonella typhimurium* strains TA98 and TA100 were 44.54%-302.06% and 57.14%-139.77%, respectively and tended to enhance the mutagenic modification effect more than those of stir fried fruit (pre-squeezed with salt) extract.

Table 8 The effect of different extracts from *Momordica charantia* on the mutagenic activity of mutagen obtained from the reaction between 1-aminopyrene and sodium nitrite (duration of presence = 0 h) on *Salmonella typhimurium* strains TA98 and TA100

Cooking method	Sample (µl/plate)	No. of Histidine revertant colonies/plate[a] (%enhancement[b])	
		TA 98	TA 100
RW[e]	0[c]	13 ± 2	121 ± 12
	0[d]	855 ± 124	945 ± 151
	25	935 ± 153 (9.54)	947 ± 98 (0.28)
	50	2098 ± 420 (147.62)	968 ± 168 (2.79)
	100	2074 ± 463 (144.77)	1093 ± 231 (18.00)
BW[f]	0[c]	13 ± 1	186 ± 19
	0[d]	683 ± 138	856 ± 122
	25	1461 ± 117 (116.12)	1358 ± 186 (74.93)
	50	2241 ± 288 (232.59)	1381 ± 154 (78.46)
	100	2948 ± 282 (338.06)	1592 ± 248 (109.95)
HB[g]	0[c]	17 ± 5	140 ± 21
	0[d]	1267 ± 230	878 ± 146
	25	1800 ± 248 (42.61)	941 ± 171 (8.59)
	50	2359 ± 314 (87.31)	1015 ± 194 (18.62)
	100	3028 ± 422 (140.85)	1448 ± 141 (77.29)

Table 8 The effect of different extracts from *Momordica charantia* on the mutagenic activity of mutagen obtained from the reaction between 1-aminopyrene and sodium nitrite (duration of presence = 0 h) on *Salmonella typhimurium* strains TA98 and TA100 (Cont.)

Cooking method	Sample (µl/plate)	No. of Histidine revertant colonies/plate[a] (%enhancement[b])			
		TA 98		TA 100	
SS[h]	0[c]	13 ± 3		172 ± 19	
	0[d]	905 ± 69		585 ± 45	
	25	1113 ± 120	(23.38)	663 ± 92	(18.89)
	50	1349 ± 219	(49.80)	737 ± 120	(36.80)
	100	3009 ± 380	(235.97)	971 ± 128	(93.38)
SF[i]	0[c]	12 ± 2		118 ± 12	
	0[d]	839 ± 110		723 ± 84	
	25	1207 ± 170	(44.54)	1068 ± 73	(57.14)
	50	3017 ± 288	(263.44)	1306 ± 68	(96.47)
	100	3337 ± 219	(302.06)	1567 ± 268	(139.77)

[a]Data were expressed as the mean ± standard deviation (SD) of six plates from two different determinations.

[b]The extracts enhanced the mutagenicity of 1-aminopyrene reacted with sodium nitrite.

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

[d]No extract added to the standard mutagen (positive control).

[e]RW is raw fruit extract.

[f]BW is blanching water.

[g]HB is hot water extract from blanched fruit.

[h]SS is stir fried fruit (pre-squeezed with salt) extract.

[i]SF is stir fried fruit extract.

Table 9 shows the mutagenicity modification effect of the extracts from *Momordica charantia* on the mutagen forming reaction between 1-aminopyrene and nitrite (duration of presence = 4 h) (MF) using *Salmonella typhimurium* strains TA98 and TA100. Results indicated that the RW, BW, HB, SF, and SS showed mutagenicity enhancing effects on MF with a dose-response relationship. RW showed a negligible to strong (4.97%-129.31%) degree of enhancement on *Salmonella typhimurium* strain TA98 and a negligible to weak (1.38%-37.85%) enhancing effect on *Salmonella typhimurium* strain TA100.

