ผลของสารสกัดจากผักใบเขียวในการปรับเปลี่ยนฤทธิ์ก่อกลายพันธุ์ของอะมิโนพัยรีน ที่ทำปฏิกิริยากับไนไทรต์และซุปไก่สกัดที่ทำปฏิกิริยากับไนไทรต์ โดยวิธีทดสอบเอมส์

นางสาวธัญวรรณ นันทะโตวัฒนะ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาอาหารเคมีและโภชนศาสตร์ทางการแพทย์ ภาควิชาอาหารและเภสัชเคมี
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2552

EFFECT OF EXTRACTS FROM GREEN VEGETABLES ON MODIFICATION OF MUTAGENICITY OF NITRITE-TREATED AMINOPYRENE AND NITRITE-TREATED CHICKEN EXTRACT USING AMES TEST

Miss Thunyawan Nuntatovattana

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program
in Food Chemistry and Medical Nutrition
Department of Food and Pharmaceutical Chemistry
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2009

Thesis Title	EFFECT OF EXTRACTS FROM GREEN VEGETABLES ON MODIFICATION OF MUTAGENICITY OF NITRITE-TREATED AMINOPYRENE AND NITRITE-TREATED CHICKEN EXTRACT USING AMES TEST
By	Miss Thunyawan Nuntatovattana
Field of Study	Food Chemistry and Medical Nutrition
Thesis Advisor	Assistant Professor Linna Tongyonk, D.Sc

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Pharmaceutical Sciences

(Associate Professor Pintip Pongpech, Ph.D.)

THESIS COMMITTEE

..... Chairman
(Associate Professor Oranong Kangsadalampai, Ph.D.)

..... Thesis Advisor
(Assistant Professor Linna Tongyonk, D.Sc.)

..... Examiner
(Associate Professor Surattana Amnuoypol, Ph.D.)

..... Examiner
(Assistant Professor Suyanee Pongthananikorn, Dr.P.H.)

..... External Examiner
(Associate Professor Kaew Kangsadalampai, Ph.D.)

ธัญวรรณ นันทะโตวัฒนะ : ผลของสารสกัดจากผักใบเขียวในการปรับเปลี่ยนฤทธิ์ก่อกลายพันธุ์ของอะมิโนพัยรีนที่ทำปฏิกิริยากับไนไทรต์และซุปไก่สกัดที่ทำปฏิกิริยากับไนไทรต์ โดยวิธีทดสอบเอมส์. (EFFECT OF EXTRACTS FROM GREEN VEGETABLES ON MODIFICATION OF MUTAGENICITY OF NITRITE-TREATED AMINOPYRENE AND NITRITE-TREATED CHICKEN EXTRACT USING AMES TEST) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร.ลินนา ทองยงค์, 81 หน้า.

สารสกัดจากใบดิบและลวกของชะพลู (*Piper sarmentosum* Roxb.), ยอ (*Morinda citrifolia* L.), ผักหวานบ้าน (*Sauropus androgynus* Merr.) และ ผักโขม (*Amaranthus lividus* Linn.) ไม่แสดงฤทธิ์ก่อการกลายพันธุ์ในเชื้อแบคทีเรีย *Salmonella typhimurium* TA98 และ TA100 เมื่อทดสอบด้วยวิธีเอมส์ อย่างไรก็ตามหลังทำปฏิกิริยากับไนไทรต์ ภายใต้สภาวะกรด (pH3-3.5) ที่ไม่มีระบบเอนไซม์กระตุ้นการออกฤทธิ์พบว่ามีฤทธิ์ก่อการกลายพันธุ์ในเชื้อแบคทีเรียทั้งสองสายพันธุ์ แบบจำลองการเกิดสารก่อกลายพันธุ์มี 2 แบบคือ 1) แบบจำลองปฏิกิริยาระหว่างอะมิโนพัยรีน-ไนไทรต์ และ 2) ซุปไก่สกัด-ไนไทรต์ พบว่าสารสกัดจากผักดิบและลวกสามารถลดฤทธิ์ก่อกลายพันธุ์ของทั้ง 2 แบบจำลองในเชื้อแบคทีเรียทั้งสองสายพันธุ์ได้ สารสกัดจากผักแต่ละชนิดแสดงศักยภาพในการลดฤทธิ์ก่อกลายพันธุ์ในแบบจำลองแรกได้ดีมาก (การยับยั้งมากกว่า 60%) ในขณะที่แบบจำลองที่ 2 ยับยั้งฤทธิ์ก่อกลายพันธุ์ได้แบบอ่อนถึงปานกลาง ยกเว้นสารสกัดจากผักโขมดิบพบว่าไม่มีฤทธิ์ลดการก่อกลายพันธุ์ในเชื้อแบคทีเรียทั้งสองสายพันธุ์ในแบบจำลองที่ 2 จากผลการทดลองพบว่าฤทธิ์ในการลดการก่อกลายพันธุ์ไม่แปรตามปริมาณของสารสกัด การที่สารสกัดของผักสามารถยับยั้งฤทธิ์ก่อกลายพันธุ์ได้อาจเนื่องมาจากมีสารประกอบบางชนิดไปลบล้างฤทธิ์การก่อกลายพันธุ์โดยตรงของสารก่อกลายพันธุ์ที่เกิดขึ้นเนื่องจากการทำปฏิกิริยาระหว่างอะมิโนพัยรีน-ไนไทรต์และซุปไก่สกัด-ไนไทรต์ จากผลการศึกษาพบว่าฤทธิ์ในการต้านการก่อกลายพันธุ์ในผักลวกไม่ได้ลดลงเมื่อเปรียบเทียบกับผักดิบ อาจสรุปได้ว่าการผ่านกระบวนการที่ให้ความร้อนไม่มีผลต่อฤทธิ์ในการต้านการก่อกลายพันธุ์ในผักแต่ละชนิด จากผลการศึกษานี้แนะนำว่าการรับประทานอาหารที่มีไนไทรต์เป็นส่วนประกอบร่วมกับผักเหล่านี้ (ทั้งดิบและลวก) จะช่วยป้องกันการก่อกลายพันธุ์เนื่องจากสารก่อกลายพันธุ์โดยตรงที่เกิดขึ้นในสภาวะของกระเพาะอาหารได้

ภาควิชา...อาหารและเภสัชเคมี.....ลายมือชื่อนิสิต....ธัญวรรณ นันทะโตวัฒนะ.....

สาขาวิชาอาหารเคมีและโภชนศาสตร์ทางการแพทย์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา..2552.....

49765709 : MAJOR FOOD CHEMISTRY
KEY WORDS: MODIFICATION / MUTAGENICITY / AMES / GREEN VEGETABLES / CHA-PLU / YOR / PAK-KHOM / PAK-WAAN-BANN

THUNYAWAN NUNTATOVATTANA: EFFECT OF EXTRACTS FROM GREEN VEGETABLES ON MODIFICATION OF MUTAGENICITY OF NITRITE-TREATED AMINOPYRENE AND NITRITE-TREATED CHICKEN EXTRACT USING AMES TEST. THESIS ADVISOR: ASST. PROF. LINNA TONGYONK, D.Sc., 81 pp.

The extracts from leaves of raw and blanched vegetables, namely Cha-Plu (*Piper sarmentosum* Roxb.), Yor (*Morinda citrifolia* L.), Pak-Waan-Bann (*Sauropus androgynus* Merr.), and Pak-Khom (*Amaranthus lividus* Linn.) did not have mutagenicity when they were tested by using *Salmonella typhimurium* strain TA98 and TA100 of the Ames test. However, they were mutagenic after reacted with nitrite in an acidic condition (pH3-3.5) without activating system on both strains. The standard mutagen models were 1-aminopyrene treated with nitrite and chicken extract treated with nitrite. The results revealed that the extracts from raw and blanched vegetables could reduce the direct-acting mutagenicity of compounds that occurred in the two models on both tester strains. The antimutagenicity of each extract added to the first model was strong (>60% inhibition) while it was moderate to weak in the second model, except the extract from raw Pak-Khom showed no active on both strains in second model. The results showed that the potency of antimutagenicity was not dose-dependent. Antimutagenicity of compounds in vegetable extracts might counteract the direct-acting mutagenicity of nitrite treated 1-aminopyrene and nitrite treated chicken extract. The results indicated that antimutagenic effect of extracts from blanched vegetables was not reduced compared with raw vegetables. It was concluded that heat processing had not an effect on antimutagenic activity of each vegetable. This experiment suggested that consumption of nitrite-containing foods with these vegetables (raw and blanched) could prevent the mutagenicity of direct mutagen occurring in gastric like condition.

Department.. Food and Pharmaceutical Chemistry Student's signature..Thunyawan Nuntatovattana

Field of study Food Chemistry and Medical Nutrition Advisor's signature..Linna Tongyonk

Academic year..2009.....

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and deep appreciation to my advisor, Assistant Professor Dr. Linna Tongyonk for her valuable advice, guidance, and encouragement throughout my graduate study.

I am very much obliged and honored to the members of the thesis committee, Associate Professor Dr. Oranong Kangsadalampai, Associate Professor Dr. Kaew Kangsadalampai, Associate Professor Dr. Surattana Amnuoypol, and Assistant Professor Dr. Suyanee Pongthananikorn for their valuable helpful suggestion.

I would like to thank all staff members of the Department of Food Chemistry and my friends for their supportive and encouragement.

I am really thankful to the Faculty of Graduate studies, Chulalongkorn University for the supporting scholarship which enabled me to undertake this study.

Finally, my special gratitude is expressed to my beloved family for their love, care, support and much encouragement throughout the period of my graduate study.

CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

LIST ABBREVIATIONS

°C	degree Celsius
g	gram
h	hour
i.e.	id est (this is)
mg	milligram
mM	millimolar
ml	millilitre
µl	microlitre
ppm	part per million
min	minute
M	Molar
MI	Mutagenicity Index
N	Normality
SD	standard deviation
et al.	*et alia* (and others)
1-AP	1-aminopyrene

CHAPTER I
INTRODUCTION

1.1 Background and Significance of the Study

Cancer in Thailand is becoming a significant health problem. It is the leading cause of death in Thailand (Vatanasapt *et al*., 2002). Three major factors for human carcinogenesis are cigarette smoking, infection and inflammation, and nutrition and dietary carcinogens (Doll and Peto, 1981; Sugimura, 2000). Social, economic and cultural factors strongly influence individual choices about diet and physical activity. Block *et al*. (1992) found the relationship between fruit and vegetable intake and the incidence of cancer and a statistically significant protective effect in 128 of 156 dietary studies. Greater consumption of vegetables, fruits, or both together has been associated in the majority of epidemiological studies with a lower risk of lung, oral, esophageal, stomach, and colon cancers. (Byers *et al*., 2002).

Studies have shown a possible increased cancer risk posed by the formation of polycyclic aromatic hydrocarbons and heterocyclic amines when high-heat cooking methods such as grilling, broiling, barbecuing and smoking meats are used (Wu *et al*., 2001). The Ames test of chicken extracts, smoked and charcoal-broiled foods treated with nitrite in acid condition have shown mutagenicity on *Salmonella typhimurium* strains TA98 and TA100 (Kangsadalampai *et al*. 1996; Peerawong and Kangsadalampai, 1998).

Vegetables are an important supplementary source of food and nutrition. They enrich the local diet with essential nutrients such as vitamins and minerals. Thailand utilizes vegetables in different ways: the uncooked vegetables are commonly used as a side dish; cooked vegetables are often eaten as a side dish or mixed in stew with meat, fish and other foods; other variations include curried, fried, broiled or baked vegetables. A common Thai meal consists of rice, cooked vegetables, and a curry dish. Vegetables are often cooked with water, oil, coconut milk and sometimes wine, blanched and served with traditional chili sauces, and even eaten in the raw form. In this research selected four green leafy vegetables namely Cha-Plu [*Piper sarmentosum* Roxb.(ชะพลู) Piperaceae], Yor [*Morinda citrifolia* L. (ยอ) Rubiaceae], Pak-Waan-Bann [*Sauropus androgynus* Merr.

(ผักหวานบ้าน) Euphorbiaceae] and Pak-Khom [*Amaranthus lividus* Linn. (ผักโขม) Amaranthaceae] are local vegetables in the central of Thailand. They are usually eaten in the raw and blanched forms.

Therefore, this study was aimed to investigate the mutagenic and antimutagenic effects of some green leafy vegetables either raw or blanched forms, including Cha-Plu [*Piper sarmentosum* Roxb.(ชะพลู) Piperaceae], Yor [*Morinda citrifolia* L. (ยอ) Rubiaceae], Pak-Waan-Bann [*Sauropus androgynus* Merr. (ผักหวานบ้าน) Euphorbiaceae] and Pak-Khom [*Amaranthus lividus* Linn. (ผักโขม) Amaranthaceae]. The assay used in this study was the Ames test using histidine-dependent *Salmonella typhimurium* strains TA 98 and TA 100.

1.2 Objectives of the Study

The s objectives of the present study were using the Ames test to determine the mutagenicity of raw and blanched selected vegetables namely Cha-Plu [*Piper sarmentosum* Roxb.(ชะพลู) Piperaceae], Yor [*Morinda citrifolia* L.(ยอ) Rubiaceae], Pak-Waan-Bann [*Sauropus androgynus* Merr. (ผักหวานบ้าน) Euphorbiaceae] and Pak-Khom [*Amaranthus lividus* Linn. (ผักโขม) Amaranthaceae] and their modulating effect on the mutagenicity of nitrite treated 1-aminopyrene and nitrite treated chicken extract.

1.3 Benefits of the Study

This study provides the information regarding the mutagenicity of raw and blanched selected vegetables mentioned above. Their modulating effect on the mutagenicity of nitrite treated 1-aminopyrene and nitrite treated chicken extract was also provided. The information obtained from this study should be used to justify that which vegetables are beneficial to consumer's health.

CHAPTER II
LITERATURE REVIEW

Cancer is a leading cause of death worldwide. World Health Organization (WHO) reported the disease accounted for 7.4 million deaths (or around 13% of all deaths worldwide) in 2004.The main types of cancer leading to overall cancer mortality each year are lung, stomach, colorectal, liver and breast cancer. Cancer is a multi-factorial, multi-stage and multi-mechanistic complex process with multiple risk factors that involve interplay between genetic and environmental components. Diet is also a major risk factor (Giovannucci, 1999). The carcinogens in diet may be exogenous in origin or formed endogenously by interact of food components; for instance, heterocyclic amines (Stavric, 1994). Doll and Peto (1981) estimated that dietary components may be responsible for about 35% of all cancer mortality. Many dietary components have been considered as etiologic factors for cancers of various sites. Rafter *et al*. (2004) suggested that we can significantly decrease cancer incidence through dietary recommendations and a change in dietary habits in populations.

2.1 Dietary Antimutagens

Diet is not only a source of mutagens and carcinogens but also a source of antimutagens or anticarcinogens. Several epidemiological studies highlighted the role of vegetables, fruits, herbs and spices in reducing the risk of cancer in many organs and tissues (Block *et al*., 1992; Potter and Steinmetz, 1996). There are many substances in fruits and vegetables that show anticarcinogenic effect, so that the entire effect is not very likely to be due to any single nutrient or photochemical. Steinmetz and Potter (1996) list possible antimutagen elements: dithiolthiones, isothiocyanates, indole-32-carbinol, allium compounds, isoflavones, protease inhibitors, saponins, phytosterols, inositol hexaphosphate, vitamin C, D-limonene, lutein, folic acid, beta carotene (and other carotenoids), lycopene, selenium, vitamin E, flavonoids, and dietary fiber. Mechanisms of dietary antimutagen are as shown in Table 1.

Table 1 Mechanisms by which dietary antimutagen could protect against mutation (Ferguson *et al*.,2004).

(1) Extracellular mechanisms	Examples of dietary antimutagens
(1.1) Inhibition of mutagen uptake	Dietary fibres, probiotics
(1.2) Inhibition of endogenous formation	
(1.2.1) Inhibition of nitrosation	Vitamins (ascorbic acid, α-tocopherol), sulphur compounds (cysteine, glutathione, *N*-acetyl cysteine), phenols (cinnamic acid, chlorogenic acid, butylated hydroxyanisole)
(1.2.2) Modification of the intestinal flora	Prebiotics, probiotics
(1.3) Complexation and/or deactivation	Dietary fibres, hemin, chlorophyllin
(1.4) Favouring absorption of protective agents	Vitamin D3 and analogues
(2) Cellular mechanisms	
(2.1) Blocking or competition	
(2.1.1) Scavenging of reactive oxygen species	Provitamins and vitamins (ß-carotene, ascorbic acid, α-tocopherol), diterpenes (sarcophytol a), polyphenols including epigallocatechin gallate and various anthocyanins
(2.1.2) Protection of DNA nucleophilic sites	Ellagic acid, retinoids polyamines
(2.2) Stimulation of trapping and detoxifycation in non-target cells	*N*-acetyl cysteine
(2.3) Modification of transmembrane transport	Short chain fatty acids (caproate, caprylate), acylglycosylsterols, dietary calcium
(2.4) Modulation of xenobiotic metabolizing enzymes	
(2.4.1) Inhibition of promutagen activation	Isothiocyanates, monocyclic monoterpenoids (limonene, methol, carveol), retinoids, flavonoids, wheat bran
(2.4.2) Induction of detoxification pathways	Polyphenols, indoles, diterpene esters, riboflavin 5'-phosphate, *S*-allyl-*L*-cysteine, allylic sulphides

Table 1 (continued)

(2.5) Modulation of DNA metabolism and repair	Cinnamaldehyde, vanillin, umbelliferone
(2.6) Enhancement of apoptosis	Retinoids, butyric acid, flavonoids
(2.7) Maintenance of genomic stability	Vitamins (folic acid, B12), minerals (selenium, zinc), polyphenols

2.2 Natural Components in Green Leafy Vegetables

Green leafy vegetables are good sources of many vitamins and minerals, they also rich in chlorophyll and carotenoids which are natural antioxidant. Many researches suggest that the nutrients found in dark green vegetables may prevent certain type of cancer and promote heart health (Hayatsu *et al*., 1993; Riboli and Norat, 2003)

2.2.1 Chlorophyll

All green plants contain chlorophyll, the light-collecting molecule. Chlorophyll is unstable in the presence of acid and light. Sunlight or fluorescent will degrade chlorophyll to pheophytin and pheophorbides. Chlorophyll is soluble in organic solvents such as ether, acetone, methanol, chloroform and pyridine. Pheophytins and pheophorbides are chlorophyll derivatives, are readily soluble in warm acetic acid and formic acids, and also in aqueous hydrochloric acids (Stokes, 1864). The degradation of the green chlorophyll pigments involves a number of reactions all of which are important because of the human dietary intake of vegetables which contain chlorophyll (Cano, 1991). Specific reactions in the degradation of chlorophyll entail the loss of green color during breakdown. Interestingly, plants have their chlorophyll molecules associated with proteins, carotenoids and tocopherols giving them stability (Hendry, 1996).

Chlorophyll and its derivatives are very effective at binding polycyclic aromatic hydrocarbons (carcinogens largely from incomplete combustion of fuels), heterocyclic amines (generated when grilling foods), aflatoxin (a toxin from molds in foods which causes liver cancer), and other hydrophobic molecules. The chlorophyll-carcinogen complex is much harder for the body to absorb, so most of it is swept out with the feces (Donaldson, 2004). Chlorophyll is of considerable interest as an anticarcinogenic

substance because it is so abundant in the green vegetables that human consume. Chlorophyll also has shown no toxic effects to human (Arbogast *et al*., 1995). Because of the cancer prevention effect of chlorophyll in foods, dark green vegetables, which contain high chlorophyll content, should be recommend to consuming in daily meal.

2.2.2 Carotenoids

Carotenoids are present in yellow and orange vegetables and fruits and in dark-green leafy vegetables. Green leafy vegetables, such as spinach, kale, broccoli, Brussels sprouts, and cabbage are moderately high in ß-carotene, but the predominant carotenoids in these vegetables are the oxygenated carotenoids (xanthophylls) (Steinmetz and Potter, 1991). Carotenoids are destroyed to some extent by cooking vegetables and the oxygenated carotenoids are destroyed to a greater extent than ß-carotene.

Carotenoids are nonpolar compounds, which are divided into two subclasses, i.e., more polar compounds called xanthophylls, or oxycarotenoids, and the nonpolar hydrocarbon carotenes. Examples of xanthophylls include lutein, zeaxanthin, capsanthin, canthaxanthin, astaxanthin, echionine and β-cryptoxanthin. Hydrocarbon carotenes include ß-carotene, α-carotene, γ-carotene and lycopene (See Figure 1).

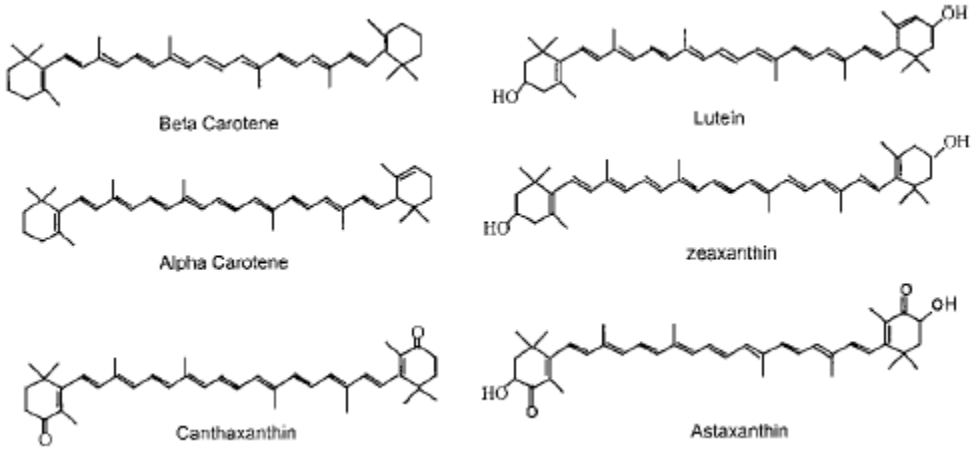


Figure 1 Chemical structures of the selected carotenoids (Zaripheh and Erdman, 2002).

Epidemiological evidence showed a link between intake of carotenoids from fruits and vegetables and relatively low incidence of various cancers. One of the mechanisms by which carotenoids may help protect against cancer is via its conversion to vitamin A. The functions of vitamin A include a role in the regulation epithelial cell differentiation.

Because lack of differentiation is a feature of cancer cells, and etiologic role for inadequate vitamin A is plausible. Wattenbuerg (1985) in a review of potential chemopreventive agents, categorized retinoids as inhibitors that act both as suppressing agents that retard development of neoplasia in cells that have already been exposed to carcinogenic agents and as inhibitors of tumor promotion. Additionally, an anti-cancer role of carotenoids may be related to their ability to quench singlet oxygen. These antioxidant effects may protect cells against oxidative DNA damage. ß-carotene may enhance immunologic function (Steinmetz and Potter, 1991).

2.2.3 Folic acid

Folic acid is in the dark green leafy vegetable vitamin. It has an integral role in DNA methylation and DNA synthesis. Folic acid works in conjunction with vitamin B-6 and vitamin B-12 in the single carbon methyl cycle. If insufficient folic acid, uracil is substituted for thymidine in DNA, which leads to DNA strand breakage. Folate may be more important for rapidly dividing tissue, like the colonic mucosa. Therefore, the cancer risk associated with low folate intake is probably higher for colon cancer than breast cancer (Donaldson, 2004).

2.2.4 Vitamin C

Vitamin C is present in the greatest amounts in fruits and vegetables, including citrus fruits and juices, broccoli, green peppers, tomatoes, strawberries, melons, cabbage and leafy green vegetables (Steinmetz and Potter, 1991). In a review of potential cancer-inhibiting agents, Wattenberg (1985) categorized vitamin C as preventing the formation of carcinogens from precursor compounds. One mechanism by which vitamin C may prevent cancer, especially in the stomach, is through its ability to scavenge and reduce nitrite, thus reducing the substrate for the reaction with secondary amines to form nitrosamines. Furthermore, vitamin C is an antioxidant and plays a role in the immune system.

2.2.5 Vitamin E

Vitamin E has a fundamental role in protecting the body against the damaging effects of reactive oxygen species that are formed metabolically or encountered in the environment. Vitamin E occurs in food as compounds called tocopherols and tocotrienols. Vitamin E protects polyunsaturated fatty acids in cell membrane from oxidation by

scavenging free radicals and terminating free-radical chain reactions (Gallgher, 2004). Vitamin E also functions to keep carotenoids in a reduced state, thereby enhancing their antioxidant capacity and decreases the formation of nitrosamines in the stomach (Bertram *et al*., 1987 and Fiala *et al*., 1985).

2.2.6 Flavonoids

Flavonoids are one of the plant phytochemicals. These universal plant pigments are responsible for the colors of flowers, fruits and sometimes leaves (Hollman *et al*., 1997). Flavonoids are a group of polyphenolic antioxidant compounds with cancer blocking or suppressing properties. They scavenge free radical compounds, such as superoxide anion and singlet oxygen, and sequester metal ions, thus they may protect tissues against free oxygen radicals and lipid peroxidation (Mathai, 2004).

2.3 Study Vegetables

Thailand has a variety of vegetables and Thai people utilize them in different ways. The uncooked vegetables are commonly used as a side dish; cooked vegetables are often eaten as a side dish or mixed in stew with meat, fish and other foods; other variations include curried, fried, broiled or baked vegetables. Vegetables are often cooked with water, oil, coconut milk and sometimes wine, blanched and served with traditional chili sauces, and even eaten in the raw form. Various kinds of vegetables are commonly consumed in Thailand. Some of them also have diverse medicinal properties. This research selected some green leafy vegetables that usually eaten in the central of Thailand namely, Cha-Plu [*Piper sarmentosum* Roxb.(ชะพลู) Piperaceae], Yor [*Morinda citrifolia* L. (ยอ) Rubiaceae], Pak-Khom [*Amaranthus lividus* Linn. (ผักโขม) Amaranthaceae] and Pak-Waan-Bann [*Sauropus androgynus* Merr. (ผักหวานบ้าน) Euphorbiaceae] (อุไร จิรมงคลการ , 2547). However, there is little information available at the present time regarding their antimutagenic and anticarcinogenic effects of these Thai vegetables.

2.3.1 Cha-Plu (*Piper sarmentosum* Roxb.) a plant in the Piperaceae family, is used as food ingredient in many Southeast Asian cuisines (Figure 2). Cha-plu leaves are usually eaten raw. In Thai cuisine, it is used to wrap miang kam, a tasty traditional snack originally from Central and Northern of Thailand. It contains calcium, ferrous, niacin and

high fiber. It is also used in traditional medicines for health promotion, expectorant, anti-flatulant and element tonic (อุไร จิรมงคลการ, 2547).

Figure 2 *Piper sarmentosum* (Cha-plu)

Subramaniam *et al*.(2003) found that methanolic leave extract of *Piper sarmentosum* has shown high antioxidant activity by using the Xanthine/Xanthine Oxidase (X/XOD) Superoxide Scavenging assay.. The cause of high antioxidant property of this plant may be an effect of naringenin, a highly potent natural antioxidant compound. Thus this plant could be considered as antioxidant food.

2.3.2 Yor or noni (*Morinda citrifolia* L.) belongs to family Rubiaceae (Figure 3). It is an evergreen shrub whose ripe fruit has a strong butyric smell and flavor. The leaves and especially fruit are consumed in different forms by various communities. Yor leaves are usually eaten blanched and served with chili paste or cooked with vegetables soup. The root is used as a dye. Yor is the source of calcium, vitamin A, vitamin B1, vitamin B2, niacin and fiber. It is used in folk medicines for health promotion, blood tonic, anti-diarrhea, anti-flatulant, anti-nausea and vomiting. (อุไร จิรมงคลการ, 2547).

Figure 3 *Morinda citrifolia* L.(Yor or noni)

Several classes of compounds have been isolated from Yor including amino acids, anthraquinones, coumarins, fatty acids, flavonoids, lignans and polysaccharides (Chan-Blanco *et al*., 2006). Many reports on antioxidative activity of Yor itself have been published. Zin *et al*. (2002) have evaluated the antioxidative activity of extracts from different parts of *Morinda citrifolia* measured by ferric thiocyanate method (FTC) and thiobarbituric acid test (TBA). They found that the methanol extract of Yor root exhibited high antioxidative activity, while the methanol extracts of fruit and leaf showed negligible activities. On the other hand, the ethyl acetate extract of all parts of Yor exhibited significant antioxidative activity. Furthermore, Murakami *et al*. (1995) found that methanol extract of Yor leaves have shown strongly active anti-tumor promoting activity (*in vitro*).

2.3.3 Pak-Khom (*Amaranthus lividus* Linn. Synonym: *Amaranthus blitum*) is a shrub plant in the Amaranthaceae family (Figure 4). Leaves are usually eaten boiled and blanched and served with chili paste as a side dish. It is used in traditional medicines for detoxify, anti-amoebiasis, anti-rash and hemorrhoids (กัญจนา ดีวิเศษ, 2542).

Figure 4 *Amaranthus lividus* Linn. (Pak-khom)

Amin *et al*. (2006) determined the antioxidant activity of *Amaranthus* sp. They reported that the extract of *Amaranthus blitum* has shown a highest free radical-scavenging activity.

2.3.4 Pak-Waan-Bann (*Sauropus androgynus* Merr.) belongs to family Euphorbiaceae (Figure 5). It is a perennial shrub found growing in South East Asia (ฐิติ เอื้ออังกูร, 2547). In Thailand people cook it with meat, coconut milk and other vegetables to make soup. Leaves are blanched to serve with chili paste. It is used in traditional medicines for antipyretic and anti-inflammation (กัญจนา ดีวิเศษ, 2542).

Figure 5 *Sauropus androgynus* Merr. (Pak-Waan-Bann)

Sauropus androgynus leaf was reported to contain high nutrition value nutrients such as carotene, thiamine, riboflavin, vitamin C, calcium, phosphorous and iron and also contain high levels of flavonoids and total phenols (Padmavathi and Rao, 1990; Subhasree *et al*., 2009). Liu *et a*l. (2007) found that leaf of this vegetable had high levels of lutein and zeaxanthine. This may be the scientific rationale in support of believes of the local populations, that these leafy vegetables are good for eye health and visions. However, leaf of *Sauropus* was reported to contain amount of the alkaloid papaverine. Excessive consumption of the leaf reportedly caused dizziness, drowsiness, constipation, etc. (Padmavathi and Rao, 1990).

2.4 Mutagens in Foods

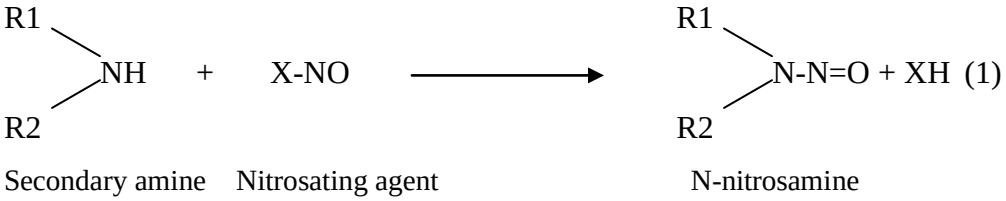
Many of the cancers common in the Western world, including colon, prostate and breast cancers, are thought to relate to dietary habits (Ferguson *et al*., 2004). High intakes of total calories and fat enhance cancer development, excessive intake of sodium chloride promotes carcinogenesis in the stomach (Wakabayashi *et al*., 1992). Foods also contain mutagenic and/or carcinogenic substances as very minor components such as nitrosamines, heterocyclic amines and polycyclic aromatic hydrocarbons. Excessive intake of these carcinogenic substances may be a leading cause of cancer.

2.4.1 Nitrite as a Converter for Direct mutagens

Nitrates and nitrites have received attention because of their relationship with nitrosamines, which are potent carcinogens in various species. Nitrates are present in a variety of foods, but the main dietary sources are vegetables and drinking water (Eldridge, 2004). The most common high-nitrate vegetables are listed in Table 2.

Sodium and potassium nitrate are used in the processes of salting, pickling and curing foods (Eldridge, 2004). Nitrates and nitrites in preserved meats (bacon, cold cuts) can prevent growth of *Clostridium botulinum*, the organism that can produce the potent botulinum toxin. High dietary nitrate intake was suggested to pose a health risk, causing infantile methemoglobinemia and gastric/intestinal cancer through the production of carcinogenic N-nitrosamines (Duncan *et al*., 1997). Nitrite as such is not a nitrosating agent, but is readily oxidized to a series of nitrosating substances, viz. N_2O_3, N_2O_4, H_2ONO^+ and NO^+, depending on the acidity of the medium. N-nitroso compounds form

when a nitrosating agent reacts with secondary nitrogen groups under acidic condition (pH<4). If the nitrosatable compound is a secondary amine, the product is a N-nitrosamine (Equation 1).



R1 and R2 could be any of a large number of chemical groups (Marais, 1997). Reduction of nitrate to nitrite is a common reaction for bacteria in the GI tract. Usually, the GI effect on nitrite is preceded by nitrate being reduced to nitrite by microflora of mouth saliva. Nitrosamine formation can occur under the acidic condition of the GI tract. Also, the nitrosation reaction can occur during the frying of nitrite-cured bacon. Certain nitrosamines induce cancers in the liver, kidney, bladder, GI tract, pancreas and respiratory tract (Omaye, 2004).

Table 2 The nitrate content of the major high-nitrate vegetables (Marais, 1997).

Vegetable	Botanical name	Nitrate content (mg kg^{-1} fresh)
Beetroot	*Beta vulgaris*	900
Brinjal	*Solanum melongena*	1300
Cabbage	*Brassica oleracea*	810
Cauliflower	*Brassica oleracea*	1310
Celery	*Apium graveolens*	1200
Fennel	*Foeniculum vulgare*	2000
Kohlrabi	*Brassica oleracea*	1060
Lettuce (butterhead)	*Lactuca sativa*	5360
Chinese mustard	*Brassica juncea*	5670
Radish	*Raphanus sativus*	1100
Spinach	*Spinacea oleracea*	3560
Turnip	*Brassica rapa*	970
Watercress	*Nasturtium officinale*	1300

Many investigators examined the diets eaten mostly in Eastern European countries where the gastric cancer rate was high (Marqauard *et al*., 1977). Ohshima *et al*. (1989) found that smoked foods, a frequent consumed food item, was associated with increased risk of gastric cancer ; they were revealed to be direct acting genotoxicity after nitrosation *in vitro*. Münzner and Wever (1984) found that the products formed by the reaction of beef extract with nitrite were assayed in the *Salmonella*/microsome mutagenicity test on strains TA1538, TA98 and TA100. The products exhibited mutagenicity activity towards all tester strains with and without metabolic activation. Marqauard *et al*. (1977) indicated that fish, beans and borscht showed the formation of one or more mutagenic on *Salmonella typhimurium* TA1535 after treated with 5000 ppm nitrite at pH 3.0. In addition, broiled chicken, pork, mutton, beef and sun-dried sardine were found to yield direct acting mutagenicity on TA98 and TA100 without metabolic activation (Yano *et al*., 1988) (Table 3).

Table 3 Mutagenicity of cooked foods after nitrite treatment (Yano *et al*., 1988).

Food	Revertants/gm original material	
	TA98	TA100
Chicken	33,300	12,800
Beef	22,600	7,400
Mutton	43,600	5,700
Pork	15,000	3,800
Sun-dried sardine	20,200	17,900

Cooked meats, smoked food and charcoal-broiled foods are common foods which polycyclic aromatic hydrocarbon (PAH) were detected and quantified. The nitrite treated products of polycyclic aromatic hydrocarbon (PAH) extracts from smoked fish, skin of fresh water catfish, charcoal-broiled chicken wing, rice pork sausage, pork (medium fat) were mutagenic towards both strains TA98 and TA100 (Kangsadalampai *et al*., 1996). New substances were not *N*-nitroso compound but might be nitro-polycyclic aromatic hydrocarbons (nitro-PAHs).

Japan also has a high incidence of gastric cancer which may relate to dietary habit. Japanese fish, soy sauce and Chinese cabbage, which are favorites of the Japanese showed direct acting mutagenicity on *Salmonella typhimurium* TA100 after nitrite treatment. Wakabayashi *et al*. (1985) have made extensive studied on the appearance of direct-acting mutagenicity of various foodstuffs produced in Japan and Southeast Asia, such as the Philippines and Thailand, on nitrite treatment. After nitrite treatment, various kinds of pickled vegetable and sun-dried fishes produced in Japan showed direct-acting mutagenicity on *Salmonella typhimurium* TA100. In addition, soy sauces widely used, as a seasoning in Southeast Asia and Japan were strongly mutagenic to *Salmonella typhimurium* TA100 after being interacted with 50 mM nitrite.

Various foods produced in Asia were reported on their direct-acting mutagenicity after nitrite treatment. Kimchis, sun-dried fishes, sun-dried squid, soy sauces, fish sauces, bean pastes and shrimp paste produced in Korea, the Philippines and Thailand showed direct acting mutagenicity after nitrite treatment (Wakabayashi *et al*., 1985). Palli (1996) indicated that salted/smoked and pickled/preserved foods (rich in salt, nitrites and performed nitroso compounds) were associated with an increased risk of gastric cancer. Salted pickled cabbage eaten by Korean three times a day contained high levels of total *N*-nitroso compounds (1,173 µg/kg) after treatment with nitrite under simulated human stomach conditions. Additionally, the extracts of raw and pickled vegetables and fruits, namely garlic, cabbage, shallot, mushroom, cucumber, ginger, Chinese mustard, bamboo shoot and mango were treated with nitrite in the absence of metabolic activation. All of them exhibited direct-acting mutagenicity in *Salmonella* assay (Hankimhun, 1997).

The interaction of orally administered drugs with nitrite under mildly acidic conditions has been considered from a safety point of view of the drugs. Common drugs including aminopyrine (tertiary amines) react with nitrite to form dimethylnitrosamine (or dialkylnitrosamines) (Lijinsky *et al*., 1972; Lijinsky, 1974). Phenolic drugs including bamethan, acetaminophen and etilefrin also became mutagenic on nitrite treatment under mildly acidic condition (Kikugawa *et al*., 1987; Ohta *et al*., 1988; Kikugawa *et al*., 1989). Therefore. Several nitrosable mutagen precursors in foods taken by people in high-risk areas might be the etiological factor of gastric cancer, investigation must be continued to

elucidate whether nitrosable compounds are involved in the development of human cancer, particularly of the stomach.

2.4.2 Heterocyclic Amines (HCAs)

Food is essential to provide nutrients but may also be an important factor in the genesis of human diseases, for example, cancer. (Doll and Peto, 1981). Commonly eaten meat products prepared from beef, pork, mutton and chicken all show some level of mutagenic activity following cooking. Food preparation methods have a significant influence on the formation of the mutagenic activity, and many studies have been devoted to the mutagenic activity in fried/broiled food. Smoking, frying, broiling, barbecuing (flame-grilling), heat processing and pyrolysis of protein-rich foods induce the formation of potent mutagenic and carcinogenic compounds (Felton *et al*., 2000; Sugimura, 2000). Creatine and creatinine, free amino acids and sugars have been shown as precursors of HCAs (Felton *et al*., 2000; Omaye, 2004). Several investigators have proposed that non-enzymatic browning (Maillard reaction) had an important role in the formation of mutagens and possible carcinogens, without specifying a reaction route (Powrie *et al*., 1982; Shibamoto *et al*., 1981; Wei *et al*., 1981). The Maillard reaction takes place in foods through the reaction of carbonyl compounds (aldehydes and ketones), notably reducing sugar, such as glucose, fructose, etc. with compounds possessing free amino groups, such as amino acids, peptides and proteins. The reaction is of great important for the development of flavors, texture and brown pigments during heat treatment of foodstuffs, thereby contributing to the palatability of cooked foods.

More than 20 HCAs have been isolated as mutagens from various heated materials, and structures of 19 of these compounds are now known. Among these, ten HCAs have been studied for their chemical and biological properties including their respective carcinogenicities (Wakabayashi and Sugimura, 1998). Full names and abbreviations of HCAs are listed in Table 4 and their structures are shown in Figure 6.

Table 4 Full names and common abbreviations of HCAs (Sugimura *et al*., 2004).