The ranges of percentages of mutagenicity enhancement of BW on *Salmonella typhimurium* strains TA98 and TA100 were 74.21%-348.69% and 13.10%-74.97%, respectively, while HB showed lower enhancing values of 37.19%-57.46% and 3.54%-34.66%, for TA98 and TA100, respectively. In addition, SF and SS showed a mutagenicity enhancing effect when these extracts were added at the beginning of the standard mutagen forming reaction.

Table 9 The mutagenic modification effect of the extracts from *Momordica charantia* on the mutagen forming reaction between 1-aminopyrene and sodium nitrite (duration of presence = 4 h) on *Salmonella typhimurium* strains TA98 and TA100

Cooking method	Sample (µl/plate)	No. of Histidine revertant colonies/plate[a] (%enhancement[b])			
		TA 98		TA 100	
RW[e]	0[c]	13 ± 2		121 ± 12	
	0[d]	875 ± 160		990 ± 96	
	25	918 ± 142	(4.97)	1020 ± 161	(3.41)
	50	1108 ± 224	(27.01)	1002 ± 116	(1.38)
	100	1990 ± 432	(129.31)	1319 ± 174	(37.85)
BW[f]	0[c]	13 ± 1		186 ± 19	
	0[d]	641 ± 117		812 ± 110	
	25	1164 ± 210	(83.18)	958 ± 186	(23.32)
	50	1107 ± 174	(74.21)	894 ± 147	(13.10)
	100	2832 ± 336	(348.69)	1281 ± 117	(74.97)
HB[g]	0[c]	17 ± 5		140 ± 21	
	0[d]	1675 ± 151		781 ± 93	
	25	2523 ± 228	(51.15)	909 ± 68	(19.92)
	50	2292 ± 230	(37.19)	804 ± 72	(3.54)
	100	2628 ± 329	(57.46)	1003 ± 132	(34.66)

Table 9 The mutagenic modification effect of the extracts from *Momordica charantia* on the mutagen forming reaction between 1-aminopyrene and sodium nitrite (duration of presence = 4 h) on *Salmonella typhimurium* strains TA98 and TA100 (Cont.)

Cooking method	Sample (µl/plate)	No. of Histidine revertant colonies/plate[a] (%enhancement[b])	
		TA 98	TA 100
SS[h]	0[c]	13 ± 3	172 ± 19
	0[d]	1216 ± 177	548 ± 88
	25	1694 ± 241 (39.76)	620 ± 68 (19.06)
	50	1893 ± 175 (56.30)	719 ± 109 (45.30)
	100	2472 ± 446 (104.41)	881 ± 122 (88.48)
SF[i]	0[c]	12 ± 2	118 ± 12
	0[d]	1208 ± 196	682 ± 96
	25	1667 ± 175 (38.43)	699 ± 109 (2.90)
	50	2205 ± 212 (83.36)	789 ± 105 (18.85)
	100	2931 ± 375 (144.12)	1004 ± 154 (57.09)

[a]Data were expressed as the mean ± standard deviation (SD) of six plates from two different determinations.

[b]The extracts enhanced the mutagenicity of 1-aminopyrene reacted with sodium nitrite.

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

[d]No extract added to the standard mutagen (positive control).

[e]RW is raw fruit extract.

[f]BW is blanching water.

[g]HB is hot water extract from blanched fruit.

[h]SS is stir fried fruit (pre-squeezed with salt) extract.

[i]SF is stir fried fruit extract.

CHAPTER V

DISCUSSION

5.1 Mutagenic activities of *Momordica charantia* extracts

The result of the present study showed that raw *Momordica charantia* fruit extract had no mutagenic activity on *Salmonella typhimurium* strains TA98 and TA100. This finding was consistent with research presented by Basaran et al.[105] and Chiampanichayakul et al.[106], who found that raw *Momordica charantia* did not demonstrate mutagenic activity using the Ames test with reaction or no reaction with S-9 mix. However, no detailed were reported about the mutagenic activity of this raw fruit extract when reacted with nitrite as well as cooked fruit. Results of previous studies showed that the cooking processes of *Momordica charantia* affected levels of phytochemical constituents such as phenolic compounds, flavonoids, tannins and carotenoids[107-109]. Here, we found that all sample extracts that reacted or did not react with sodium nitrite showed no mutagenic activity on both strains of *Salmonella typhimurium*, suggesting that the extracts of raw bitter gourd and cooked bitter gourd did not contain any mutagenic compounds. Moreover, no polar compounds in bitter gourd showed mutagenic activity after reaction with sodium nitrite. According to this information, consumption of *Momordica charantia* can, therefore, be considered safe from mutagenicity using the Ames test.

5.2 Mutagenic modification effects of *Momordica charantia* extracts

After the reaction between 1-aminopyrene and sodium nitrite was completed, the raw bitter gourd extract was added into the finished product in order to assess if the extract affected the mutagenic activity of the newly prepared mutagen. The result showed that raw fruit extract (RW) exhibited a mutagenicity enhancing effect on *Salmonella typhimurium* strain TA98. However, after testing on *Salmonella typhimurium* strain TA100, the RW did not show any mutagenicity enhancing effect. There was no report of the effect of raw bitter gourd extract on the mutagenic activity of 1-aminopyrene reacted with sodium nitrite on both strains of *Salmonella typhimurium*. However, Chiampanichayakul et al.[106] reported that raw *Momordica charantia* extract promoted the mutagenic activity of some standard mutagenic agents with S-9 mix including 3-amino-1,4-dimethyl-*5H*-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-*5H*-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-*9H*-pyrido[2,3-*b*]indole (AαC) and 2-amino-3-methyl-*9H*-pyrido[2,3-*b*]indole (MeAαC), on *Salmonella typhimurium* strain TA98. In addition, Triteeradej et al.[110] found that the fully ripe pulp and aril of *Momordica cochinchinensis*, which belongs to the Cucurbitaceae family, the same family as *Momordica charantia*, showed enhancing effect on the mutagenicity of 1-aminopyrene reacted with nitrite on *Salmonella typhimurium* strain TA100.

The same experimental condition was conducted to evaluate the modifying activity of blanching water (BW), hot water extract from blanched fruit (HB), stir fried fruit extract (SF), and stir fried fruit (pre-squeezed with salt) extract (SS) on the mutagenic activity of newly prepared mutagen (MB). Results showed that percentage enhancing values of BW tended to be higher than those obtained from HB. Donya et al.[111] reported that momordicoside L, a bitter substance contained in bitter gourd was leached out with hot water. In addition, Kumar et al.[112] reported that the bitter gourd contained luteolin which belongs to a group of naturally occurring compounds called flavonoids. This compound had the pro-oxidative catechol structural element (hydroxyl group at positions 3 and 4 of the B ring) which demonstrated mutagenic activity without metabolic activation. The auto-oxidation of luteolin to luteolin semiquinone radical generated the superoxide anion radical. Furthermore, auto-oxidation of catechol-type flavonoids to quinones and their isomeric quinone methides produced electrophiles that may cause DNA alkylating[113]. These compounds may stimulate the *O*-acetyltransferase and nitroreductase enzymes within the cells of *Salmonella typhimurium*[114]. These enzymes are involved in the activating systems of direct-acting mutagens including 1-nitropyrene present in bacterial cells[115]. Moreover, some compounds in bitter gourd may alter the permeability of bacterial membranes or modify some extracellular physical and chemical interactions between flavonoids and mutagens responsible for enhancing

mutagenicity[114, 116]. However, the detailed mechanisms of action may need further investigation.

Stir frying is another procedure for cooking bitter gourd. In this study, preparation of the extracts from stir fried bitter gourd was divided into two portions; the extract from stir fried bitter gourd and the extract from stir fried bitter gourd that had been pretreated by squeezing with salt and rinsing with water. The mutagenicity modification activities of these two extracts were determined separately. The percentages of mutagenicity enhancement of SF on *Salmonella typhimurium* strains TA98 and TA100 were higher than those of SS. It is possible that squeezing with salt may cause the plant cell membrane to change in composition, structure or function[117]. In addition, rinsing bitter gourd with water after squeezing with salt may eliminate certain substances that can increase mutagenic activity. Our results suggested that pretreatment of bitter gourd before cooking, either blanching before boiling or squeezing with salt before stir frying, is a method that not only reduces the bitterness of the fruit but also helps to reduce the mutagenic enhancing effect of this fruit.