Common abbreviation	Full name
IQ	2-amino-3-methylimidazo[4,5-*f*]quinoline
MeIQ	2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline
IQx	2-amino-3-methylimidazo[4,5-*f*]quinoxaline
MeIQx	2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
DiMeIQx	2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline
7,8-DiMeIQx	2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline
4-CH_2OH-8-MeIQx	2-amino-4-hydroxymethyl-3,8-dimethylimidazo[4,5-*f*]quinoxaline
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
4´-hydroxy-PhIP	2-amino-6-(4-hydroxyphenyl)-1-methylimidazo[4,5-*b*]pyridine
Trp-P-1	3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole
Trp-P-2	3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole
AαC	2-amino-9*H*-pyrido[2,3-*b*]indole
MeAαC	2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole
Glu-P-1	2-amino-6-methyldipyrido[1,2-*a*:3´,2´-*d*]imidazole
Glu-P-2	2-aminodipyrido[1,2-*a*:3´,2´-*d*]imidazole

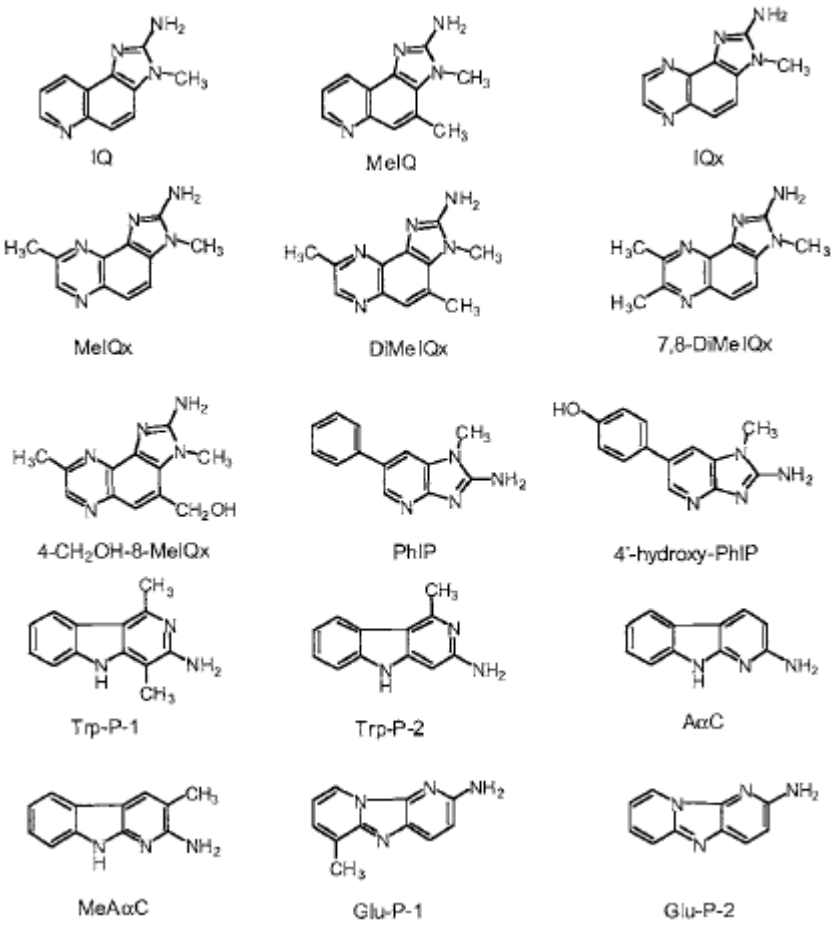


Figure 6 Structures of HCAs (Sugimura *et al*., 2004).

Heterocyclic amines are classified into two groups by treatment with 2 mM sodium nitrite for example Aminoimidazoazaarene and Carbolines (Robbana-Barnat *et al*., 1996; Sugimura *et al*., 2004).

2.4.2.1. Aminoimidazoazaarene (AIAs) (IQ-type, polar)

AIAs compounds have a 2-aminoimidazo group fused to a quinoline (IQ and MeIQ), a quinoxaline (MeIQx, and DiMeIQx), or a pyridine (PhIP) ring. The amino group is not changed by treatment with 2 mM sodium nitrite but it converted to a nitro group, which is a direct carcinogenic compound, with 50 mM sodium nitrite. The main precursors of the AIAs found in meat and fish are creatine/creatinine, free amino acids and sugars. The discovery that reducing sugars, amino acids, and creatine are AIA precursors led to speculation that the Maillard reaction plays an important role in the formation of these mutagens.

2.4.2.2. Carbolines (Non-IQ-type, non-polar)

Non-IQ type is produced by pyrolysis of amino acids and protein, being produced at higher temperature than IQ type (Tsuda *et al*. 1985). The carbolines and their analogues are the type "non-IQ" contrary to the AIAs. They include the aminopyridoindoles (Trp-P-1, Trp-P-2, AαC, MeαC), the aminopyridoimidazoles (Glu-P-1, Glu-P-2, Lys-P-1, Orn-P-1), and an aminophenyl-pyridine (Phe-P-1). On 2 mM nitrite treatment, carbolines group lose their mutagenicity through conversion of amino to hydroxyl groups, while amino group of aminoimidazoazaarene group is not changed.

International Agency for Research on Cancer (IARC) has rated many chemicals into the categories of human carcinogen, possible human carcinogen and non-human carcinogen, based on information regarding chemical nature and pharmacological, as well as animal experimental and epidemiological data. IQ is in Group 2A “probably carcinogenic to humans” and MeIQ, MeIQx and PhIP are in Group 2B “possibly carcinogenic to humans”.

HCAs requires metabolic activation before its carcinogenicity is expressed. The proximate carcinogenic intermediate, *N*-hydroxylamino derivatives, is formed by hydroxylation of the exocyclic amino group, a reaction catalyzed by micosomal cytochrome P450 (CYP1A2) in S9 mix. Further esterification of *N*-hydroxylamino derivatives by phase II enzymes such as *N*-acetyltransferase, arylsulfotransferase, prolyl tRNA synthase and phosphorylase (ATP-dependent kinase), greatly enhances its reactivaty with DNA (Schut *et al*., 1997). The ester moieties serve as leaving groups giving rise to putative electrophilic arylnitrinium ion intermediates considered for many years to be involved in arylamine-DNA adduct formation. Although to a much lesser extent, arylnitrinium ions may also be generated directly from the *N*-hydroxylamine metabolite following the protonation of the *N*-hydroxylamino group. This reaction mechanism explains the DNA adduction of certain *N*-hydroxy-AIAs, such as *N*-hydroxyl-IQ, in the absence of esterification. The arylnitrinium ion is generally considered to be the ultimate carcinogenic form of the Aminoimidazoazaarenes (AIAs) responsible for the formation of AIA-DNA adducts. IQ, MeIQ, MeIQx, PhIP have been reported to formed mainly adducts at the C-8 position of the guanine base in DNA (Wakabayashi *et al*., 1993).

2.4.3 Polycyclic Aromatic Hydrocarbons (PAHs)

Many cooking methods involve intense heat with limited availability of oxygen, both favorable conditions for mild browning or pyrolytic (300°C) decomposition of the food's fat and protein components. Various browning products, polycyclic aromatic hydrocarbon and heterocyclic amines are the major decomposition products of such cooking. PAHs are produced during cooking, mainly by pyrolysis of fats, whereas HCAs are pyrolysis products of amino acids. PAHs can be from environmental sources, such as those occurring in wood-burning stoves, diesel exhaust, and oil-burning heaters. PAHs are formed when fat from food drips into the hot coals and is incinerated. The resulting PAHs in smoke can subsequently adsorb to the cooking food. PAHs most likely to pose human health problems are benzo(α)pyrene (3,4 benzpyrene) and 7,12-dimethylbenz-anthrene (DMBA). Although most PAHs, particularly the low molecular weight compounds, are non carcinogenic, both PAHs and other similar compounds are potent carcinogens that are active after metabolic conversion to electrophilic epoxide derivatives (Omaye, 2004).

The major site for PAH-induced carcinogenesis is the skin. Tumors can be produced at other sites following ingestion, but the concentrations required are quite high. Inhaled PAHs (tobacco smoke) have been implicated in cancer of the respiratory system, and the magnitude of this disease is likely to be more than that by the dietary route (Omaye, 2004). Lowering exposure to PAHs include stopping smoking, reducing the use of wood-burning stoves, and decreasing consumption of smoked and char-broiled foods.

2.5 Direct-Acting Mutagens Models

2.5.1 Chicken Extract-Nitrite Mutagenicity Model

Peerawong and Kangsadalampai (1998) reported that chicken extracts ,which contain HCAs, treated with nitrite in acid condition (pH 3.0-3.5, 37°C) have shown mutagenicity on *S.typhimurium* strains TA98 and TA100. Agreed with previous study, Nagao (2000) found that various HCAs are mutagenic toward *Salmonella typhimurium* as shown in Table 5. Solyakov and Skog (2002) have suggested that the content of HCAs in chicken cooked in various ways is low if prepared at low temperatures, and increases with increasing cooking temperature.

Table 5 Mutagenicity of HCAs in *Salmonella typhimurium* TA98 and TA100 with S9 mix (Nagao, 2000).

HCA	Revertants/µg	
	TA98	TA100
MeIQ	661,000	30,000
IQ	433,000	7,000
DiMeIQx	183,000	8,000
7,8- DiMeIQx	163,000	9,900
MeIQx	145,000	14,000
Trp-P-2	104,200	1,800
4-CH_2OH-8-MeIQx	99,000	3,000
IQx	75,400	1,500
Glu-P-1	49,000	3,200
Trp-P-1	39,000	1,700
Glu-P-2	1,900	1,200
PhIP	1,800	120
AαC	300	20
MeAαC	200	120
4´-hydroxy-PhIP	2	no data available

2.5.2 1-Aminopyrene-Nitrite Mutagenicity Model

1-Aminopyrene is a derivative of 1-nitropyrene found in human gastrointestinal tract. Anaerobic bacteria metabolize 1-nitropyrene to 1-aminopyrene. 1-Nitropyrene is generally a product of incomplete combustion and is the predominant nitro-PAHs emitted in diesel engine exhaust, exhaust of kerosene heaters, petroleum gas burners and food products as a result of pyrolysis of fat in meat during barbecuing (Rosenkranz and Mermelstein, 1983; Handa *et al*., 1983; Tokiwa *et al*., 1985; Kinouchi *et al*., 1986 and Edenharder *et al*., 1993). The most primary route of potential human exposure to 1-nitropyrene is inhalation.

The metabolism of 1-nitropyrene occurs through cytochrome P450-mediated ring C-oxidation to epoxides, with subsequent rearrangement to nitropyrenols or hydration to

dihydrodiols, or through nitroreduction to 1-nitrosopyrene, *N*-hydroxy -1-aminopyrene or 1-aminopyrene (Figure 7) (Howard *et al*., 1995). Metabolites are presumably further metabolized via hydrolysis by epoxide hydrolases and/or by conjugation with glutathione, glucuronic acid, sulphate and then excreted as conjugates as shown in Figure 7 (Djuric *et al*., 1987; Kataoka *et al*., 1991).

Nitroreduction of nitro-PAHs *in vivo* occurs mainly by bacteria in the intestinal tract (El-Bayoumy *et al*., 1982; Moller *et al*., 1988; Ball *et al*., 1991). Nitroreduction in mammalian cells is catalysed by xanthine oxidase, aldehyde oxidase, or NADPH cytochrome P450 reductase. Complete nitroreduction of nitro-PAHs results, through a *N*-hydroxylarylamine intermediate, in the formation of the corresponding arylamine. Arylamines can be reactivated through *N*-oxidation by a cytochrome P450-mediated reaction to form the *N*-hydroxylarylamine (Shimada *et al*., 1989). Although reactive arylnitrenium ion that can bind to DNA is formed when the hydroxyl group leaves the *N*-hydroxylarylamine, a more reactive species is formed through esterification of the *N*-hydroxylarylamine by acetyltransferases or sulfotransferases (Miller *et al*., 1981; Rosser *et al*., 1996).

1-Nitropyrene is C-oxidized by cytochromes P450 to 1-nitropyren-4,5-oxide, which is hydrolyzed via epoxide hydrolase to 1-niropyrene-4,5-dihydrodiol. Cytochrome P450 C-oxidation at the other K-region results in the formation of 1-nitropyren-9,10-oxide, which is hydrolyze via epoxide hydrolase to 1-nitropyrene-9,10-dihydrodiol. Three non-K-region phenolic metabolites of 1-nitropyrene are formed and include 1-nitropyren-3-ol, 1-nitropyren-6-ol and 1-nitropyren-8-ol. Nitroreduction of 1-nitropyrene can occur in one or two electron steps to form 1-nitrosopyrene, *N*-hydroxy-1-aminopyrene or 1-aminopyrene, the latter of which is acetylated to form *N*-acetyl-1-aminopyrene.

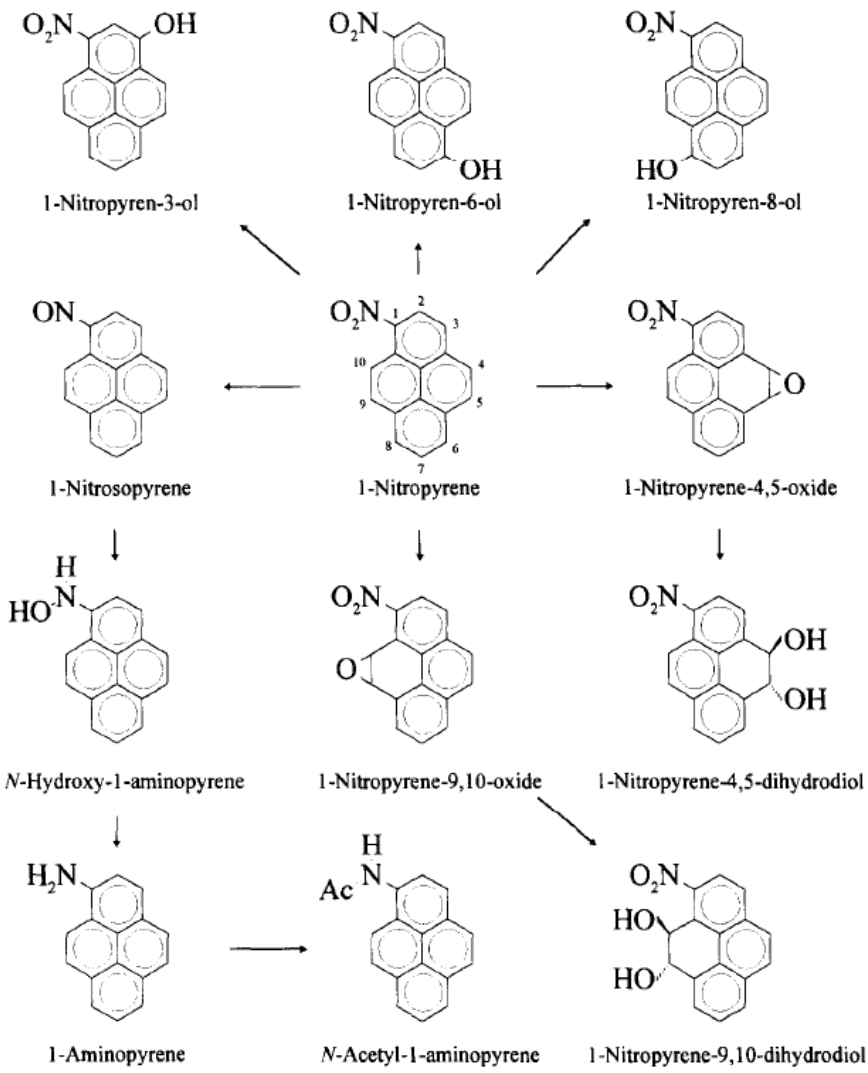


Figure 7 Scheme for the metabolism of 1-nitropyrene in mice (Howard *et al*., 1995).

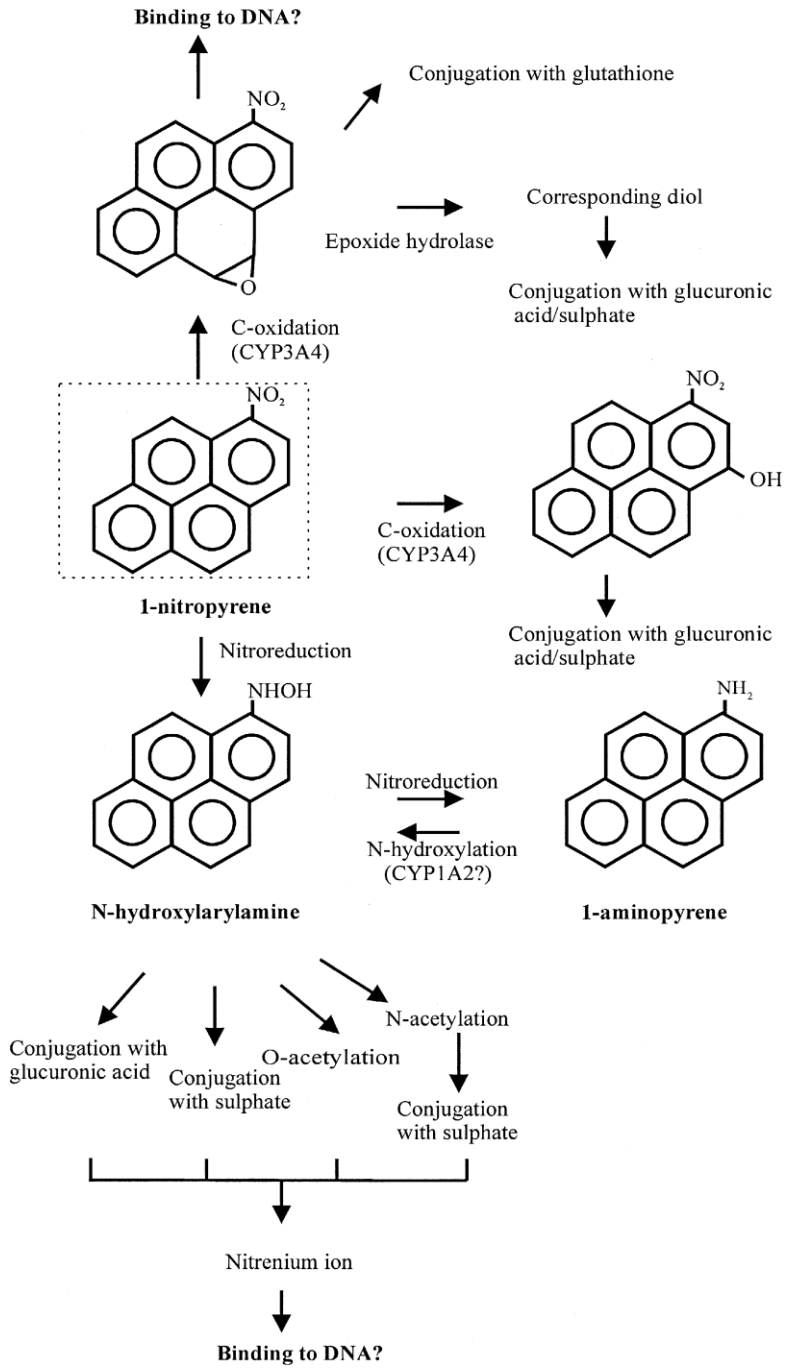


Figure 8 Potential pathways of 1-nitropyrene detoxification and metabolic activation in human and rat (Howard *et al*., 1983; Kataoka *et al*., 1991)

1-Aminopyrene was known to be non-mutagenic when it was tested without metabolic activation (Kinouchi *et al*., 1986). Kato *et al*. (1991) demonstrated that 1-aminopyrene treated with nitrite at pH 3.0 and 37°C showed mutagenicity to *Salmonella*

typhimurium strains TA98 and TA100 without metabolic activation. The results agreed with the work of Kangsadalampai *et al*. (1996), which stated that nitrite-treated 1-aminopyrene exhibited stronger mutagenicity than the authentic aminopyrene towards *Salmonella typhimurium* strains TA98 (frame-shift mutation) and TA100 (base-pair substitution), in the absence of metabolic activation. The mutation appear to be due to the presence of nitroreductases (IARC, 1989) and *O*-acetyltransferase (Mermelstein *et al*., 1981) which are the two activating systems presented in bacterial cells for nitrite-treated 1-aminopyrene (supposed to be 1-nitropyrene). Such enzymes metabolize 1-nitropyrene to be arylhydroxylamine, which is active to interact with DNA. Evidences had been shown that 1-nitropyrene induced tumors in experimental animals (El-Bayoumy *et al*., 1982; Rosenkranz and Mermelstein, 1983; Busby *et al*., 1994). Thus, the mutagenicity of 1-aminopyrene and nitrite in acid condition has been established as a model for antimutagenicity study of some chemical concerning the phenomenon occurred during stomach digestion.

2.6 The *Salmonella* Mutagenicity Test (Ames test)

Bacterial mutagenicity assays, especially the Ames test (*Salmonella typhimurium* his^+ reversion assay) have been used worldwide, in research laboratories. This test is a short-term tests used for screening of potential mutagens and carcinogens. They combine a high sensitivity for genotoxins with relative technical ease, rapidity and economy. According to current understanding of chemically induced carcinogenesis, it was premature to expect that one *in vitro* mutagenesis test could have the predictive power for chemical carcinogenicity. In addition, there are several mechanisms of chemical carcinogenesis that mechanistically would not be detected in the Ames test. Nevertheless, the Ames test is the only short-term test considered to be thoroughly validated and remains a very widely used and powerful method for detecting mutagens and potential carcinogens, especially when conducted in a battery of complementary short-term tests. As with all methods, the Ames test is most valuable when the investigator is knowledgeable of its limitations (Barile, 1994).