The final study was to evaluate whether the extracts of raw and cooked *Momordica charantia* had any effect on the reaction of 1-aminopyrene and nitrite which generated the mutagen (duration of presence = 4 h) (MF). The percentage of enhancing values obtained from this test (duration of presence = 4 h) showed a pattern that was similar to the values obtained from MB. All extracts of *Momordica*

charantia at 50 and 100 µl showed a trend of enhancing effect on the mutagenic activity of this mutagen forming reaction model. Considering the percentage of enhancing values, it was found that pretreatment before cooking was one factor that could alter values of cooked sample extracts. In the case of boiled fruit, bitter gourd was pretreated by means of blanching first and then boiled. BW showed a higher enhancement effect than HB. Although the condition of adding the extract to the reaction mixture was different, the results obtained were similar to MB. It is possible that extract added at the beginning of the reaction may not have the effect of modifying the reaction between 1-aminopyrene and sodium nitrite as the extract remained in the system. When the reaction ended, a new mutagen was formed. The extract may increase the mutagenicity of this new mutagenic compound through the mechanism of action to stimulate the *O*-acetyltransferase and nitroreductase enzymes proposed in MB. Therefore, further study is required in order to investigate the mechanisms of this action in more detail.

For stir fried fruit, bitter gourd was pretreated by squeezing with salt first and then stir fried. No obvious differences were found between the percentages of enhancing values of extracts between stir fried fruit and pre-squeezed stir fried fruit when tested on *Salmonella typhimurium* strain TA98. When testing was conducted on strain TA100, SS showed a higher percentage of enhancing values compared to the values obtained from SF; however, the mechanism of action was not clear and further research is required. The results of this study suggest that *Momordica*

charantia fruit should first be cooked with pretreatment by blanching or pre-squeezing with salt. The pretreatment process of bitter gourd cooking may allow consumers to eat bitter gourd more safely.

CHAPTER VI

CONCLUSION

This study aimed to determine the mutagenic activity and mutagenic modification activity of various types of extracts from *Momordica charantia* including raw fruit extract, blanching water, hot water extract from blanched fruit, stir fried fruit extract, and stir fried fruit (pre-squeezed with salt) extract on *Salmonella typhimurium* strains TA98 and TA100 in acidic conditions. Results showed that all of the extracts either with or without sodium nitrite treatment showed no mutagenic activity on both strains of *Salmonella typhimurium*. However, when each sample extract was added at the beginning of the 1-aminopyrene-sodium nitrite reaction this promoted the mutagenicity of this model. In addition, a similar result was found when the extract was added after the end of the 1-aminopyrene-sodium nitrite reaction. It is interesting to note that pretreatment before cooking, such as blanching or squeezing with salt, could reduce mutagenicity enhancing effects. However, further studies on the mutagenicity enhancing reaction mechanisms of *Momordica charantia* extracts are required. Moreover, all extracts in this study did not use dimethyl sulfoxide (DMSO) as a solvent; therefore, the extracts contained only substances in the polar group. This was a limitation. Our findings suggested that raw or cooked *Momordica charantia* are safe for consumption in consideration of

mutagenicity. However, consumers should avoid eating bitter gourds with foods containing nitrites such as ham and sausages.

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APPENDIX

APPENDIX

1. Preparation of stock solutions and media

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

Ingredient	**100 ml**
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	1 g
Citric acid monohydrate	10 g
Potassium phosphate, dibasic (anhydrous) (K_2HPO_4)	50 g
Sodium ammonium phosphate ($NaNH_4HPO_4 \cdot 4H_2O$)	17.5 g
Distilled ultrapure water	67 ml

Add salts in the order indicated above to warm water and dissolve each salt completely before adding the next. Adjust to volume, filter the solution into the glass bottle with screw caps and then autoclave for 20 minutes at 121 °C.