The strains and genotypes of bacteria most widely used for mutation assays are listed in Table 6.

Table 6 Genotypes of bacteria strains widely used in reverse-mutation assays (Venitt, 1984).

Strain	Amino acid marker			Other relevant mutations		Plasmid
	Histidine mutation	Type of mutation	Main DNA target[a]	Cell-wall	DNA-repair	
			Salmonella typhimurium			
TA1535	*hisG46*	Base pair substitution	GC	*rfa*	*uvrB*	No
TA100	*hisG46*	Base pair substitution	GC	*rfa*	*uvrB*	pKM101
TA1537	*hisC3076*	Frame shift	GC	*rfa*	*uvrB*	No
TA1538	*hisD3052*	Frame shift	GC	*rfa*	*uvrB*	No
TA98	*hisD3052*	Frame shift	GC	*rfa*	*uvrB*	pKM101
TA97	*hisD6610*	Frame shift	GC	*rfa*	*uvrB*	pKM101
TA102	*hisG428*	Base pair substitution	AT	*rfa*	+	pKM101 pAQ1 (multicopy carries *hisG428* mutation)

[a]Mutation induction at suppressor loci may allow detection of agents which react at other sites.

The *Salmonella* histidine reverse mutation assay is based on the use of several selected histidine dependence (auxotrophy) to histidine independence (prototrophy) at an increased frequency of a mutagen. The assays are normally conducted with and without S9 activation. The test detects a wide variety of mutagens, including many that require an exogenous metabolic activation system. The test is used as a screen for mutagenic activity of pure compounds, complex mixtures, and body fluids. This identifies not only those compounds requiring metabolism to express mutagenic activity, but also identifies chemicals which are detoxified by metabolisms (Barile, 1994). At present the most commonly used *Salmonella* strains are TA 1535, TA 1537, TA 1538, TA 98 and TA 100 (Maron and Ames 1983). All strains contain some type of mutation in the histidine operon, while additional mutations increase their sensitivity to mutagens. For instance, the *rfa* mutation causes partial lose of the lipopolysaccharide surface coating of bacteria and thus increases the permeability to large molecules that do not normally penetrate the cell wall. Another mutation (*uvrB*), greatly increases the sensitivity of the bacteria

towards mutagens by deleting the gene coding for DNA excision repair. Many of the standard tester strains contain the R-factor plasmid, pKM101. these R-factor containing strains are sensitive to a number of mutagens that are inactive or weakly active in the non-R-factor parent strains. This is apparently due to the enhancement of an error-prone repair system which is normally present in these organisms (Barile, 1994).

McCann and Ames (1977) discussed several aspects of the experimental basis for their current assessment of the value of the test as useful predictive tool:

1. The predictive value of the test as an indicator of carcinogenic potential, including both the strengths and weaknesses of the test at this stage in its development.

2. Current applications of the test method to problems that were not approachable using conventional animal test methods.

3. Some of the environmental chemicals that have already been pinpointed as potential carcinogens by the test and the current status of carcinogenicity tests of these chemicals in animals.

4. The evidence that the correlation between carcinogenicity and mutagenicity in the *Salmonella* test reflected more than a useful coincidence and fitted into a compelling collection of evidence supporting a central role for somatic mutation in the initiation of human cancer.

2.7 The Mutagenicity Test (Preincubation Method) Using *Salmonella typhimurium*

Some mutagens, such as dimethylnitrosamine and diethylnitrosamine are poorly detected in the standard plate incorporation assay and should be tested using a modification of the standard procedure. The most widely used test modification is the pre-incubation assay first describe by Yahagi *et al*. (1975), in which carcinogenic azo dyes were found to be mutagenic. They incubated the mutagen and bacteria for 20-30 min at 37°C and then added the top agar. The assay has been also used to detect the mutagenicity of 10 carcinogenic nitrosamines (Yahagi *et al*., 1977) and several carcinogenic alkaloids (Yamanaka *et al*., 1979). The mutagenic activity of aflatoxin B1, benzidine, benzo[a]pyrene and methylmetane sulfonate has been determine using both plate incorporation and pre-incubation procedures and in all cases the pre-incubation assay is of equal or greater sensitivity than the plate incorporation assay (Matsushima *et*

al., 1980). The increased activity is attributed to the fact that the test compound and bacteria are incubated at higher concentration in the pre-incubation assay than in the standard plate incorporation test. The procedure described below is based on recommendation of Matsushima *et al*. (1980).

The pre-incubation modification can be used routinely or when in conclusive results are obtained in the standard plate incorporation assay. This assay requires an extra step and therefore involves more work than the standard test. Nevertheless, many laboratories use it routinely because of the increased sensitivity towards some compounds. Its use in screening assays has been recommended by De Serres and Shelby (1979).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals for Ames Test

1-Aminopyrene, ammonium sulfamate, dimethylsulfoxide, d-biotin, sodium dihydrogen phosphate, and sodium nitrite were purchased from Sigma Chemical (St Louis, USA). Bacto agar, crystal violet, dipotassium hydrogen phosphate anhydrous, histidine monohydrochloride, hydrochloric acid 34% w/v, magnesium sulphate heptahydrate, and potassium chloride were purchased from Merck (Darmstadt, Germany). Citric acid, d (+) – glucose anhydrous were purchased from VWR International (Poole,England). Disodium hydrogen phosphate dehydrate, and sodium ammonium hydrogen phosphate tetrahydrate were purchased from Fluka (Buchs, Switzerland). Acetonitrile was purchased from J.T. Baker (Phillipsburg, USA). Ampicillin sodium salt was purchased from M&H Manufacturing (Samutprakarn, Thailand). Oxoid nutrient broth No.2 was purchased from Oxoid (Hampshire, England). Sodium chloride was purchased from BDH Laboratory Supplies (Poole, England).

1-Aminopyrene from Sigma Chemical (St Louis, USA) as well as commercial chicken concentrate from Bestfoods Ltd. (Esher, UK) were used as the representative of polycyclic aromatichydrocarbon and IQ-type heterocyclic amine mixture, respectively. After nitrite treatment described below, each gave rise to direct mutagens. The chicken concentrate, as indicated on the label, consisted of chicken extract, dehydrated chicken, water, maltodextrin, salt, flavor enhancers (monosodium glutamate and disodium-5'-ribonucleotide), yeast extract, stabilizer (sodium carboxy methyl cellulose), waxy maize starch, onion powder, flavouring, dehydrated parsley, spice extracts, antioxidant (BHA), niacin, thiamin, riboflavin, folic acid and vitamin B_{12}.

3.1.2 Samples

Cha-Plu [*Piper sarmentosum* Roxb.(ชะพลู) Piperaceae], Yor [*Morinda citrifolia* L. (ยอ) Rubiaceae], Pak-Waan-Bann [*Sauropus androgynus* Merr. (ผักหวานบ้าน) Euphorbiaceae] and Pak-Khom [*Amaranthus lividus* Linn. (ผักโขม) Amaranthaceae] were purchased from local markets in Bangkok, Thailand.

3.2 Methods

3.2.1 Experimental Design

Determination of the mutagenicity modifying activity of vegetable extracts on the standard mutagen (1-aminopyrene treated with nitrite) and nitrite treated chicken extract were carried out according to the experimental design shown in Figure 9.

The mutagenicity of 1-aminopyrene, chicken extract and of each vegetable extract as well as their products after nitrite treatment was assayed using the Ames test in the absence of metabolic activation. The antimutagenicity of each vegetable extract against nitrite treated 1-AP or nitrite treated chicken extract was then investigated.

3.2.2 Preparation of Vegetable Extracts

Each sample was cleaned with tap water and dry at room temperature. One part (100 g) was kept raw while the other part (100 g) was blanched with hot water for 3 minutes, cool in tap water and left at room temperature. It was chopped and soaked in 400 ml of acetone for 3 days. The acetone extract of each sample was filtered through filter paper No.541. The extraction process was repeated twice. The combined filtrate was evaporated to dryness under vacuum in a rotary evaporator at 45°C and was steriled by autoclaving at 121°C for 20 minutes. Each sample was diluted with DMSO to obtain appropriate concentrations and stored below 5°C.

3.2.3 The Preparation of Bacteria Tester Strains

Salmonella typhimurium tester strains used in this study were histidine-dependent strains (His^+) TA98 and TA100, which were capable of detecting frameshift mutation and base-pair substitution respectively. The both strains were kindly provided by associate professor Dr. Kaew Kangsadalampai, Nutrtion Institute, Mahidol University. The tester strains were manipulated as shown in appendix. Overnight cultures of bacteria inoculated

from frozen stock culture in Oxoid nutrient broth no.2 at 37°C was used for mutagenicity assay within 24 hours.

3.2.4 Nutrient Agar Preparation

3.2.4.1 Preparation of minimal agar plates

Minimal agar containing 1.5% Bacto agar was autoclaved at 121°C 20 minutes and then mixed with 2% sterile glucose and Vogel-Bonner mediun E stock salt solution (VB salt) (see in appendix). Approximately 30 ml of molten agar was poured into the sterile Petri dish. It was left until solidified and stored at 37°C in the incubator for 48 hours before using.

3.2.4.2 Preparation of top agar

Top agar containing 0.6% Bacto agar and 0.5% sodium chloride was autoclaved at 121°C for 20 minutes. Ten percent (v/v) of a sterile solution of 0.5 mM histidine and biotin were added to the molten top agar and then it was maintained at 45°C in a water bath.

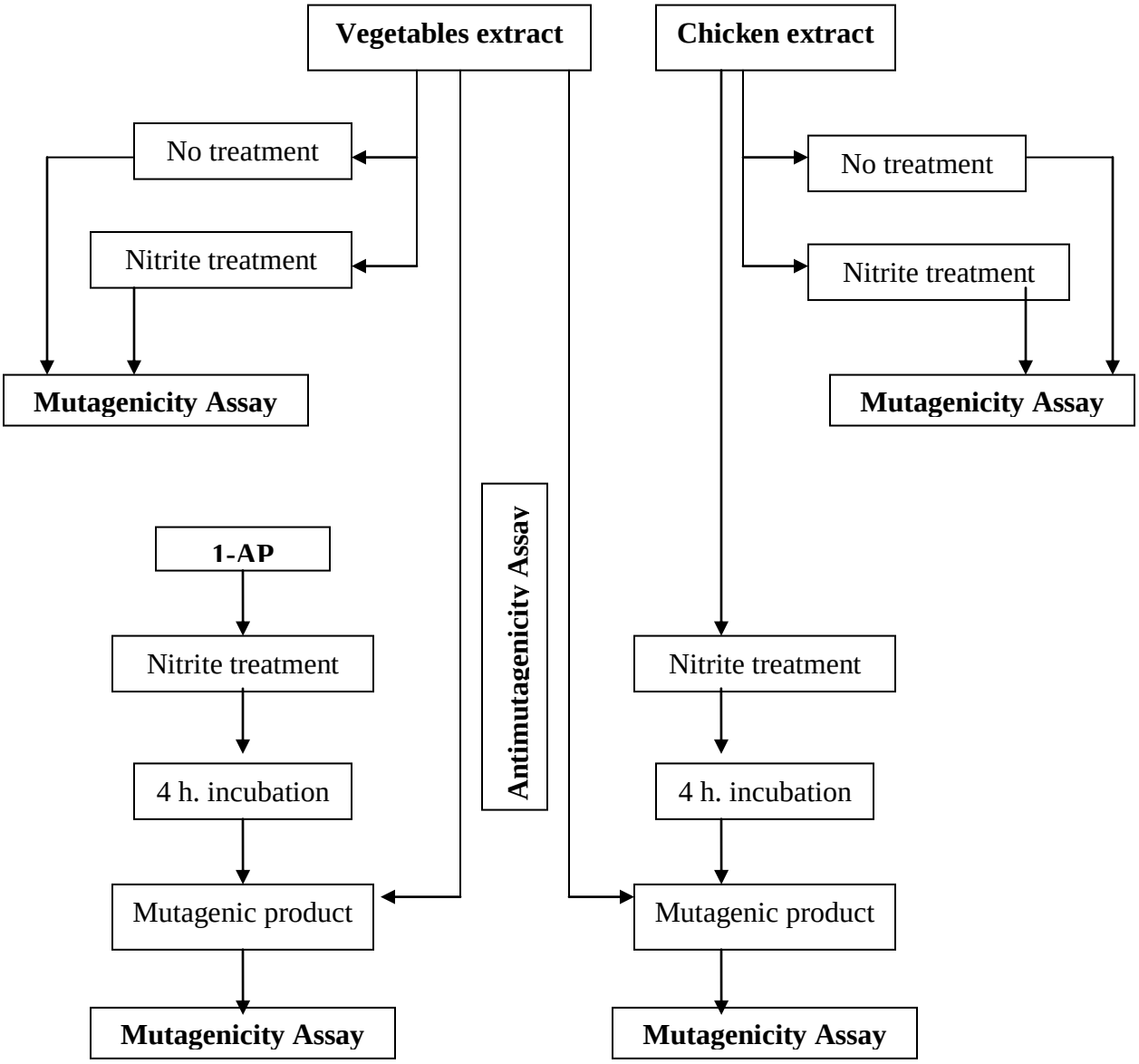


Figure 9 Experimental designs for determining the mutagenicity modification of vegetable extracts on the mutagenicity of nitrite treated 1-aminopyrene and nitrite treated chicken extract.

3.3 Mutagenicity of 1-Aminopyrene-Nitrite and Chicken Extract-Nitrite

1-AP treated with nitrite in acid solution was used as a standard mutagen since it was shown to give direct-action mutagnicity in the condition similar to that occurred during stomach digestion (Kangsadalampai, Butryee, and Manoonphol, 1996). Briefly explain, 10 µl (tested on TA98) or 20 µl (tested on TA100) of 1-AP (0.0375 mg/ml) was added into a tube fitted with a plastic stopper and then mixed with 0.74 or 0.73 ml 0.2 N hydrochloric acid (sufficient to acidify the reaction mixture to pH 3.0-3.5) and added 0.25 ml of 2 M sodium nitrite to the reaction mixture. The final concentration of nitrite was 0.5 M. The reaction tube was shaken in a water bath at 37°C for 4 hours and the reaction was stopped by placing the tube in an ice bath for 1 minute. In order to decompose the residual nitrite, 0.25 ml of 2 M ammonium sulfamate was added to the reaction mixture and the tube was allowed to stand for 10 minutes in an ice bath. The reaction mixture was mixed with 0.5 ml phosphate buffer (pH 7.4), and 100 µl of fresh overnight culture of the tester strain and incubated at 37°C in a shaking water bath for 20 minutes. After incubation, 2 ml of molten top agar (45°C) was added. It was mixed well and poured onto a minimal glucose agar plate. The plate was rotated to achieve uniform colony distribution and incubated at 37°C for 48 hours. After incubation, His^+ revertant colonies were counted.

The other positive direct-acting mutagen used in this study was chicken extract treated with nitrite. The chicken extract was treated with and without nitrite in acidic condition (Figure 10 and Figure 11 respectively) as follows. A solution of commercial chicken extract was prepared in distilled water (ratio 2g:1ml) and centrifuged at 4000 rpm for 30 minutes. The supernatant was used and sterile by autoclaved at 121°C 20 minutes. The chicken extract solution was diluted with distilled water to obtain the concentration of 0.8, 1.6 and 3.2 mg/plate which were calculated from residue weight and was mixed with 550 µl of 0.1 N HCl (sufficient to acidify the reaction mixture to pH 3.0-3.5) and 250 µl of 2 M $NaNO_2$ or distilled water. The final concentration of nitrite was 0.5 M. The reaction tube was shaken in a water bath at 37°C for 4 hours and the reaction was stopped by placing the tube in an ice bath for 1 minute. The nitrosated product (100 µl) was mixed with 500 µl phosphate buffer (pH 7.4), and 100 µl of overnight cultures of the tester strain and incubated at 37°C in a shaking water bath for 20 minutes. After

incubation, 2 ml of molten top agar (45°C) was added. It was mixed well and poured onto a minimal glucose agar plate. Then it was evenly distributed by rotating the plate. After solidification, the plate was inversely placed in an incubator at 37°C for 48 hours. After incubation, His^+ revertant colonies were counted. Each set of experiment was performed at least twice of triplicate plates.

3.4 Mutagenicity of Vegetable Extracts

Each extract from vegetable was also treated with or without nitrite and determined for its mutagenicity. Briefly, different volumes (25, 50, 100, and 200 µl) of each sterile vegetable extract was measured into a sterile test tube fitted with a plastic stopper and adjusted volume to 200 µl with dimethylsulfoxide. Next, the tube was mixed with 550 µl of 0.2 N hydrochloric acid (sufficient to acidify the reaction mixture to pH 3.0-3.5) and 250 µl of dimethylsulfoxide (Figure 10) or 2 M sodium nitrite (Figure 11). The reaction tube was shaken at 37°C for 4 h. Then, it was placed in an ice bath to stop the reaction. Added 250 µl of 2 M ammonium sulfamate to the reaction mixture, for decomposing nitrite, or 250 µl of dimethylsulfoxide), the final volume was 1250 µl, and allowed to stand for 10 min in an ice bath. The reaction mixture (100 µl) was mixed with 500 µl phosphate buffer (pH 7.4), and 100 µl of overnight cultures of the tester strain and incubated at 37°C in a shaking water bath for 20 minutes. After incubation, 2 ml of molten top agar (45°C) was added. It was mixed well and poured onto a minimal glucose agar plate. After solidification, the plate was inversely placed in an incubator at 37°C for 48 hours. After incubation, His^+ revertant colonies were counted. Each set of experiment was performed at least twice of triplicate plates.

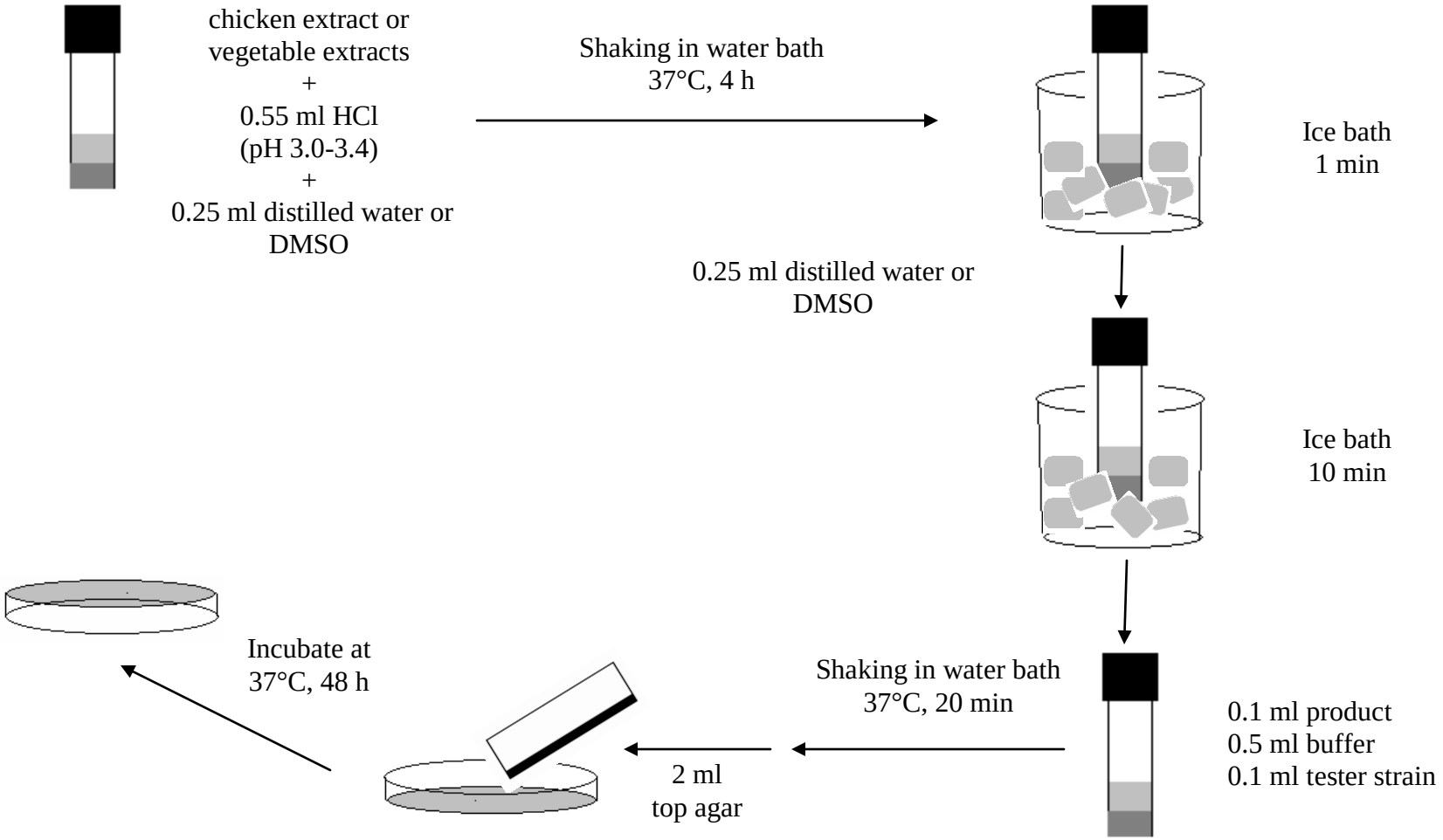


Figure 10 Steps to determine the mutagenicity of the sample extracts using the Ames mutagenicity test (pre-incubation modification) in the absence of metabolic activation.

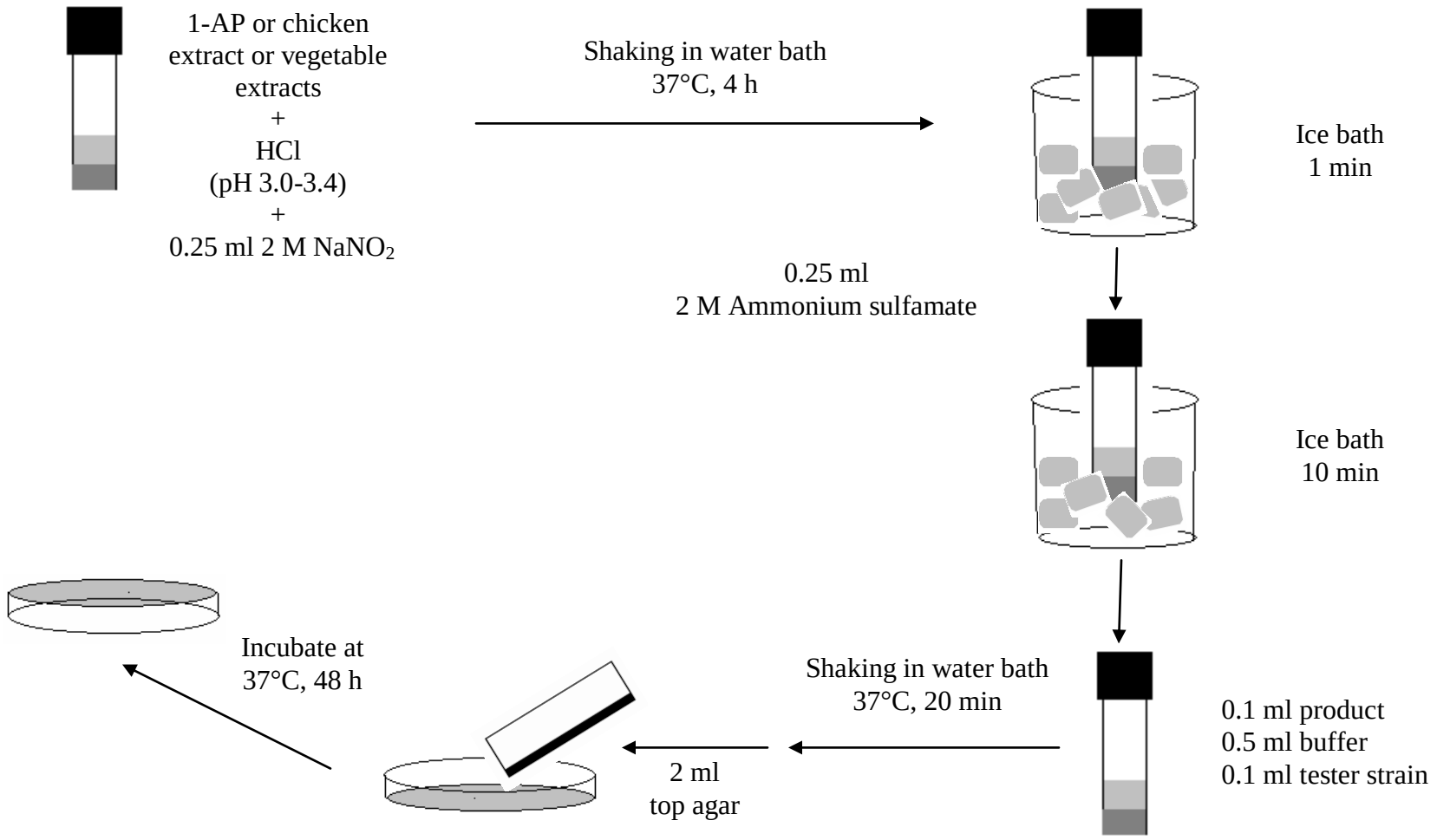


Figure 11 Steps to determine the mutagenicity of the nitrite treated sample extracts using the Ames mutagenicity test (pre-incubation modification) in the absence of metabolic activation.

3.5 Antimutagenicity of Vegetable Extracts

The pre-incubation method as suggested by Yahagi (1975) was used to investigate antimutagenicity property of the vegetable extracts. An aliquot of 100 µl of nitrite treated chicken extract (32 mg of chicken extract/plate) or nitrite treated 1-AP was used as a standard mutagen. The procedure was performed as follows (Figure 12). An aliquot of 0.1 ml of nitrosated product, 0.5 ml of phosphate buffer, 0.1 ml of the tester strain and 0.1 ml of each vegetable extracts were mixed together in a test tube and incubated at 37°C for 20 minutes. After incubation, 2 ml of the molten agar (45°C) was added. The contents of the tube were well mixed and poured onto the minimal agar plate. Then it was evenly distributed by rotating the plate. After solidification, the plate was inversely placed in an incubator at 37°C for 48 hours. After incubation, the number of His^+ revertant colonies was determined.

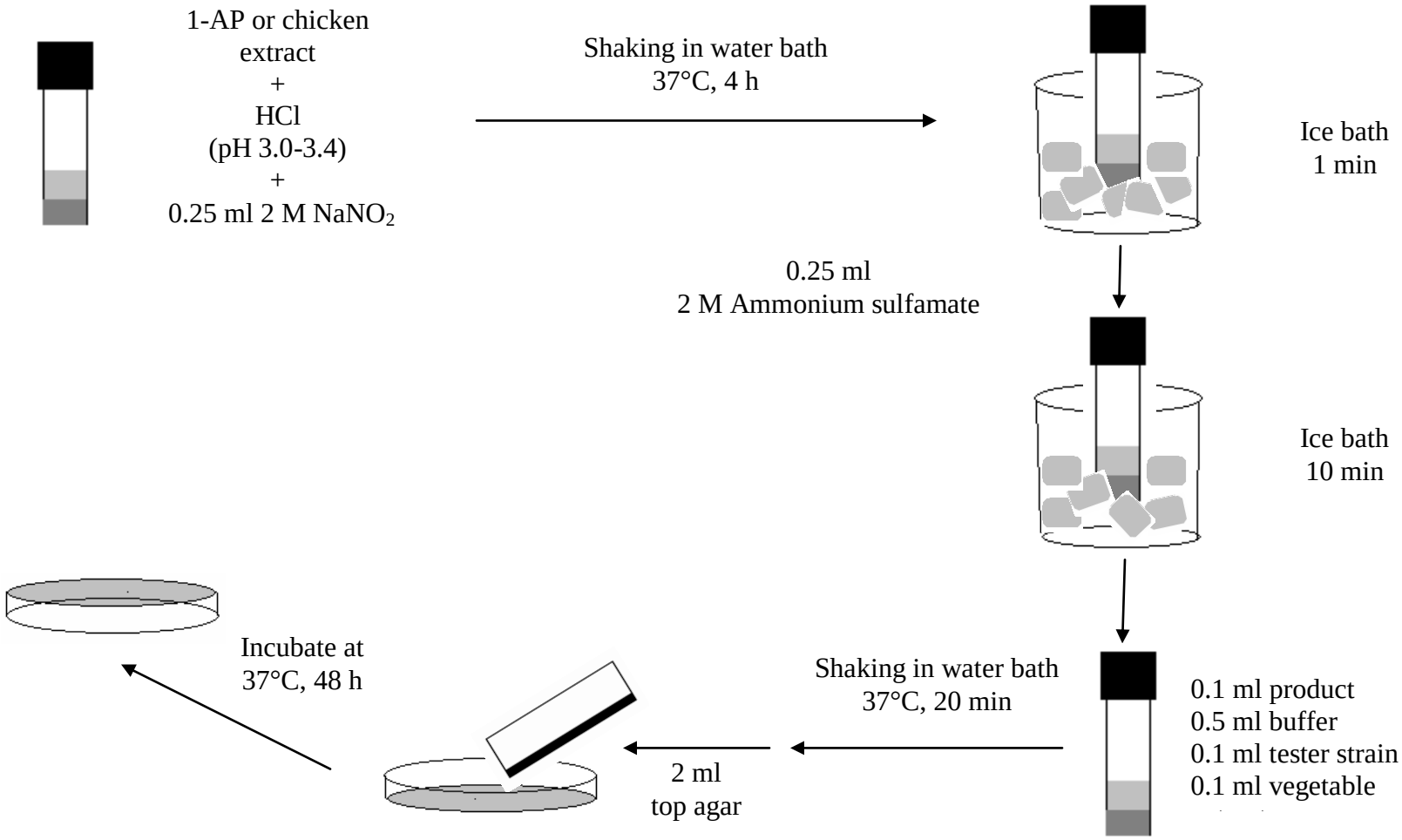


Figure 12 Steps to determine the antimutagenicity of four selected vegetable extracts on nitrite treated 1-AP or nitrite treated chicken extract using the Ames mutagenicity test (pre-incubation modification) in the absence of metabolic activation.

3.6 Data Manipulation

The mutagenicity of each sample was presented as a number of histidine revertants per plate. The data were reported as means with standard deviation of six plates from two experiments. To compare the degree of mutagenicity, the results were expressed as a mutagenicity index (MI) (equation 2):

$$MI = \frac{\text{Average (N)}}{\text{Average (S)}} \qquad (2)$$

N = a number of histidine revertants per plate of the sample

S = a number of spontaneous revertants per plate of the negative control

The mutagenicity of samples was determined by number of histidine revertants at least one concentration which should be higher than 2 times of spontaneous revertants with a dose-response relationship. A percentage of modification (either increase or decrease in mutagenicity of sample) was calculated as following (equation 3):

$$\% \text{ modification} = \frac{(A - B)}{(A - C)} \; X\,100 \qquad (3)$$

A = a number of histidine revertants induced by nitrite treated 1-aminopyrene or nitrite treated chicken extract

B = a number of histidine revertants induced by nitrite treated 1-aminopyrene or nitrite treated chicken extract in the presence of vegetable extract

C = a number of spontaneous histidine revertants per plate.

Percentage of modification as follows (Calomme *et al*. 1996):

> + 60% inhibition : strongly active

+ 41-60% inhibition : moderately active

+ 21-40% inhibition : weakly active

+ 0-20% inhibition : no active

Enhancement of mutagenicity was ranked in a similar way:

0-(-20%)	enhancement	: no active
(-21)-(-40%)	enhancement	: weak enhancement
(-41)-(-60%)	enhancement	: moderately enhancement
> - 60%	enhancement	: strong enhancement

CHAPTER IV
RESULTS

In the present study, The *Salmonella* mutagenicity assay was used to determine the mutagenic and antimutagenic activity of extracts from four vegetables namely Cha-Plu [*Piper sarmentosum* Roxb.(ชะพลู) Piperaceae], Yor [*Morinda citrifolia* L. (ยอ) Rubiaceae], Pak-Waan-Bann [*Sauropus androgynus* Merr. (ผักหวานบ้าน) Euphorbiaceae] and Pak-Khom [*Amaranthus lividus* Linn. (ผักโขม) Amaranthaceae]. 1-AP and chicken extract being treated with nitrite in acid condition were used as the standard mutagens.

4.1 Positive Control

Nitrite Treated Chicken Extract

The study on the mutagenicity of nitrite treated chicken extract in the absence of metabolic activation on *S. typhimurium* TA98 and TA100 (Table 7) showed that the higher the amount of the nitrite treated chicken extract, the higher the mutagenicity index (MI) obtained from both TA98 and TA100. The 32 mg/plate concentration of nitrite treated chicken extract was chosen as a standard for antimutagenic study.

Table 7 Mutagenicity of the chicken extract treated with and without nitrite in acid condition pH 3.0-3.5 on *S. typhimurium* strains TA98 and TA100 in the absence of metabolic activation.

Chicken extract	Amount weight of chicken extract (mg/plate)	Number of His^{+} [a] (revertants/plate)		Mutagenicity Index (MI) [b]	
		TA98	TA100	TA98	TA100
With nitrite	0	26±9	113±11	1.00	1.00
	8	40±15	339±134	1.54	3.00
	16	71±18	470 ±75	2.73	4.16
	32	93±16	514±87	3.58	4.55
Without nitrite	0	26±9	113±11	1.00	1.00
	8	27±12	137±14	1.04	1.21
	16	34±16	124±16	1.31	1.10
	32	37±15	154±25	1.42	1.36

[a] Data are expressed as mean ± SD of six plates from two experiments.
[b] Mutagenicity Index (MI) is calculated from the average value of histidine revertants/plate of the chicken extract divided by that of spontaneous revertants.

4.2 Mutagenicity of the Vegetable Extracts

The mutagenicity of the extracts from raw and blanched vegetables evaluated on *S. typhimurium* TA98 are shown in Tables 8 and 9 respectively. The mutagenicity of the extracts from raw and blanched vegetables evaluated on *S. typhimurium* TA100 are shown in Tables 10 and 11 respectively. The results show that all nitrite treated vegetable extracts were mutagenic on both strains. Without being treated with nitrite, the mutagenicity was not observed.

Table 8 Mutagenicity of the extracts from raw vegetables in acid solution pH 3.0-3.5 on *S. typhimurium* strains TA98 (frameshift mutation) without metabolic activation.

Vegetable extracts	Amount [a] (mg/plate)	TA98			
		Number of His^+ [b] (revertants/plate)		Mutagenicity Index (MI) [c]	
		w/o nitrite	w/nitrite	w/o nitrite	w/nitrite
Cha-Plu	0	14±5	14±5	1.00	1.00
	0.4	11±1	136±30	0.79	9.71
	0.8	15±6	235±77	1.07	16.79
	1.6	15±4	366±93	1.07	26.14
	3.2	13±8	310±107	0.93	22.14
Yor	0	16±3	16±3	1.00	1.00
	0.4	14±3	88±9	0.87	5.50
	0.8	16±2	154±10	1.00	9.62
	1.6	14±3	209±16	0.87	13.06
	3.2	20±3	209±8	1.25	13.06
Pak-Waan-Bann	0	22±1	22±1	1.00	1.00
	0.4	27±15	70±17	1.23	3.18
	0.8	22±13	122±8	1.00	5.54
	1.6	23±10	194±38	1.04	8.82
	3.2	22±9	154±11	1.00	7.00
Pak-Khom	0	16±5	16±5	1.00	1.00
	0.4	14±7	42±6	0.87	2.62
	0.8	14±1	75±1	0.87	4.69
	1.6	17±3	177±23	1.06	11.06
	3.2	21±1	246±38	1.31	15.37

[a] Amount per plate of vegetable extract calculated on the residue.
[b] Data are expressed as mean ± SD of six plates from two experiments.
[c] Mutagenicity Index (MI) is calculated from the average value of histidine revertants/plate of each vegetable extract divided by that of spontaneous revertants.

Table 9 Mutagenicity of the extracts from blanched vegetables in acid solution pH 3.0-3.5 on *S. typhimurium* strains TA98 (frameshift mutation) without metabolic activation.

Vegetable extracts	Amount [a] (mg/plate)	TA98			
		Number of His^{+} [b] (revertants/plate)		Mutagenicity Index (MI) [c]	
		w/o nitrite	w/nitrite	w/o nitrite	w/nitrite
Cha-Plu	0 [d]	14±1	14±1	1.00	1.00
	0.4	18±8	39±8	1.28	2.79
	0.8	20±1	87±31	1.43	6.21
	1.6	23±1	150±56	1.64	10.71
	3.2	21±1	200±68	1.50	14.29
Yor	0 [d]	12±2	12±2	1.00	1.00
	0.4	11±6	105±29	0.92	8.75
	0.8	13±2	126±35	1.08	10.50
	1.6	14±1	200±36	1.17	16.67
	3.2	16±2	179±3	1.33	14.92
Pak-Waan-Bann	0 [d]	30±5	30±5	1.00	1.00
	0.4	40±9	43±12	1.33	1.43
	0.8	35±4	51±7	1.17	1.70
	1.6	42±5	56±12	1.40	1.87
	3.2	38±3	71±11	1.27	2.37
Pak-Khom	0 [d]	23±15	23±15	1.00	1.00
	0.4	24±20	36±15	1.04	1.56
	0.8	21±21	46±27	0.91	2.00
	1.6	26±17	53±20	1.13	2.30
	3.2	26±13	82±36	1.13	3.56

[a] Amount per plate of vegetable extract calculated on the residue.
[b] Data are expressed as mean ± SD of six plates from two experiments.
[c] Mutagenicity Index (MI) is calculated from the average value of histidine revertants/plate of each vegetable extract divided by that of spontaneous revertants.

Table 10 Mutagenicity of the extracts from raw vegetables in acid solution pH 3.0-3.5 on *S. typhimurium* strains TA100 (base-pair substitution) without metabolic activation.

Vegetable extracts	Amount [a] (mg/plate)	TA100			
		Number of His^+ (revertants/plate)		Mutagenicity Index (MI)	
		w/o nitrite	w/nitrite	w/o nitrite	w/nitrite
Cha-Plu	0 [d]	118±43	118±43	1.00	1.00
	0.4	133±38	532±30	1.13	4.51
	0.8	135±49	751±120	1.14	6.36
	1.6	128±70	804±76	1.08	6.81
	3.2	64±3	808±267	0.54	6.85
Yor	0 [d]	98±1	98±1	1.00	1.00
	0.4	104±14	430±25	1.06	4.39
	0.8	100±13	645±25	1.02	6.58
	1.6	127±26	909±47	1.30	9.27
	3.2	117±28	953±3	1.19	9.72
Pak-Waan-Bann	0 [d]	83±1	83±1	1.00	1.00
	0.4	85±9	490±54	1.02	5.90
	0.8	98±8	848±62	1.18	10.22
	1.6	96±12	934±85	1.16	11.25
	3.2	99±11	1037±17	1.19	12.49
Pak-Khom	0 [d]	87±7	87±7	1.00	1.00
	0.4	68±4	247±27	0.78	2.84
	0.8	94±4	446±11	1.08	5.13
	1.6	82±3	746±83	0.94	8.57
	3.2	82±6	806±44	0.94	9.26

[a] Amount per plate of vegetable extract calculated on the residue.
[b] Data are expressed as mean ± SD of six plates from two experiments.
[c] Mutagenicity Index (MI) is calculated from the average value of histidine revertants/plate of each vegetable extract divided by that of spontaneous revertants.

Table 11 Mutagenicity of the extracts from blanched vegetables in acid solution pH 3.0-3.5 on *S. typhimurium* strains TA100 (base-pair substitution) without metabolic activation.

Vegetable extracts	Amount [a] (mg/plate)	TA100			
		Number of His^+ (revertants/plate)		Mutagenicity Index (MI)	
		w/o nitrite	w/nitrite	w/o nitrite	w/nitrite
Cha-Plu	0	102±39	102±39	1.00	1.00
	0.4	119±23	331±49	1.17	3.24
	0.8	119±21	474±46	1.17	4.65
	1.6	125±28	694±51	1.22	6.80
	3.2	135±25	903±9	1.32	8.85
Yor	0	112±18	112±18	1.00	1.00
	0.4	106±13	343±44	0.95	3.06
	0.8	113±25	431±119	1.01	3.85
	1.6	102±16	674±93	0.91	6.02
	3.2	120±3	768±108	1.07	6.86
Pak-Waan-Bann	0	89±8	89±8	1.00	1.00
	0.4	87±7	167±13	0.98	1.88
	0.8	92±8	196±13	1.03	2.20
	1.6	110±14	250±24	1.24	2.81
	3.2	105±3	394±20	1.18	4.43
Pak-Khom	0	87±7	87±7	1.00	1.00
	0.4	72±11	154±3	0.83	1.77
	0.8	79±9	215±11	0.91	2.47
	1.6	83±12	278±11	0.95	3.19
	3.2	76±2	516±70	0.87	5.93

[a] Amount per plate of vegetable extract calculated on the residue.
[b] Data are expressed as mean ± SD of six plates from two experiments.
[c] Mutagenicity Index (MI) is calculated from the average value of histidine revertants/plate of each vegetable extract divided by that of spontaneous revertants.