1.2 40% Glucose

Ingredient	**100 ml**
Glucose	40 g
Distilled ultrapure water q.s. to	100 ml

Dissolve glucose in distilled ultrapure water and then autoclave for 20 minutes at 121 °C.

1.3 Minimal glucose agar plate (MGA plate)

Ingredient	**200 ml**	**300 ml**
Agar agar	3 g	4.5 g
VB salts	4 ml	6 ml
40% glucose	10 ml	15 ml
Distilled ultrapure water	186 ml	279 ml

Add agar to distilled ultrapure water in Erlenmeyer flask and then autoclave for 20 minutes at 121 °C. When the solution has cooled slightly, add sterile VB salts and sterile 40% glucose. Mix and pour 30 ml into sterile petri dish. MGA plate are kept in incubator at 37 °C before using.

1.4 Oxoid nutrient broth No.2

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

Dissolve Oxoid nutrient broth No.2 2.5 g in Distilled ultrapure water 100 ml, transfer 12 ml to Erlenmeyer flask with gauze and then autoclave for 20 minutes at 121 °C.

1.5 0.1 M histidine hydrochloride

Ingredient	**10 ml**
L-histidine HCl	0.1552 g
Distilled ultrapure water q.s. to	10 ml

Dissolve 0.1552 g of L-histidine HCl (Molecular weight = 155.2 g/mol) in distilled ultrapure water, adjust to 10 ml and then autoclave for 20 minutes at 121 °C.

1.6 1 mM histidine hydrochloride

Ingredient	**100 ml**
0.1 M histidine HCl	1 ml
Distilled ultrapure water q.s. to	99 ml

Pipette 1 ml of 0.1 M histidine HCl into volumetric flask, adjust with distilled ultrapure water to 100 ml and then autoclave for 20 minutes at 121 °C.

1.7 1 mM biotin

Ingredient	**100 ml**
Biotin	0.0244 g
Distilled ultrapure water q.s. to	100 ml

Dissolve 0.0244 g of biotin (Molecular weight = 244.3 g/mol) in distilled ultrapure water, adjust to 100 ml and then autoclave for 20 minutes at 121 °C.

1.8 0.5 mM histidine hydrochloride / biotin

Ingredient	**200 ml**
1 mM histidine HCl	100 ml
1 mM biotin	100 ml

Mix and autoclave for 20 minutes at 121 °C.

1.9 Top agar

Ingredient	**110 ml**
Agar agar	0.6 g
Sodium chloride (NaCl)	0.5 g
0.5 mM histidine HCl/biotin	10 ml
Distilled ultrapure water	100 ml

Dissolve ingredients in distilled ultrapure water and autoclave for 20 minutes at 121 °C. Add 0.5 mM histidine HCl/biotin 10 ml and then mix by thoroughly by swirling.

1.10 ampicillin solution 8 mg/ml (aseptic technique)

Ingredient	**10 ml**
Ampicillin sodium	0.0800 g
Distilled ultrapure water	10 ml

Dissolve ampicillin sodium 0.0800 g in distilled ultrapure water 10 ml.

1.11 0.1% crystal violet (aseptic technique)

Ingredient	**100 ml**
Crystal violet	0.0100 g
Distilled ultrapure water	10 ml

Dissolve crystal violet 0.0100 g in distilled ultrapure water 10 ml.

1.12 0.9% sodium chloride

Ingredient	**100 ml**
Sodium chloride (NaCl)	0.9 g
Distilled ultrapure water q.s. to	100 ml

Dissolve sodium chloride 0.9 g in distilled ultrapure water 100 ml and then autoclave for 20 minutes at 121 °C.

1.13 0.2 N hydrochloric acid (aseptic technique)

Ingredient	**100 ml**
Conc. hydrochloric acid (conc. HCl)	1.67 ml
Distilled ultrapure water q.s. to	100 ml

Pipette 1.67 ml of conc. hydrochloric acid into volumetric flask with appropriate volume of distilled ultrapure water and then adjust to 100 ml.