4.3 Antimutagenicity of the Vegetable Extracts.

Tables 12 and 13 reveal the effects of extracts from raw and blanched vegetables on the mutagenic activity of nitrite treated 1-AP using *S. typhimurium* TA98 in the absence of metabolic activation. The results show that the extracts from raw and blanched vegetables exhibited the strongly inhibition effect (>60% inhibition) on this tester strain.

The effects of raw and blanched vegetable extracts on the mutagenic activity of nitrite treated 1-AP on *S. typhimurium* TA100 are shown in Tables 14 and 15 respectively. The results also show that the extract from raw and blanched vegetables had expressed exhibited strongly inhibition effect (>60% inhibition) on this tester strain.

Tables 16 and 17 reveal the effects of extracts from raw and blanched vegetables on the mutagenic activity of nitrite treated chicken extract on *S. typhimurium* TA98 in the absence of metabolic activation. The results show that the extracts from raw and blanched Cha-Plu and Pak-Waan-Bann inhibited the mutagenicity of the reaction mixture of nitrite treated chicken extract. Whereas the extract from raw leaf of Yor was weakly (21-40% inhibition) to moderately active (40-60% inhibition), but the modulating effect of blanched extract of Yor on nitrite treated chicken extract model was not active (0-20% inhibition) to weakly active (21-40% inhibition) on this tester strain. The results of the extract from raw Pak Khom show that it was not active on *S. typhimurium* TA98, whereas the extract from blanched Pak Khom was weakly to moderately active on this tester strain.

Tables 18 and 19 reveal the effects of extracts from raw and blanched vegetables on the mutagenic activity of nitrite treated chicken extract on *S. typhimurium* TA100. The results show that extracts from raw and blanched Cha-Plu and Pak-Waan-Bann inhibited the mutagenicity of the reaction mixture of nitrite treated chicken extract model weakly active (21-40% inhibition), except extract of raw Cha-Plu (3.2 mg/plate) show moderate inhibition (40-60%). The modulating effects of extracts from raw and blanched of Yor were not active (0-20% inhibition) to weakly active (21-40% inhibition) on this tester strain. The results of the extract from raw Pak Khom show that it was not active on *S. typhimurium* TA100, whereas the extract from blanched Pak Khom was weakly active on this tester strain.

Table 12 Effect of the extracts from raw vegetables on the mutagenicity of nitrite treated 1-aminopyrene on *S. typhimurium* strains TA98 without metabolic activation.

Vegetable extracts	**Amount** [a] **(mg/plate)**	**Number of His$^+$** [b] **(revertants/plate)**	**%Modification**	**Classification**
Cha-Plu	Spontaneous [c]	20±11		
	0 [d]	1737±159		
	0.8	234±77	+87	strong
	1.6	132±44	+93	strong
	3.2	74±24	+97	strong
Yor	Spontaneous [c]	19±15		
	0 [d]	872±377		
	0.8	115±37	+89	strong
	1.6	74±15	+93	strong
	3.2	66±30	+94	strong
Pak-Waan-Bann	Spontaneous [c]	27±10		
	0 [d]	970±13		
	0.8	113±10	+91	strong
	1.6	68±18	+96	strong
	3.2	55±18	+97	strong
Pak-Khom	Spontaneous [c]	26±11		
	0 [d]	844±165		
	0.8	92±27	+92	strong
	1.6	78±13	+94	strong
	3.2	65±6	+95	strong

[a] Amount per plate of vegetable extract calculated on the residue.
[b] Data are expressed as mean ± SD of six plates from two experiments.
[c] Spontaneous mutation.
[d] No extract was added to the standard mutagen, namely nitrite treated 1-aminopyrene (0.03 µg/plate for *Salmonella typhimurium* strain TA98).

Table 13 Effect of the extracts from blanched vegetables on the mutagenicity of nitrite treated 1-aminopyrene on *S. typhimurium* strains TA98 without metabolic activation.

Vegetable extracts	**Amount** [a] **(mg/plate)**	**Number of His$^+$** [b] **(revertants/plate)**	**%Modification**	**Classification**
Cha-Plu	Spontaneous [c]	9±1		
	0 [d]	2482±194		
	0.8	297±9	+88	strong
	1.6	172±25	+93	strong
	3.2	87±4	+97	strong
Yor	Spontaneous [c]	10±1		
	0 [d]	556±71		
	0.8	73±6	+88	strong
	1.6	54±2	+92	strong
	3.2	54±19	+92	strong
Pak-Waan-Bann	Spontaneous [c]	27±10		
	0 [d]	970±13		
	0.8	57±23	+97	strong
	1.6	51±27	+97	strong
	3.2	37±15	+99	strong
Pak-Khom	Spontaneous [c]	26±11		
	0 [d]	844±165		
	0.8	78±1	+94	strong
	1.6	61±16	+96	strong
	3.2	52±8	+97	strong

[a] Amount per plate of vegetable extract calculated on the residue.
[b] Data are expressed as mean ± SD of six plates from two experiments.
[c] Spontaneous mutation.
[d] No extract was added to the standard mutagen, namely nitrite treated 1-aminopyrene (0.03 µg/plate for *Salmonella typhimurium* strain TA98).

Table 14 Effect of the extracts from raw vegetables on the mutagenicity of nitrite treated 1-aminopyrene on *S. typhimurium* strains TA100 without metabolic activation.

Vegetable extracts	**Amount [a] (mg/plate)**	**Number of His^{+} [b] (revertants/plate)**	**%Modification**	**Classification**
Cha-Plu	Spontaneous [c]	104±23		
	0 [d]	1011±119		
	0.8	269±6	+82	strong
	1.6	233±23	+86	strong
	3.2	129±27	+97	strong
Yor	Spontaneous [c]	126±44		
	0 [d]	461±38		
	0.8	183±37	+83	strong
	1.6	184±13	+83	strong
	3.2	176±41	+85	strong
Pak-Waan-Bann	Spontaneous [c]	145±76		
	0 [d]	519±73		
	0.8	223±106	+79	strong
	1.6	222±108	+79	strong
	3.2	193±108	+87	strong
Pak-Khom	Spontaneous [c]	139±68		
	0 [d]	448±28		
	0.8	182±50	+86	strong
	1.6	167±82	+91	strong
	3.2	173±86	+89	strong

[a] Amount per plate of vegetable extract calculated on the residue.
[b] Data are expressed as mean ± SD of six plates from two experiments.
[c] Spontaneous mutation.
[d] No extract was added to the standard mutagen, namely nitrite treated 1-aminopyrene (0.06 µg/plate for *Salmonella typhimurium* strain TA100).

Table 15 Effect of the extracts from blanched vegetables on the mutagenicity of nitrite treated 1-aminopyrene on *S. typhimurium* strains TA100 without metabolic activation.

Vegetable extracts	**Amount [a] (mg/plate)**	**Number of His^{+} [b] (revertants/plate)**	**%Modification**	**Classification**
Cha-Plu	Spontaneous [c]	105±43		
	0 [d]	1180±87		
	0.8	328±59	+79	strong
	1.6	278±3	+84	strong
	3.2	179±22	+93	strong
Yor	Spontaneous [c]	141±23		
	0 [d]	375±83		
	0.8	211±16	+70	strong
	1.6	187±47	+80	strong
	3.2	195±40	+77	strong
Pak-Waan-Bann	Spontaneous [c]	145±76		
	0 [d]	519±72		
	0.8	179±98	+91	strong
	1.6	157±88	+97	strong
	3.2	145±85	+100	strong
Pak-Khom	Spontaneous [c]	139±68		
	0 [d]	448±28		
	0.8	194±61	+82	strong
	1.6	155±68	+95	strong
	3.2	161±70	+93	strong

[a] Amount per plate of vegetable extract calculated on the residue.
[b] Data are expressed as mean ± SD of six plates from two experiments.
[c] Spontaneous mutation.
[d] No extract was added to the standard mutagen, namely nitrite treated 1-aminopyrene (0.06 µg/plate for *Salmonella typhimurium* strain TA100).

Table 16 Effect of the extracts from raw vegetables on the mutagenicity of nitrite treated chicken extract on *S. typhimurium* strains TA98 without metabolic activation.

Vegetable extracts	**Amount [a] (mg/plate)**	**Number of His^{+} [b] (revertants/plate)**	**%Modification**	**Classification**
Cha-Plu	Spontaneous [c]	10±1		
	0 [d]	74±14		
	0.8	48±11	+41	moderate
	1.6	49±16	+39	weak
	3.2	47±10	+42	moderate
Yor	Spontaneous [c]	13±6		
	0 [d]	72±2		
	0.8	43±1	+49	moderate
	1.6	50±3	+37	weak
	3.2	50±4	+37	weak
Pak-Waan-Bann	Spontaneous [c]	20±5		
	0 [d]	67±6		
	0.8	44±1	+49	moderate
	1.6	46±1	+45	moderate
	3.2	40±1	+57	moderate
Pak-Khom	Spontaneous [c]	19±1		
	0 [d]	70±3		
	0.8	62±4	+16	not active
	1.6	60±8	+20	not active
	3.2	60±5	+20	not active

[a] Amount per plate of vegetable extract calculated on the residue.
[b] Data are expressed as mean ± SD of six plates from two experiments.
[c] Spontaneous mutation.
[d] No extract was added to the standard mutagen, namely nitrite treated chicken extract.

Table 17 Effect of the extracts from blanched vegetables on the mutagenicity of nitrite treated chicken extract on *S. typhimurium* strains TA98 without metabolic activation.

Vegetable extracts	**Amount [a] (mg/plate)**	**Number of His^{+} [b] (revertants/plate)**	**%Modification**	**Classification**
Cha-Plu	Spontaneous [c]	9±1		
	0 [d]	66±3		
	0.8	41±4	+44	moderate
	1.6	39±1	+47	moderate
	3.2	40±1	+46	moderate
Yor	Spontaneous [c]	15±6		
	0 [d]	61±9		
	0.8	52±6	+20	not active
	1.6	48±3	+28	weak
	3.2	53±11	+17	not active
Pak-Waan-Bann	Spontaneous [c]	20±5		
	0 [d]	67±6		
	0.8	43±2	+51	moderate
	1.6	42±9	+53	moderate
	3.2	46±3	+45	moderate
Pak-Khom	Spontaneous [c]	19±1		
	0 [d]	70±3		
	0.8	47±1	+45	moderate
	1.6	56±1	+27	weak
	3.2	57±3	+25	weak

[a] Amount per plate of vegetable extract calculated on the residue.
[b] Data are expressed as mean ± SD of six plates from two experiments.
[c] Spontaneous mutation.
[d] No extract was added to the standard mutagen, namely nitrite treated chicken extract.

Table 18 Effect of the extracts from raw vegetables on the mutagenicity of nitrite treated chicken extract on *S. typhimurium* strains TA100 without metabolic activation.

Vegetable extracts	**Amount [a] (mg/plate)**	**Number of His^{+} [b] (revertants/plate)**	**%Modification**	**Classification**
Cha-Plu	Spontaneous [c]	97±32		
	0 [d]	556±3		
	0.8	400±14	+34	weak
	1.6	403±2	+33	weak
	3.2	313±42	+53	moderate
Yor	Spontaneous [c]	127±42		
	0 [d]	571±31		
	0.8	486±7	+19	not active
	1.6	493±2	+18	not active
	3.2	476±23	+21	weak
Pak-Waan-Bann	Spontaneous [c]	193±8		
	0 [d]	679±42		
	0.8	498±22	+37	weak
	1.6	483±47	+40	weak
	3.2	501±30	+37	weak
Pak-Khom	Spontaneous [c]	173±19		
	0 [d]	627±31		
	0.8	596±27	+7	not active
	1.6	572±12	+12	not active
	3.2	583±30	+10	not active

[a] Amount per plate of vegetable extract calculated on the residue.
[b] Data are expressed as mean ± SD of six plates from two experiments.
[c] Spontaneous mutation.
[d] No extract was added to the standard mutagen, namely nitrite treated chicken extract.

Table 19 Effect of the extracts from blanched vegetables on the mutagenicity of nitrite treated chicken extract on *S. typhimurium* strains TA100 without metabolic activation.

Vegetable extracts	**Amount [a] (mg/plate)**	**Number of His^{+} [b] (revertants/plate)**	**%Modification**	**Classification**
Cha-Plu	Spontaneous [c]	105±43		
	0 [d]	592±47		
	0.8	475±50	+24	weak
	1.6	486±41	+22	weak
	3.2	478±61	+23	weak
Yor	Spontaneous [c]	120±52		
	0 [d]	580±44		
	0.8	477±5	+22	weak
	1.6	449±36	+28	weak
	3.2	497±1	+18	not active
Pak-Waan-Bann	Spontaneous [c]	179±28		
	0 [d]	657±73		
	0.8	521±72	+28	weak
	1.6	507±98	+31	weak
	3.2	517±112	+29	weak
Pak-Khom	Spontaneous [c]	173±19		
	0 [d]	627±31		
	0.8	473±10	+34	weak
	1.6	516±8	+24	weak
	3.2	508±16	+26	weak

[a] Amount per plate of vegetable extract calculated on the residue.
[b] Data are expressed as mean ± SD of six plates from two experiments.
[c] Spontaneous mutation.
[d] No extract was added to the standard mutagen, namely nitrite treated chicken extract.

CHAPTER V
DISCUSSION

Gastric cancer prevention is the most desirable route for cancer control, while chemoprevention is a promising alternative to reduction of the inevitable human exposure to dietary carcinogens. Much of the evidence for the role of diet in cancer has been interpreted as evidence for the presence of carcinogenic factors in the diet (Lynnette, 1999). Particularly nitrite intake and gastric cancer mortality in various countries has been documented. For example, the reaction between nitrite and dietary amines and amides under the acidic conditions of the stomach could lead to the formation of nitrosated products. It is possible to the development of gastric cancer in human (Mirvish, 1983). On the other hand, it is well known that ingredients in diet including edible plants, fruits and seeds may exert anticarcinogenic and antimutagenic activities (Hartman and Shankel, 1990; Liu, 2003). Therefore, in this study, the protective effect of the extracts from selected four green leafy vegetables either raw or blanched forms against mutagenicity induced by nitrite treated 1-aminopyrene and nitrite treated chicken extract formed was examined using the Ames test.

5.1 Mutagenicity of Nitrite Treated 1-Aminopyrene and Nitrite treated Chicken Extract

1-Aminopyrene (1-AP), a derivative of pyrene, is a major metabolite during biotransformation of 1-nitropyrene by microflora in natural environment and in the guts of animals and humans (Balarezo *et al*., 2002). In Ames test, 1-AP treated with the excess of sodium nitrite in acid solution (pH 3.0) for 4 h was used as a positive mutagen and as an example of mutagen occurring during the gastric digestion. The products of nitrite treated 1-AP, 1-nitropyrene and unidentified nitro-introduced compounds revealed their direct mutagenicity towards *S. typhimurium* TA98 and TA100 (Kato *et al*., 1991; Kangsadalampai *et a*l., 1996). Since the products of the model did not require metabolic activation before expressing their mutagenicity, it was used to determine the counteracting activity of the sample on the mutagen that may cause mutation to the gastric cells. Such a model was used to determine whether there were some antimutagens from various substances against the mutagen in the gastric like pH solution.

The product of nitrite treated chicken extract was selected because it showed the mutagenicity towards *S. typhimurium* both TA98 and TA100 strains in the absence of metabolic activation. The products formed during the reaction of the chicken extract and sodium nitrite in the gastric-liked condition were suggested to be several heterocyclic amines and were direct-acting mutagenic towards both strains TA98 and TA100 (Peerawong and Kangsadalampai, 1998). The mutagenic products might be derived from the reaction between IQ-type heterocyclic amines which occurred during boiling meat and nitrite as proposed by previous studies (Sasagawa *et al*., 1988; Lin *et al*., 1992; Peerawong and Kangsadalampai, 1998). Sasagawa *et al*. (1988) reported that treatment of IQ type heterocyclic amines with 50 mM nitrite resulted in conversion of amino groups to nitro groups. The nitro derivatives of imidazoquinoline and imidazoquinoxaline showed almost the same mutagenicity both in the absence and presence of metabolic activation (Sugimura *et al*., 1993).

5.2 Mutagenicity of Vegetables Extracts

In this study, all of the extracts from raw and blanched vegetables i.e. Cha-Plu (*Piper sarmentosum* Roxb.), Yor (*Morinda citrifolia* L.), Pak-Waan-Bann (*Sauropus androgynus* Merr.), and Pak-Khom (*Amaranthus lividus* Linn.) were not mutagenic on both *S. typhimurium* TA98 and TA100 in the absence of metabolic activating system. This result supported that Thai indigenous vegetables are safe for consuming. On the other hand, nitrite treated extracts of raw and blanched vegetables showed their mutagenicity on both tester strains. The compounds in plant material extracted by acetone are generally phenolic compounds (Jaroszyńska, 2003); therefore, it is proposed that such compounds could be interacted with nitrite to form genotoxic substances. Simple phenolic compounds such as phenol, 3-methoxycatechol, catechol and vanillin were identified as the precursors of the genotoxic substances. These phenolic compounds exhibited direct-acting genotoxicity after nitrosation (Ohshima *et al*., 1989). Therefore, it should be the precaution to the health concerning consumers in simultaneously consuming such vegetables along with nitrite containing food items.

5.3 Effect of Vegetables Extracts on Mutagenicity of Nitrite Treated 1-Aminopyrene and Nitrite Treated Chicken Extract

The present study is the attempt to elucidate the protective effect of the extracts from selected vegetables i.e. Cha-Plu (*Piper sarmentosum* Roxb.), Yor

(*Morinda citrifolia* L.), Pak-Waan-Bann (*Sauropus androgynus* Merr.), and Pak-Khom (*Amaranthus lividus* Linn.) on the tester strains against mutation induced by nitrite treated 1-AP and nitrite treated chicken extract. The extracts from all vegetables, both raw and blanched, showed the strong antimutagenic effect on the mutagenicity of the 1-aminopyrene model. The result of chicken extract model indicated that the extracts from all vegetables were not good antimutagens, especially raw Pak-Khom which did not inhibit the mutagenicity of nitrite treated chicken extract on both strains of *S. typhimurium*. The result indicated that the direct acting mutagens formed during interaction between nitrite treated 1-AP and nitrite treated chicken extract in acid solution could be suppressed by some components in the extracts. The plant chemicals such as carotenoids, flavonoid and phenolic compounds are the contributors to the antioxidant activity to the plants and also found in these vegetables (Chan-Blanco *et al*., 2006; Chanwitheesuk *et al*. 2005; Maisuthisakul *et al*., 2008; Sato *et al*., 2002).

Phenolic compounds such as flavonoids might inhibit enzyme in the bacterial cell, nitroreductase and/or O-acetyltransferase in *Salmonella* cell as the activating enzymes of many direct mutagens. Flavonoids, the active antioxidant compounds, were found commonly in green leafy vegetables (Balakrishnan *et al*., 2009; Mathai, 2004). Kuo *et al.* (1992) found that flavonoids could inhibit nitropyrene reductase. In the bacterial tester strains (TA98 and TA100) flavonoids inhibited the activity of mutagens identified in foods whether it was direct acting (e.g. 2-aminofluorene) or it required metabolic activation [2-amino-1-methyl-6-pheny limidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3-methylimi-dazo[4,5-*f*]quinoline (IQ) and 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (Trp-P-1)] (Edenharder *et al*., 1997; Calomme, 1996; Weisburger *et al*., 1998; Miyazawa *et al*., 1999).

Acetone extract component in green leafy vegetables, namely chlorophyll (Rüdiger and Schoch, 1988; Talbot and Sauer, 1997) has been shown antimutagenic activity. The potency of the inhibitory activity was found to correlate with the chlorophyll content of the vegetables (Lai *et al*., 1980). Possible mechanisms of activity of chlorophyll are antioxidative properties (Sato *et al*., 1977) and trapping with carcinogens, by which mutagens become unavailable to target cells or organisms (Sarkar *et al*., 1994). Hayatsu *et al*. (1993) reported that chlorophyll and chlorophyllin could inhibit selectively the activity of mutagens having polycyclic structures by complex formation. Therefore, the precursors that could interact with sodium nitrite

of the two models such as 1-aminopyrene, a polycyclic aromatic hydrocarbons compound, and chicken extract which has heterocyclic amines might be trapped by chlorophyll to form complexes. This may describe the mechanism how extract of green leafy vegetables in the present study inhibited the mutagenesis of bacterial tester strains in the two models. However, the potencies of antimutagenic activity are not dose-dependent, this may possibly be due to variation in chemical components responsible for vegetables activities or killing effect.

The study obtained from chicken extract model revealed that the extract from Cha-Plu and Pak-Waan-Bann showed antimutagenic potency stronger than that of Yor and Pak-Khom. This may be caused by some constituents found in Cha-Plu such as naringenin that is a highly potent natural antioxidant compound (Subramaniam *et al*., 2003). Francis *et al*. (1989) found that naringenin, a plant flavonoid, has ability to suppress mutagenesis in *Salmonella typhimurium* strain TA100 NR induced by the direct-acting carcinogen *N*-methyl-*N´*-nitro-*N*-nitrosoguanidine. Pak-Waan-Bann also has high levels of flavonoids and total phenols (Subhasree *et al*., 2009).

5.4 Effect of Heat Treatment on the Inhibitory Effect

The present result indicated that the heat treatment showed no effect on antimutagenic activity compared with the raw samples. The antimutagenic components extracted from vegetables seemed to be the heat stable components. As it was known that washing, trimming, wilting and blanching have been reported to reduce the levels of yield and nutrients such as ascorbic acid, carotenoids and chlorophyll of the vegetables (Akpapunam, 1984; Onayemi and Badifu, 1987; Negi and Roy, 2000). Onayemi and Badifu (1987) reported higher losses of ascorbic acid in amaranth through water blanching followed by sun drying than steam blanching and cabinet drying. Ascorbic acid and β-carotene were sensitive to heat and oxidation during blanching and drying. Loss of vitamins and chlorophyll in leafy vegetables during blanching necessitates a proper combination of time and temperature for blanching (Negi and Roy, 2000). Conversely, Dewanto *et al*. (2002) showed that thermal processing elevated total antioxidant activity and bioaccessible lycopene content in tomatoes and produced no significant changes in contents of the total phenolics and flavonoids. Oboh (2005) found that blanching cause a significant increase in the total phenol content of the green leafy vegetables. The basis of the increase could not be categorically stated, however it could be attributed to the

possible breakdown of the tannins present in the vegetables during blanching to simple phenol. This is the reason why antimutagenic effect of extract from blanched vegetables were not reduced compared with raw vegetables in this study.

The further study may be used the variety of vegetables or change method of blanching such as conventional and microwave blanching. In addition, the antimutagenicity of vegetable extracts should be performed on other testing systems in order to warrant the effect of the vegetables found in the present experiment. Finally, the differences between bacterial cells (*in vitro*) and mammalian tissues (*in vivo*) might be limit in the interpretation of such benefit of vegetables; therefore, examinations for their antimutagenic activity in animal model are recommended.

CHAPTER VI
CONCLUSION

This study aimed to determine the antimutagenicity of extracts from raw and blanched vegetables, namely Cha-Plu (*Piper sarmentosum* Roxb.), Yor (*Morinda citrifolia* L.), Pak-Waan-Bann (*Sauropus androgynus* Merr.), and Pak-Khom (*Amaranthus lividus* Linn.), on *S. typhimurium* TA98 and TA100 in gastric-like pH condition.

The results showed that all of the extracts from raw and blanched vegetables in this study are safe for consumption. The extracts have variation in antimutagenic effect using the two models namely nitrite treated 1-aminopyrene model and nitrite treated chicken extract model. All of the extracts were good antimutagens in first model. Conversely, the second model were not. It was proposed that compounds in vegetable extracts might counteract the direct acting mutagenicity of nitrite treated 1-aminopyrene and nitrite treated chicken extract in many mechanisms. However, in this study, heat processing did not influence on antimutagenic activity of each vegetable.

It was proposed that compounds extracted from vegetables such as chlorophyll, flavonoids and other phytochemicals might play important role as antioxidant and antimutagens during the mutation of *S. typhimurium* in each model. The finding from this experiment suggested that consumption of nitrite-containing foods with these vegetables (raw and blanched) should prevent the mutagenicity of direct mutagen in gastric like condition, but the prevention from nitrite treated chicken extract should be more investigated. In the future, these vegetables will be promoted as functional foods to optimize the health benefit for consumers.

REFERENCES

THAI

กัญจนา ดีวิเศษ. 2542. ผักพื้นบ้านภาคกลาง. กรุงเทพฯ: โรงพิมพ์องค์การสงเคราะห์ทหารผ่านศึก.

ฐิติ เอื้ออังกูร. 2547. พืช ผัก ผลไม้ที่ใช้เป็นยารักษาโรค. กรุงเทพฯ: สำนักพิมพ์นานาบุ๊ค.

อุไร จิรมงคลการ. 2547. ผักพื้นบ้าน 1. พิมพ์ครั้งที่ 2. กรุงเทพฯ: สำนักพิมพ์บ้านและสวน.

ENGLISH

Akpapunam, M.A. 1984. Effects of wilting, blanching and storage temperatures on ascorbic acid and total carotenoids content of some Nigerian fresh vegetables. Qual Plant 34: 177-180.

Amin, I., Norazaidah, Y., Hainida, K.I.E. 2006. Antioxidant activity and phenolic content of raw and blanched *Amaranthus* species. Food Chem 94:47-52.

Arbogast, D., Bailey, G., Breinholt, V., Hendricks, J., and Pereira, C. 1995. Dietary chlorophyllin is apotent inhibitor of aflatoxin B1 hepatocarcinogenesis in rainbow trout. Cancer Res 55: 57-62.

Balakrishnan, S., Baska, R., Keerthana, R.L., Rajan, L.S., and Rajasekaran, P. 2009. Evaluation of antioxidant potential in selected green leafy vegetables. Food Chem 115: 1213-1220.

Balarezo, A.L., Jones, V.N., Yu, H., and Hwang, H. 2002. Influence of humic acid on 1-aminopyrene ecotoxicity during solar photolysis process. Int J Mol Sci 3: 1133-1144.

Ball, L.M., Raft, J.J., Gustafsson, J.A., Gustafsson, B.E., Kohan, M.J., and Lewtas, J. 1991. Formation of mutagenic urinary metabolites from 1-nitropyrene in germ free and conventional rats: role of the gut flora. Carcinogeneis 12: 1-5.

Barile, F.A. 1994. Introduction to in vitro cytotoxicology: Mechanisms and methods. USA: CRC press.

Bertram, J.S., Kolonel, L.N., and Meyskens, F.L. 1987 Rationale and strategies for chemoprevention of cancer in humans. Cancer Res 47: 3012-3031.

Block, G., Patterson, B., and Subar, A. 1992. Fruits, vegetables and cancer prevention: A review of the epidemiologic evidence. Nutr Cancer 18: 1-29.

Busby, W.F., Smith, J.H., Bishop, W.W., and Thilly, W.G. 1994. Mutagenicity of mono and dinitropyrenes in the *Salmonella typhimurium* TM677 forward

mutation assay. Mutat Res 322: 221-232.

Byers, T., Nestle, M., McTiernan, A., Doyle, C., Currie-Williams, A., Gansler, T. *et al*. 2002. American cancer society guidelines on nutrition and physical activity for cancer prevention: Reducing the risk of cancer with healthy food choices and physical activity. CA-Cancer J Clin 52: 92–119.

Calomme, M., Pieters, L., Vlietinck, A., and Berghe, DV. 1996. Inhibition of bacterial mutagenesis by Citrus flavonoids. Planta Med 62: 22-26.

Cano, M. 1991. HPLC separation of chlorophyll and carotenoid pigments of four kiwi fruit cultivars. J Agric Food Chem 39: 1786-1791.

Chan-Blanco, Y., Vaillant, F., Perez, A.M., Reynes, M., Brillouet, JM., and Brat, P. 2006. The noni fruit (*Morinda citrifolia* L.): A review of agricultural research, nutritional and therapeutic properties. J Food Compos Anal 19: 645-654.

Chanwitheesuk, A., Teerawutgulrag, A. and Rakariyatham, N. 2005. Screening of antioxidant activity and antioxidant compounds of some edible plants of Thailand. Food Chem 92: 491-497.

De Serres, F., and Shelby, M.D. 1979. Recommendation on data production and analysis using the *Salmonella*/microsome muatgenicity assay. Mutat Res 64: 159-165.

Dewanto, V., Wu, X., Adom, K.K., and Liu, R.H. 2002 Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J Agr Food Chem 50: 3010-3014.

Djuric, Z., Coles, B., Fifer, E.K., Ketterer, B., and Beland, F.A. 1987. *In vivo* and *in vitro* formation of glutathione conjugates from K-region epoxides of 1-nitropyrene. Carcinogenesis 8: 1781-1786.

Doll, R., and Peto, R. 1981. The cause of cancer: Quantitative estimates of avoidable risks of cancer in the United States today. J Natl Cancer Inst 66: 1191-1308.

Donaldson, M.S. 2004. Nutrition and cancer: A review of the evidence for an anti-cancer diet. Nutrition J 3: 19.

Duncan, C., Li, H., Dykhuizen, R., Frazer, R., Johnston, P., MacKnight, G. *et al*. 1997. Protection against oral and gastrointestinal diseases: Importance of dietary nitrate intake, oral nitrate reduction and enterosalivary nitrate circulation. Comp Biochem Phys 118A(4): 939-948.

Edenharder, R., Rauscher, R., and Platt, K.L. 1997. The inhibition by flavonoids of 2-

amino-3-methylimidazo[4,5-*f*]quinoline metabolic activation to a mutagen: A structure-activity relationship study. Mutat Res 379: 21-32.

Edenharder, R., von Petersdorff, I., and Rauscher, R. 1993. Antimutagenic effects of flavonoids, chalcones and structurally related compounds on the activity of 2-amino-3-methylinidazo[4,5-*f*]quinoline (IQ) and other heterocyclic amine mutagens from cooked food. Mutat Res 287: 261-274.

El-Bayoumy, K., Hecht, S.S., and Hoffmann, D. 1982. Comparative tumor initiating activity on mouse skin of 6-nitrobenzo[a]pyrene, 6-nitrochrysene, 3-nitroperylene, 1-nitropyrene and their parent hydrocarbons. Cancer Lett 16(3): 333-337.

Eldridge, B. 2004. Medical nutrition therapy for cancer prevention, treatment, and recovery. In: Mahan, L.K., and Escott-Stump, S. (eds.), Krause's food, nutrition, & diet therapy, pp. 997-1023. Pennsylvania: Saunders.

Felton, J.S., Jägerstad, M., Knize, M.G., Skog, K., and Wakabayashi, K. 2000. Contents in foods, beverages and tobacco. In: Nagao, M., and Sugimura, T. (eds.), Food borne carcinogens: Heterocyclic amines, pp. 31-62. Chichester: John Wiley & Sons Ltd.

Ferguson, L.R., Philpott, M., and Karunasinghe, N. 2004. Dietary cancer and prevention using antimutagens. Toxicology 198: 147-159.

Fiala, E.S., Reddy, B.S., and Weisburger, J.H. 1985. Naturally occurring anticarcinogenic substances in foodstuffs. Annu Rev Nutr 5: 295-321.

Francis, A.R., Shetty, T.K., and Bhattacharya, R.K. 1989. Modulating effect of plant flavonoids on the mutagenesis *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. Carcinogenesis 10(10): 1953-1955.

Gallagher, M.L. 2004. Viamins. In: Mahan, L.K., and Escott-Stump, S. (eds.), Krause's food, nutrition, & diet therapy, pp. 88-90. Pennsylvania: Saunders.

Giovannucci, E. 1999. Nutritional factors in human cancers. Adv Exp Med Biol 472: 1-29.

Handa, T., Yamauchi, T., Ohnishi, M., Hisematsu, Y., and Ishii, T. 1983. Detection and average content levels of carcinogenic and mutagenic compounds from the particulates on diesel and gasoline engine mufflers. Environ Int 9: 335-341.

Hankimhun, P. 1997. Mutagenic potential of raw and pickled fruits and vegetables

treated with nitrite. Master's Thesis in Nutrition, Faculty of Graduate Studies, Mahidol University.

Hartman, P.E., and Shankel, D.M. 1990. Antimutagens and anticarcinogens: A survey of putative interception molecules. Environ Mole Mutagen 15(3): 145-182.

Hayatsu, H., Negishi, T., Arimoto, S., and Hayatsu, T. 1993. Porphyrins as potential inhibitors against exposure to carcinogens and mutagens. Mutat Res 290: 79-85.

Hendry, G.A.F. 1996. Chlorophylls and chlorophyll derivatives. In. Hendry G.A.F.,and Houghon, J.D. (eds.), Natural Food Colorants, pp. 131-156. London: London Blackie.

Hollman, P.C.H., van Trijp, J.M., Mengelers, M.J., de Vries, J.H., and Katan, M.B. 1997. Bioavailability of the dietary antioxidant flavonol quercetin in man. Cancer Lett 114: 139-140.

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

Howard, P.C., Consolo, M.C., Dooley, K.L., and Beland, F.A. 1995. Metabolism of 1-nitropyrene in mice: transport across the placenta and mammary tissues. Chem-Biol Interact 95: 309-325.

IARC (International agency for research on cancer). 1989. 1-Nitropyrene. IARC Monog Eval Carc 46: 321-358.

Jaroszyńska, J. 2003. The influence of solvent choice on the recovery of phytogenic phenolic compounds extracted from plant material. Pol J Environ Stud 12(4): 481-484.

Kangsadalampai, K., Butryee, C., and Manoonphol, K. 1996. Direct mutagenicity of the polycyclic aromatic hydrocarbon-containing fraction of smoked and charcoal-broiled foods treated with nitrite in acid solution. Food Chem Toxicol 35: 213-218.

Kataoka, K., Kinouchi, T., and Ohnishi, Y. 1991. Species differences in metabolic activation and inactivation of 1-nitropyrene in the liver. Cancer Res 51: 3919-3924.

Kato, T., Tadokoro, N., Tsutsui, M., and Kikugawa, K. 1991. Transformation of

arylamines into the direct-acting mutagens by reaction with nitrite. Mutat Res 249: 243-254.

Kikugawa, K., Kato, T., and Takeda, Y. 1987. Formation of a highly mutagenic diazo compounds from the bamethan-nitrite reaction. Mutat Res 117: 35-43.

Kikugawa, K., Kato, T., and Takeda, Y. 1989. Formation of a direct mutagen, diazo-*N*-nitrosoetilefrin, by interaction of etilefrin with nitrite. Chem Pharm Bull 37(6): 1600-1603

Kinouchi, T., Tsutsui, H., and Ohnishi, Y. 1986. Detection of 1-nitropyrene in yakitori grilled chicken. Mutat Res 171: 105-113.

Kuo, M.L., Lee, K.C., and Lin, J.K. 1992. Genotoxicities of nitropyrenes and their modulation by apigenin, tannic acid, ellagic acid and indole-3-carbinol in Salmonella and CHO systems. Mutat Res 270: 87-95.

Lai, C., Butler, M.A., and Matney, T.S. 1980. Antimutagenic activities of common vegetables and their chlorophyll content. Mutat Res 77: 245-250.

Lijinsky, W. 1974. Reaction of drugs with nitrous acid as a source of carcinogenic nitrosamines. Cancer Res 34: 255-258.

Lijinsky, W., Conard, E., and Van de Bogart, R. 1972. Carcinogenic nitrosamines formed by drug/nitrite interaction. Nature 239: 165-167.

Lin, D., Kaderlik, K.R., Turesky, R.J., Miller, D.W., Lay Jr., J.O. and Kadlubar, F.F. 1992. Identification of *N*-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine as the major adduct formed by the food-borne carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, with DNA. Chem Res Toxicol 5:691-697.

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

Liu, Y., Perera, C.O., Suresh, V. 2007. Comparison of three chosen vegetables with others from South East Asia for their lutein and zeaxanthin content. Food Chem 101:1533-1539.

Lynnette, R.F. 1999.Natural and man-made mutagens and carcinogens in the human diet. Mutat Res 443: 1-10.

Maisuthisakul, P., Pasuk, S., and Ritthiruangdej, P. 2008. Relationship between antioxidant properties and chemical composition of some Thai plants. J Food

Compos Anal 21: 229-240.

Marais, J. P. 1997. Nitrate and Oxalates. In: Felix D'Mello, J.P. (ed.), Handbook of plant and fungal toxicants, pp. 205-218. USA: CRC Press.

Maron, D.M., and Ames, B. N. 1983. Revised methods for *Salmonella* mutagenicity test. Mutat Res 444: 451-461.

Marqauard, H., Rufino, F., and Weisburger, J.H. 1977. Mutagen activity of nitrite-treated food; Human stomach cancer may be related dietary factors. Science 196: 1000-1001.

Mathai, K. 2004. Nutrition in the adults year. In: Mahan, L.K., and Escott-Stump, S. (eds.), Krause's food, nutrition, & diet therapy, pp. 302-308. Pennsylvania: Saunders.

Matsushima, T., Sugimura, T., Nagao, M., Yahagi, T., Shirai, A., and Sawamura, M. 1980. Factors modulating mutagenicity in microbial test. In: Norpoth, K.H., and Garner, R.C. (eds.), Short-term test system for detecting carcinogens, pp. 273-285. Berling: Springer.

McCann, J., and Ames, B.N. 1977. The *Salmonella*/microsome mutagenicity test: Predictive value for animal carcinogenicity. In: Hiatt, H.H., Watson, J.D., and Winsten, J.A. (eds.), Origin of human cancer, pp. 1431-1450. New York: Cold Spring Hardor Laboratory.

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

Miller, E.C., and Miller, J.A. 1981. Searches for ultimate chemical carcinogens and their reactions with cellular macromolecules. Cancer 47: 2327-2345.

Mirvish, S.S. 1983. The etiology of gastric cancer: intragastric nitrosamide formation and other theories. J Natl Cancer Inst 71(3): 629-647.

Miyazawa, M., Sakano, S., Nakamura, S., and Kosaka, H. 1999. Antimutagenic activity of isoflavones from soybean seeds (*Gyicine max* Merrill). J Agr Food Chem 47: 1346-1349.

Moller, L., Corrie, M., Midtvedt, T., Rafter, J., and Gustafsson, J.A. 1988. The role of the intestinal microflora in the formation of mutagenic metabolites from the carcinogenic air pollutant 2-nitrofluorene. Carcinogenesis 9: 823-830.

Münzner, R., and Wever, J. 1984. Mutagenic activity in stomach and small intestine

of rats after oral administration of nitrosated beef extract. Cancer Lett 25: 225-230.

Murakami, A., Jiwajinda, S., Koshimizu, K. and Ohigashi, H. 1995. Screening for in vitro anti-tumor promoting activities of edible plants from Thailand. Cancer Lett 95: 139-146.

Nagao, M. 2000. Mutagenicity. In: Nagao, M., and Sugimura, T. (eds.), Food borne carcinogens: Heterocyclic amines, pp. 163-195. Chichester: John Wiley & Sons Ltd.

Negi, P.S., and Roy, S.K. 2000. Effect of blanching and drying methods on β-carotene, ascorbic acid and chlorophyll retention of leafy vegetables. Lebensm Wiss Technol 33: 295-298.

Oboh, G. 2005. Effect of blanching on the antioxidant properties of some tropical green leafy vegetables. Lebensm Wiss Technol 38: 513-517.

Ohshima, H., Friesen, M., Malavelle, C., Brouet, I.,Hautefeuille, A., and Bartsch, H. 1989. Formation of direct acting genotoxic substances in nitrosate smoked fish and meat products: Identification of simple phenolic precursors and phenyldiazonium ions as reactive products. Food Chem Toxicol 27: 193-203.

Ohta, T., Oribe, H., Kameyama, T., Goto, Y., and Takitani, S. 1988. Formation of diazoquinone-type mutagen from acetaminophen treated with nitrite under acidic conditions. Mutat Res 209: 95-98.

Omaye, S.T. 2004. Polycyclic aromatic hydrocarbons and other processing products. Food and nutritional toxicology, pp. 273-281. New York: CRC Press.

Onayemi, O., and Badifu, G.I.O. 1987. Effect of blanching and drying methods on the nutritional and sensory quality of leafy vegetables. Plant Foods for Human Nutrition 37: 291-298.

Padmavathi, P., and Rao, M.P. 1990. Nutritive value of *Sauropus androgynus* leaves. Plant Food Hum Nutr 40: 107-113.

Palli, D. 1996. Epidemiology of gastric cancer. Ann Ist Super Sanita 32(1): 85-99.

Peerawong, K. and Kangsadalampai, K. 1998. Mutagenic potential of various chicken extracts after nitrosation. Mahidol J 5(1): 5-8.

Potter, J.D., and Steinmetz, K.A. 1996. Vegetables, fruits and phytoestrogens as preventive agents. IARC Sci Publ 139: 61-90.

Powrie, W., Wu, C., and Stich, H. 1982.Browning reaction system as sources of

mutagens and modulators. Cancer Lett 19: 121-133.