1.14 2 M sodium nitrite

Ingredient	**10 ml**
Sodium nitrite ($NaNO_2$)	1.38 g
Distilled ultrapure water q.s. to	10 ml

Dissolve 1.38 g of sodium nitrite (Molecular weight = 68.99 g/mol) in distilled ultrapure water, adjust to 10 ml and then autoclave for 20 minutes at 121 °C.

1.15 2 M ammonium sulfamate

Ingredient	**10 ml**
Ammonium sulfamate ($NH_2SO_3NH_4$)	2.2824 g
Distilled ultrapure water q.s. to	10 ml

Dissolve ammonium sulfamate 2.2824 g (Molecular weight = 114.12 g/mol) in distilled ultrapure water, adjust to 10 ml and then autoclave for 20 minutes at 121 °C.

1.16 1 M potassium chloride

Ingredient	**100 ml**
Potassium chloride (KCl)	7.456 g
Distilled ultrapure water q.s. to	100 ml

Dissolve potassium chloride 7.456 g in distilled ultrapure water, adjust to 100 ml and then autoclave for 20 minutes at 121 °C.

1.17 0.5 M sodium dihydrogen phosphate

Ingredient	**100 ml**
Sodium dihydrogen phosphate (NaH_2PO_4)	6 g
Distilled ultrapure water q.s. to	100 ml

Dissolve 6 g of sodium dihydrogen phosphate (Molecular weight = 120 g/mol) in distilled ultrapure water, adjust to 100 ml and then autoclave for 20 minutes at 121 °C.

1.18 0.5 M disodium hydrogen phosphate dihydrate

Ingredient	**100 ml**
Disodium hydrogen phosphate dihydrate (NaH_2PO_4)	8.9 g
Distilled ultrapure water q.s. to	100 ml

Dissolve 8.9 g of disodium hydrogen phosphate dihydrate (Molecular weight = 177.99 g/mol) in distilled ultrapure water, adjust to 100 ml and then autoclave for 20 minutes at 121 °C

1.19 0.5 M sodium phosphate pH 7.4

Ingredient	
0.5 M disodium hydrogen phosphate dihydrate	100 ml
0.5 M sodium dihydrogen phosphate	Adjust pH to 7.4

Add 0.5 M sodium dihydrogen phosphate (from 1.17) into 0.5 M sodium dihydrogen phosphate (from 1.18) until pH 7.4 and then autoclave for 20 minutes at 121 °C

1.20 Sodium phosphate – potassium chloride buffer (Na_3PO_4 – KCl buffer)

Ingredient	**3300 ml**
1 M potassium chloride	16.5 ml
0.5 M sodium phosphate pH 7.4	100 ml
Distilled ultrapure water	213.5 ml

Mix and autoclave for 20 minutes at 121 °C.

1.21 3 mg/ml 1-aminopyrene solution (aseptic technique)

Ingredient	**1 ml**
1-aminopyrene	0.0030 g
Acetonitrile	1 ml

Dissolve 1-aminopyrene 0.0030 g in acetonitrile and then store in the sterile amber glass with screw cap.

1.22 0.3 mg/ml 1-aminopyrene solution (aseptic technique)

Ingredient	**1 ml**
3 mg/ml 1-aminopyrene solution	0.1 ml
Acetonitrile	0.9 ml

Pipette 0.1 ml of 3 mg/ml 1-aminopyrene solution into sterile amber glass and then add acetonitrile 0.9 ml.

1.23 0.0375 mg/ml 1-aminopyrene solution (aseptic technique)

Ingredient	**2 ml**
0.3 mg/ml 1-aminopyrene solution	0.250 ml
Acetonitrile	1.750 ml

Pipette 0.250 ml of 0.3 mg/ml 1-aminopyrene solution into sterile amber glass, and then add acetonitrile 1.750 ml.