Rafter, J., Govers, M., Martel, P., Pannemans, D., Pool-Zobel, B., Rechkemmer, G. *et al*. 2004. PASSCLAIM[1]-Diet-related cancer. Eur J Nutr 43(Suppl 2): II/47-II/84.

Riboli, E., and Norat, T. 2003. Epidemiologic evidence of the protective effect of fruit and vegetables on cancer risk. Am J Clin Nutr 78(Suppl): 559S-569S.

Robbana-Barnat, S., Rabache, M., Rialland, E., and Fradin, J. 1996. Heterocyclic amines: Occurrence and prevention in cooked food. Environ Health Persp 104: 280-288.

Rosenkranz, H. S., and Mermelstein, R. 1983. Mutagenicity and genotoxicity of nitroarenes. All nitro-containing chemicals were not created equal. Mutat Res 114: 1217-267.

Rosser, P.F., Ramachandran, P., Sangaiah, R., Austin. R.N., Gold, A., and Ball, L.M. 1996. Role of *O*-acetyltransferase in activation of oxidized metabolites of genotoxic environmental pollutant 1-nitropyrene. Mutat Res 369: 209-220.

Rüdiger, W., and Schoch, S. 1988. Chlorophylls. In: Goodwin, T.W. (ed.), Plant Pigments, pp. 1-12. New York: Academic Press.

Sarkar, D., Sharma, A., and Talukder, G. 1994. Chlorophyll and chlorophyllin as modifiers of genotoxic effects. Mutat Res 318: 239-247.

Sasagawa, C., Muramatsu, M., and Matsushima, T. 1988. Formation of direct mutagens from amino-imidazoazaarenes by nitrite treatment. Mutat Res 203: 386-43.

Sato, M., Iguchi, N., and Murata, T. 1977. Effect of sodium copper chlorophyllin on lipid peroxidation. I. effect on lipid peroxidation in rat liver homogenates in the presence of both Fe^{2+} and L-ascorbic acid. Yakugaku Zasshi 97(3): 268-273

Sato, M., Nagata, M., and Engle, L.M. 2002. Evaluation of antioxidant activity of indigenous vegetables from South and Southeast Asia. JIRCAS Research Highlights : 10-11.

Schut, H.A., Wang, C.L., Twining, L.M., and Earle, K.M. 1997. Formation and persistence of DNA adducts of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) in CDF_1 mice fed a high ω-3 fatty acid diet. Mutat Res 378: 23-30.

Shibamoto, T., Nishimura, D., and Mihara, S. 1981. Mutagenicity of products

obtained from maltol-ammonia browning model system. J Agr Food Chem 29: 643-646.

Shimada, T., Iwasaki, M., Martin M.V., and Guengerich, F.P. 1989. Human liver microsomal cytochrome P-450 enzymes involved in the bioactivation of procarcinogens detected by umu gene response in Salmonella typhimurium TA1535 rpSK1002. Cancer Res 49: 3218-3228.

Solyakov, A., and Skog, K. 2002. Screening for heterocyclic amines in chicken cooked in various ways. Food Chem Toxicol 40: 1205-1211.

Stavric, B. 1994. Antimutagens and anticarcinogens in foods. Food Chem Toxicol 32(1): 79-90.

Steinmetz, K.A., and Potter, J.D. 1991. Vegetables, fruit and cancer. II. Mechanism. Cancer Cause Control 2: 427-442.

Steinmetz, K.A., and Potter, J.D. 1996. Vegetables, fruits and cancer prevention: A review. J Am Diet Assoc 96: 1027-1039.

Stokes. 1864. Plant pigment: Chlorophyll. Proc Roy Soc 13:144.

Subhasree, B., Baskar, R., Keerthana, R.L., Susan, R.L., and Rajasekaran, P. 2009. Evaluation of antioxidant potential in selected green leafy vegetables. Food Chem 115: 1213-1220.

Subramaniam, V., Adenan, M.I., Ahmed, A.R., and Sahdan, R. 2003. Natural antioxidants:*Piper sarmentosum* (Kadok) and *Morinda elliptica* (Mengkudu). Mal J Nutr 9(1): 41-51.

Sugimura, T. 2000. Nutrition and dietary carcinogens. Carcinogenesis 21(3): 387-395.

Sugimura, T., Wakabayashi, K., Nagao, M., and Esumi, H. 1993. A new class of carcinogens: heterocyclic amines in cooked food. In: Parke, D.V., Ioannides, C., and Walker, R. (eds.), Food, Nutrition and Chemical Toxicity, pp. 259-276. Smith-Gordon and Nishimura Ltd.

Sugimura, T., Wakabayashi, K., Nakagama, H., and Nagao, M. 2004. Heterocyclic amines: Mutagens/carcinogens produced during cooking of meat and fish. Cancer Sci 95(4): 290-299.

Talbot, M.F.J., and Sauer, K. 1997. Spectrofluorimetric method for the determination of large chlorophyll *a/b* ratios. Photosynth Res 53: 73-79.

Tokiwa, H., Nakagawa, R., and Horikawa, K. 1985. Mutagenic/carcinogenic agents

in indoor pollutant; dinitropyrenes generated by kerosene heaters and fuel gas and liquefied petroleum gas burners. Mutat Res 157: 39-47.

Tsuda, M., Negishi, C., Makino, R., Sato, S., Yamaizumi, Z., Hirayama, T. *et al*. 1985. Uses of nitrite and hypochlorite treatments in determination of the contribution of IQ-type and nonIQ-type heterocyclic amines to the mutagenicities in crude pyrolyzed materials. Mutat Res 147: 335-341.

Vatanasapt, V., Sriamporn, S., and Vatanasapt, P. 2002. Cancer control in Thailand. Jpn J Clin Oncol 32(Supplement): S82-S91.

Venitt, S., Crofton-Sleigh, C., and Forster, R. 1984. Bacterial mutation assays. In: Venitt, S., and Parry, J.M. (eds.), Mutagenicity testing: A practical approach, pp. 58-59. England: IRL press Ltd.

Wakabayashi, K., Nagao, M., Chung, T.H., Yin, M., Tahira, T., and Sugimura, T. 1985. Appearance of direct-acting mutagenicity of various foodstuffs produced in Japan and Southeast Asia in nitrite treatment. Mutat Res 158: 119-124.

Wakabayashi, K., Nagao, M., Esumi, H., and Sugimura, T. 1992. Food-derived mutagens and carcinogens. Cancer res 52(Supplement): S2092-S2098.

Wakabayashi, K., and Sugimura, T. 1998. Heterocyclic amines formed in the diet: Carcinogenicity and its modulation by dietary factors. J Nutr Biochem 9: 604-612.

Wakabayashi, K., Usiyama, H., Takahashi, M., Nukaya, H., Kim, S.B., Hirose, M. *et al*. 1993. Exposure to heterocyclic amines. Environ Health Persp 99: 129-134.

Wattenberg, L.W. 1985. Chemoprevention of cancer. Cancer Res 45: 1-8.

Wei, C.I., Kitamura, K., and Shibamoto, T. 1981. Mutagenicity of Maillard browning products obtained from a starch-glycine model system. Food Chem Toxicol 19: 749-751.

Weisburger, J.H., Dolan, L., and Pittman, B. 1998. Inhibition of PhIP mutagenicity by caffeine, lycopene, daidzein, and genistein. Mutat Res 416: 125-128

Wu, A.H., Shibata, D., Yu, M.C., Lai, M. and Ross, R.K. 2001. Dietary heterocyclic amines and microsatellite instability in colon adenocarcinomas. Carcinogenesis 22(10): 1681-1684.

Yahagi, T., Degawa, M., Seino, Y., Matsushima, T., and Nagao, M. 1975. Mutagenicity of carcinogenic azo dyes and their derivatives. Cancer Lett 1:

91-96.

Yahagi, T., Nagao, M., Seino, Y., and Matsushima, T. 1977. Mutagenicity of *N*-nitrosamines on *Salmonella*. Mutat Res 48: 121-129.

Yamanaka, H., Nagao, M., Sugimura, T., Furuya, T., Shirai, A., and Matsushima, T. 1979. Mutagenicities of pyrrolizidine alkaloids in the *Salmonella*/mammalian microsome test. Mutat Res 68: 211-216.

Yano, M., Wakabayashi, K., Tahira, T., Arakawa, N., Nagao, M., and Sugimura, T. 1988. Presence nitrosable mutagen precursors in cooked meat and fish. Mutat Res 202: 119-123.

Zaripheh, S., and Erdman, J.W. 2002. Factors that influence the bioavailability of xanthophylls. J Nutr 132: 531S-534S.

Zin, M.Z., Abdul-Hamid, A., Osman, A. 2002. Antioxidative activity of extracts from Mengkudu (*Morinda citrifolia* L.) root, fruit and leaf. Food Chem 78: 227-231.

Appendix

APPENDIX

1. Preparation of Stock Solution and Medium

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

Use : Minimal agar

Ingredient	Per liter	
Warm distilled water (45°C)	670	ml
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	10	g
Citric acid monohydrate	100	g
Potassium phosphate, dibasic (anhydrous) (K_2HPO_4)	500	g
Sodium ammonium phosphate tetrahydrate ($NaNH_4HPO_4.4H_2O$)	175	g

Salts were added to water in order that indicated above and allowed each salt to dissolve completely before adding the next. The solution were filtered and then autoclave at 121°C for 15 min.

1.2 Minimal glucose agar plate

Use : Mutagenicity assay

Ingredient	Per liter	
Bacto agar	15	g
Distilled water	930	ml
VB salts	20	ml
40% glucose	50	ml

Agar was added to distilled water in a glass bottle and then autoclave at 121°C for 15 min. When the solution has cooled slightly, sterile VB salts and sterile 40% glucose were added, mixed and pour 30 ml into each sterile petri plate. Minimal glucose agar plate were kept in incubator at 37°C before using.

1.3 Oxoid nutrient broth NO.2

Use : Growing culture

Dissolve 2.5 g of nutrient broth NO.2 in 100 ml distilled water and 12 ml of nutrient broth was transferred into each flask (covered with sterile guaze). Autoclave at 121°C for 15 min.

1.4 Top agar

Use : Mutagenic assay

Ingredient	100	ml

Bacto agar	0.6	g
Sodium chloride (NaCl)	0.5	g
Distilled water	100	ml

All ingredients were dissolved in water and stored in a glass bottle. The solution was autoclave at 121°C for 15 min. 10 ml of 0.5 mM histidine / biotin was added for 100 ml of top agar and mixed thoroughly by swirling.

1.5 0.1 M L-histidine HCl stock

Use : Fortification of minimal agar plate.

Ingredient	10	ml
L-histidine HCl	0.2096	g
Distilled water	10	ml

Dissolve 0.2096 g of L-histidine HCl (MW 209.63) in 10 ml distilled water.

1.6 1 mM L-histidine HCl stock

Use : Fortification of minimal agar plate.

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

1 ml of 0.1 M L-histidine HCl was diluted in 99 ml of distilled water.

1.7 1 mM biotin stock

Use : Fortification of minimal agar plate.

Ingredient	100	ml
Biotin	24.43	mg
Distilled water	100	ml

Dissolve 24.43 g of biotin (MW 244.3) in distilled water. Warm it until dissolve completely.

1.8 0.5 mM L-histidine / biotin solution.

Use : Mutagenicity assay (add 10 ml to 100 ml of top agar)

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

Mix and autoclave at 121°C for 15 min.

1.9 1 M potassium chloride

Use : Na_3PO_4 - KCl buffer

Ingredient	100	ml

Potassium chloride	7.456	g
Distilled water	100	ml

Potassium chloride was dissolved into distilled water.

1.10 0.5 M sodium phosphate pH 7.4

Use : Na_3PO_4 - KCl buffer for mutagenicity assay

Ingredient

0.5 M	Sodium dihydrogen phosphate (NaH_2PO_4) (MW 120) (30 g / 500 ml)
0.5 M	Disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$) (MW 177.9) (44.5 g / 500 ml)

Dissolve 44.5 g disodium hydrogen phosphate dihydrate in 300 ml of distilled water. Add 0.5 M sodium dihydrogen phosphate until pH 7.4, then adjust volume to 500 ml. Sterilize by autoclaving for 15 min at 121°C.

1.11 Na_3PO_4 – KCl buffer

Use : mutagenicity assay

Ingredient	330	ml
0.5 M Na_3PO_4 pH 7.4	100	ml
1 M KCl	16.5	ml
Distilled water	213.5	ml

Mix and autoclave at 121°C for 15 min.

2. Recipes for Some Reagents and Test Chemicals

2.1 2 M sodium nitrite

Use : Nitrosation

Ingredient	10	ml
Sodium nitrite	1.38	g
Distilled water to	10	ml

Mix and autoclave at 121°C for 15 min.

2.2 2 M ammonium sulfamate

Use : reaction mixture

Ingredient	10	ml
Ammonium sulfamate	2.28	g
Distilled water to	10	ml

Mix and autoclave at 121°C for 15 min.

2.3 0.2 N hydrochloric acid

Use : reaction mixture

Ingredient	100	ml
Conc. hydrochloric acid	1.66	ml
Sterile distilled water	98.34	ml

Dissolve conc. hydrochloric acid in sterile water. Store in sterile glass tubes or bottles with screw caps.

2.4 0.0375 mg/ml aminopyrene

Use : standard solution for mutagenicity assay

Ingredient	2	ml
0.3 mg/ml aminopyrene	250	µl
Acetonitrile	1,750	µl

Dissolve 3 mg of 1-aminopyrine in 300 µl of acetonitrile and mix; and subsequently dilute 300 µl of this solution (3 mg/ml 1-aminopyrine) in 2,700 µl of acetonitrle, the solution obtained will be 0.3 mg/ml 1-aminopyrine. Then, dilute 250 µl of 0.3 mg/ml 1-aminopyrine in 1,750 µl of acetonitrile and mix. Store all solutions in sterile glass vials with screw caps in a freezer. The preparation must be used sterile technique.

2.5 8 mg/ml ampicillin solution

Ingredient	10	ml
Ampicillin sodium	80	mg
Distilled water to	10	ml

Ampicillin was dissolved into water and stored in glass bottle with screw cap. Preparation of this solution must be used sterile technique.

2.6 0.1% crystal violet

Ingredient	10	ml
Distilled water	10	ml
Crystal violet	10	mg

Crystal violet was dissolved into water and stored in glass bottle with screw cap. Preparation of this solution must be used sterile technique.

3. Procedure for Re-isolation and Growing Culture

Tester strains, TA98 and TA100 were grown in Oxoid nutrient broth No.2 and incubated overnight in 37°C in shaking water bath. The growth period should not exceed 16 hours. These cultures were re-isolated by streaking on minimal glucose agar plates which the surface were spread with 0.1 ml of 8 mg/ml ampicillin, 0.3 ml of 0.1M histidine HCl and 0.1 ml of 1mM biotin. These plates are incubated at 37°C for 48 h. after incubation, the 4 single colonies per strain TA98 and TA100 are picked up and grown in Oxoid nutrient broth No.2 overnight at 37°C in shaking water bath. Each culture is confirmed genotypes of the strains and kept the cultures as the source of bacteria for mutagenicity testing. For each 1.0 ml of culture, add 0.09 ml of spectrophotometric grade DMSO. Combine the culture and DMSO in a sterile tube and distribute 400 µl of the culture aseptically into sterile cryotubes. Store the tubes in a freezer at -80 °C.

4. Confirming Genotype of Tester Strains

The broth of TA98 and TA100 are used to confirm genotypes in the following ways.

4.1 Histidine requirement

The his^+ character of the strains is confirmed by demonstrating the histidine requirement for growth on the minimal glucose agar plates enriched with histidine and biotin

Procedure:

Plate A: no histidine and biotin

Plate B: 0.1 ml of 1 mM biotin

Plate C: 0.3 ml of 0.1 M His-HCl

Plate D: 0.3 ml of 0.1 M His-HCl + 0.1 ml of 1 mM biotin

Four minimal glucose agar plates are required for each tester strains. Each plate is applied on the surface with 0.1 ml of 1 mM biotin or 0.3 ml of 0.1 M His-HCl or 0.3 ml of 0.1 M His-HCl plus 0.1 ml of 1 mM biotin or no application (plate b, c, d, a, respectively). Made a single streak of each strains across these plates. Four strains could be tested on the same pate. Incuated at 37°C for 24 h. The growing of bacteria on histidine plus biotin plate is the result of histidine requirement.

4.2 R-factor

The R-factor strains (TA 97, TA 98, TA 100 and TA 102) should be tested routinely for the presence of the ampicillin resistance factor because the plasmid is somewhat unstable and can be lost from bacteria.

Procedure: For each tester strain, add 0.3 ml of fresh overnight culture to a tube containing 0.1 ml of 0.1 M histidine-HCl follow by adding 20 ml of molten top agar containing 0.5 mM, mixed and poured on a minimal glucose agar plate. Rotated the plate to distribute the mixtures and allowed several minutes for agar to become firm. R-factor and rfa mutation (see the next section) are performed in the same plate by dividing the plate into 2 areas, one for R-factor and the other for rfa mutation. For R-factor, commercial ampicillin disc or filter paper disc containing 8 mg/ml ampicillin is applied on the surface of the agar by using sterile forceps. The disc is pressed lightly to embed in the overlay. The plates are incubated at 37°C for 24 h. The absence of the clear zones of inhibition around the disc indicates resistance to ampicillin.

4.3 Rfa mutation

Strains having the deep rough (rfa) character should be tested for crystal violet sensitivity.

Procedure: Pipetted 0.1% solution of crystal violet to the sterile filter paper disc (1/4 inch) and transferred the disc to plates, seeded with bacteria (the procedure is similar to R-factor). Incubated at 37°C for 48 h. The clear zone appeared around the disc indicated the presence of the rfa mutation that permitted crystal violet to enter and kill bacteria.

5. Spontaneous Reversion

Spontaneous reversion of the tester strains to histidine independence is measured routinely in mutagenicity experiments and is expressed as the number of spontaneous revertants per plate. The revertant colonies are clearly visible in a uniform background lawn of auxotrophic bacteria. Each tester strain reverts spontaneously at a frequency that is characteristic of the strain. Nevertheless, there is a variability in the number of spontaneous revertants from one experiment to another and from one plate to another, and it is advisable to include at least 2-3 spontaneous mutation control plates for each strain in a mutagenicity assay.

Procedure: 0.1 ml of DMSO was added to capped culture tube. 0.5 ml of $NaPO_4$-KCl buffer pH 7.4 and 0.1 ml of fresh overnight culture of TA98 or TA100 was added. The mixture was incubated in shaking water bath at 37°C in 20 min. After that 2.0 ml of molten top agar was added to the mixture, mixed and then poured on the incubated at 37°C for 48 hours. The his^+ revertants colonies that grown on the minimal glucose agar plate were counted.

6. The Response to Standard carcinogen.

Standard mutagens or positive mutagens are used routinely in mutagenicity experiments to confirm the reversion property and specificity of each strain. The standard mutagen, which used in this experiment, was nitrosated-aminopyrene. Tester strains which highly response to positive mutagens must be collected.

Procedure: 0.02 and 0.04 ml of 0.075 mg/ml aminopyrene in acetonitrile were pipetted to sterile capped tube. Then, 0.73 and 0.71 ml of 0.2 N HCl were added respectively, and followed by 0.25 ml of 2 M $NaNO_2$. The final concentration of nitrite was 0.5 M. The solution was mixed and shaked in water bath at 37°C for 4 hours. The tube was placed in an ice bath and 0.25 ml of 2 M ammonium sulfamate ($NH_2SO_3NH_4$) was added and standed for 10 min in ice bath. 0.1 ml of each mixture was pipetted to cap culture tube for testing the stock culture TA98 (equal to 0.12 µg of aminopyrene/plate) and TA100 (equal to 0.24 µg of aminopyrene/plate). Then, the evaluation of their mutagenicity was tested as described in spontaneous reversion. The characteristic properties of the stock culture for TA98 and TA100 as the source of bacteria for mutagenic testing are:

1. Contain R-factor (pKM 101) and rfa mutation.
2. His+ requirement.
3. Low spontaneous reversion.
4. Highly response to standard carcinogen.

The experiment was performed only when the characteristic properties of bacteria strain were done.

BIOGRAPHY

NAME	Miss Thunyawan Nuntatovattana
DATE OF BIRTH	18 November 1980
PLACE OF BIRTH	Bangkok, Thailand
INSTITUTIONS ATTENDED	Chulalongkorn University, 1999-2003; Bachelor of Science in Pharmacy (Clinical Pharmacy) Chulalongkorn University, 2006-2009; Master of Science in Pharmacy (Food Chemistry and Medical Nutrition)
ADDRESS	1/161 Raminthra Rd. Tarang Bangkhen Bangkok, Thailand 10220