2. Procedure for re-isolation and growing culture

Salmonella typhimurium TA98 and TA100 are grown in Oxoid nutrient broth No.2 12 ml and incubated overnight in a shaking water bath at 37 °C for 16 hours. Each overnight culture strains is streaked on a minimal glucose agar plate which are spread with ampicillin solution 8 mg/ml 0.1 ml, 0.1 M histidine 0.3 ml, and 1 mM biotin 0.1 ml. These plates are incubated at 37 °C for 48 hours. After that, four single colonies of each strains are picked up and grown overnight in a shaking water bath at 37 °C for 16 hours. Each overnight culture strains is confirmed genotype and kept the cultures for mutagenicity assay. Five ml of each overnight culture strains is mixed with 0.45 ml of dimethyl sulfoxide (DMSO) then transfer 0.2 ml to sterile micro-centrifuge tube before storing at -80 °C.

3. Confirming genotype of tester strain

3.1 Histidine requirement

The histidine is a requirement for *Salmonella typhimurium* growth. The histidine dependent character of the *Salmonella typhimurium* should be tested on the minimal glucose agar plate for confirmation as follows

Procedure:

Plate A: no histidine and biotin

Plate B: 0.1 ml of 1mM biotin

Plate C: 0.3 ml of 0.1M hitidine hydrochloride

Plate D: 0.1 ml of 1mM biotin and 0.3 ml of 0.1M hitidine hydrochloride

Each strain is streaked on the surface of four plates which each plate divided into 4 parts and then incubated at 37 °C for 24 hours. The growth of bacteria on plate D is the result of histidine requirement.

3.2 R-factor

The R-factor strains (TA97, TA98, TA100 and TA102) should be tested routinely for the presence of the ampicillin factor due to unstable plasmid and possibly lost from these strains.

Procedure:

For each strain, 0.3 ml of overnight culture is mixed with 0.1 ml of 0.1 M histidine hydrochloride and 2 ml of molten top agar in a sterile tube, then poured on a minimal glucose agar plate. After that, the minimal glucose agar plate is divided

into 2 parts for testing of R-factor and *rfa* mutation (see in 3.3 *rfa* mutation). For R-factor, a filter paper disc containing ampicillin solution 8 mg/ml is placed on the surface of the agar. Then, these plates are incubated at 37 °C for 24 hours. No clear zones around disc indicate the resistance to ampicillin.

3.3 *rfa* mutation

The strains containing *rfa* character should be confirmed using 0.1% crystal violet.

Procedure:

This test is performed as described in 3.2 R-factor. The filter paper disc containing using 0.1% crystal violet is placed on the surface of the agar. Then, these plates are incubated at 37 °C for 24 hours. Clear zones around disc indicate the presence of *rfa* mutation which permits larges molecule to enter the bacteria.

4. Spontaneous reversion

Spontaneous reversion of each strain is conducted routinely in mutagenicity assay and reported as the number of spontaneous reversion colonies per plate. The number of spontaneous reversion colonies is variability from one experiment to another, and one plate to another, and it is recommended to make at least 3 plates of spontaneous reversion plates for each strain in the mutagenicity assay.

Procedure:

0.1 ml of sterile distilled ultrapure water is mixed with 0.5 ml of Na_3PO_4 - KCl buffer (pH 7.4) in a tube with a plastic cap, added 100 ml of overnight culture,

shaken in a water bath at 37 °C for 20 minutes, gently mixed with 2 ml of molten Top agar (45 °C), carefully poured onto a minimal glucose agar plate, and incubated at 37 °C for 48 hours.

5. The response to standard mutagen

Confirmation of reversibility and specificity of each strain in the mutagenicity assay, the standard mutagen are used for routine testing. In this study, sodium nitrite reacted with 1-aminopyrene in acidic condition is used as a standard mutagen in the Ames test.

Procedure:

The procedure is performed according to *4. Spontaneous reversion* except 0.1 ml of sterile distilled ultrapure water is replaced by 0.1 ml of the product from 1-aminopyrene reacted with sodium nitrite (1-aminopyrene 0.0375 mg/ml, 10 µl and 40 µl for *Salmonella typhimurium* TA98 and TA100, respectively).

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